Shamsul Hayat · Aqil Ahmad
Mohammed Nasser Alyemeni *Editors*

Salicylic Acid

Plant Growth and Development

Springer

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the Institutions and Nations
Where We Work*

Preface

The healthy plant canopy that we recognise is the result of integrated metabolic functions administered by a number of factors, including hormones, of which six (Auxins, Gibberellins, Cytokinins, Abscissic acid, Ethylene and Brassinosteroids) are well recognised for their regulatory functions. However, the others (Salicylic acid, Polyamines and Jasmonates) can not be excluded from the list of hormones because of their well-recognised involvement in plant metabolism and growth.

This book is providing recent information related with Salicylic acid (SA), that was first noticed to be a major component in the extract from Salix (willow) bark and was used as an anti-inflammatory drug. It belongs to the phenolic group and is ubiquitous in plants. SA is involved in signal transduction, pondering over the plant resistance to stress and generates significant impact on photosynthesis, transpiration, uptake and transport of ions and plant growth and development. However, the observations related with this presumed plant hormone are very much scattered. It was, therefore, decided to compile all in the form of a book, based on 16 chapters written by various experts, working in this field. A total of 47 experts have explained their results based on the practical work carried over by them and of others on various selected aspects of plants under stress. After going through these chapters it may be concluded that this hormone has a wide range of actions mediated through genes and/or the cell membranes and can be grouped as a stress hormone. It is the second revised edition of the book.

With great pleasure, we extend our sincere thanks to all the contributors for their timely preparation of excellent and up-to-date contributions and also for their consistent support and cooperation. The first two editors (Hayat and Ahmad) are thankful to Aligarh Muslim University, Aligarh, India that gave us the employment and the seat to work. Whereas, Hayat and Alyemeni are also thankful to King Saud University, Riyadh, Saudi Arabia for their present assignment. Thanks are also due to Springer, The Netherlands for expeditious acceptance of our proposal and completion of the review process. Subsequent cooperation and understanding of their staff, especially, Malanie van Overbeek, publishing assistant, is also gratefully acknowledged.

We express our sincere thanks to the members of our family for all the support they provided and the neglect, what so ever, they suffered during the preparation of this book.

Finally we are thankful to the Almighty God.

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Chapter 1
Salicylic Acid: An Update on Biosynthesis and Action in Plant Response to Water Deficit and Performance Under Drought

Hanna Bandurska

Abstract Salicylic acid (SA) and its derivatives are the most widely known drugs in the world used to reduce pain and fever, helping to treat many inflammatory diseases, in the prevention of coronary heart disease and heart attacks, and in tumor suppression. This substance is also characterized by a high metabolic and physiological activity, which enables it to perform regulatory functions in plant development and reaction to biotic and abiotic stress factors. Under non-stress conditions, SA is present in plant tissues in quantities of several mg to several ng in one g of fresh mass. Its level substantially increases in plants exposed to water deficit. The accumulation of SA may result from its *de novo* synthesis through activation of enzymes involved in the synthesis of SA from phenylalanine, i.e. phenylalanine ammonia lyase (PAL) and benzoic-acid-2-hydroxylase (BA2H). SA accumulated in plants growing under the conditions of water shortage may be involved in the regulation of mechanisms responsible for resistance to drought through the control of water balance and activation of antioxidant system. Large body of evidences revealed that exogenous application of SA was effective in modeling plant responses to water deficit. Plant pre-treatment with SA resulted in higher tissue water content, increased activity of antioxidant enzymes, decreased level of lipid peroxidation and membrane injury and it also protected nitrate reductase activity against inhibition under water deficit conditions. These changes enable plants to survive under drought and play an essential role in countering the adverse effects of stress on growth and yield.

Keywords Drought · Salicylic acid · Water deficit · Stress resistance

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1 Introduction

Salicylic acid (SA) or *ortho*-hydroxybenzoic acid (Fig. 1), belongs to a varied group of phenolic compounds well known in the plant kingdom.

SA is present in plants as a free phenolic acid and as a conjugate form, which may be generated by glucosylation, methylation or hydroxylation of the aromatic ring (Raskin 1992; Lee et al. 1995). The best known natural SA derivative is salicin (ß-glucoside salicylic alcohol), occurring in white willow (*Salix alba*) and other willow species including *S. purpurea*, *S. daphnoides* and *S. fragilis*. The highest salicin content in that plant is observed during spring or summer and the lowest in winter (Foster and Tyler 1999). The highest content of free SA was monitored in bark of *S. laponum* (3 mg·g^{-1}fr. mass) and in the branches of *S. purpurea* and *S. plantifolia* (about 2.1 mg·g^{-1}fr. mass) (Peterek et al. 2007). The name salicylic acid is derived from the Latin word for willow tree (Salix), from whose bark Johan Bűchner isolated in 1828 a small amount of salicin (Raskin 1992). Later, in 1838 the Italian scientist Rafaele Piria obtained SA from flower buds of Meadowsweet (*Spiraea ulmaria* known as *Filipendula ulmaria*). At the end of the nineteenth century, in 1899 the Bayer Company formulated a new drug, acetylsalicylic acid, and called it at first acetyl-spiric-acid (from *Spiraea*) and then aspirin (Ansari and Misra 2007). Recently, Blazics et al. (2010) showed that *Filipendula ulmaria* is a rich source of salicylic acid, which is present in its herb and flowers at an amount of about 1.4 mg·g^{-1} dry mass. A large amount of salicylates also occurs in tissues of wintergreen (*Gaultheria procumbens*), known as checkerberry or teaberry. The salicylate predominantly present in that plant is a derivatized form of methyl salicylate called gaultherin (Rybnicky et al. 2003). SA acid and its derivatives are the most widely known drugs in the world used to reduce pain, fever, helping to treat many inflammatory diseases and in the prevention of coronary heart disease and heart attacks. Lately, large body of evidences suggests that salicylates also affect tumor suppression (Brummelkamp et al. 2003; Ansari and Misra 2007; Elwood et al. 2009). These therapeutic substances for humans also play an important role in the plant kingdom. SA is characterized by a high metabolic and physiological activity, which performs regulatory functions in plant cells. For over twenty years, this compound has been under investigation for its role in the response of plants to various stress factors (Horváth et al. 2007; San-Vincente and Plasencia 2011). Under optimal conditions SA content in leaves of *Arabidopsis*, tobacco, corn, and rape is lower than 0.1 µg·g^{-1} fresh mass.

Fig. 1 Structure of salicylic acid
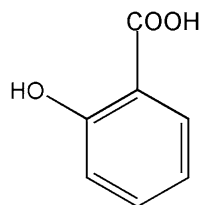
Higher SA levels were detected in leaves of tomato (0.27 $\mu g \cdot g^{-1}$ fr. mass), bean (0.86 1 $\mu g \cdot g^{-1}$ fr. mass), barley (2.13 $\mu g \cdot g^{-1}$ fr. mass) and the highest in rice, which varied from 5 to 30 $\mu g \cdot g^{-1}$ fr. mass, depending on the cultivar (Raskin et al. 1990; Yang et al. 2004). The increased level of endogenous SA after pathogen infection, and its participation in plant responses to biotic stresses, has been confirmed in numerous studies and presented in several papers (Durner et al. 1997; Pieterse and Loon 1999; Metraux 2001; Shah 2003; Vlot et al. 2009). SA has also been recognized as a regulatory molecule, mediating plant responses to abiotic stress factors. The present work focuses on the results of studies examined the role of SA in the regulation of plant responses to water deficit.

2 Biosynthesis of Salicylic Acid and Accumulation Under Water Deficit Conditions

SA is synthesized in plants through two (Fig. 2) distinct enzymatic routes, which require chorismate as a primary metabolite (Wildermuth 2006; Chen et al. 2009). One is chloroplast-localized SA synthesis from chorismate via isochorismate in a two-step reaction catalyzed by isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Wildermuth et al. 2001). It was shown that SA synthesized through the isochorismate route has an important role in plant defense against pathogen infection as well UV- or ozone-treated *Arabidopsis thaliana* L., *Nicotiana benthamiana* and tomato (Ogawa et al. 2005; An and Mou 2011). An alternative SA biosynthetic pathway is the phenylalanine route localized in the cytoplasm. Chorismate-derived phenylalanine (Phe) is converted to trans-cinnamic acid (t-CA) by phenylalanine ammonia lyase (PAL) and later t-CA is oxidized to benzoic acid (BA). The hydroxylation of the aromatic ring of BA catalyzed by benzoic-acid-2-hydroxylase (BA2H) leads to SA formation (Leon et al. 1993; Lee et al. 1995). This pathway was confirmed to operate in ozone-exposed tobacco leaves, heat-treated pea plants, and salt-stressed rice seedlings (Ogawa et al. 2005; Sawada et al. 2006; Pan et al. 2006). According to Chong et al. (2001) conversion of benzoic acid to SA requires the presence of hydrogen peroxide (H_2O_2), which stimulates the activity of BA2H. A number of studies have shown that water deficit induces an increase in the activity of PAL in leaves of wheat and white clover seedlings, and in fruits of pepper (Sung 2005; Tian and Lei 2007; Lee et al. 2007). Recently we examined the effect of water deficit on SA accumulation and the activity of two enzymes involved in the synthesis of SA from phenylalanine, i.e. PAL and BA2H. Moderate water deficit increased the activity of both enzymes and triggered an increase of SA level in leaves and roots of barley seedlings (Bandurska and Cieślak 2012). So, these studies indicate that the cinnamic acid → benzoic acid → SA route is activated in plants exposed to water deficit.

Taking into account the presence in plant tissue of conjugated forms of SA and its precursors another possibility is that the release of free SA from conjugates

Fig. 2 Schematic diagram of SA biosynthetic pathways. Abbreviations: *PAL* phenylalanine ammonia lyase, *ICS* isochorismate synthase, *IPL* isochorismate pyruvate lyase, *BA2H* benzoic-acid-2-hydroxylase
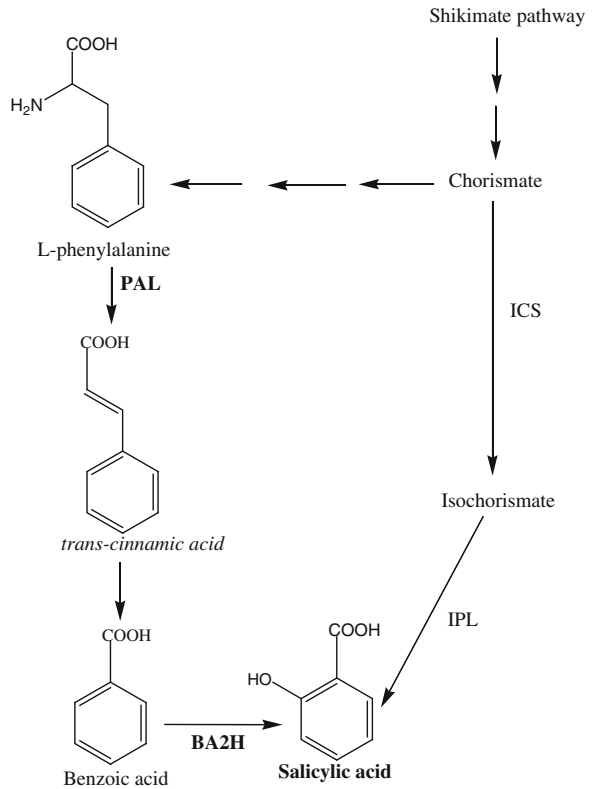
takes place under water deficit conditions. This route may proceed via decarboxylation of conjugated forms of t-CA to conjugated forms of BA followed by its hydroxylation to salicyl-glucose ester and salicyl-CoA and then release of free SA, as was found in tobacco plants infected with mosaic virus (Lee et al. 1995; Chong et al. 2001).

3 Effect of Salicylic Acid on Plant Resistance to Water Deficit

Three experimental approaches are commonly used in studies focus on the role of SA in plant response to stress factors. One is to examine the stress resistance of plants treated with various concentrations of SA. Another approach is to study the effect of certain stress factors on the level of SA in plant tissues and its relationship with other biochemical and physiological parameters responsible for resistance. The third approach consists in testing the resistance of transgenic plants, which

have increased or decreased levels of SA, to a particular stress factors. Yang et al. (2004) revealed that SA-deficient transgenic rice exhibited increased susceptibility to oxidative stress and suggested that SA may play an important role in plant resistance to oxidative stress caused by biotic and abiotic stress agents. In the available literature there are no data with regard to transgenic plants having modified capacity for SA synthesis in terms of the role of this regulator in resistance to water deficit. However, we have shown that SA may play a role in the mechanism of *cross-resistance* in plants subjected to the combined action of UV-B and water deficit. UV-B applied before water deficit induced an increase of endogenous SA level in the leaves of barley seedlings and alleviated the damaging effect of water deficit on cell membranes and leaf hydration. This was evidenced by the lack of membrane damage as well as the lack of a decrease in leaf water content of plants pre-treated with UV-B before water deficit imposition, in spite of occurrence of such damage after the sole action of water deficit (Bandurska and Cieślak 2012).

In the studies related to the involvement of SA in plant responses to water deficit most of the investigations are concentrated on the impact of exogenous application of SA on plant resistance to stress. However, some research also focuses on the interplay between changes in the level of SA in water deficit treated plant and its resistance to water deficit.

3.1 Effect of Water Deficit on SA Level in Plant Tissues and Stress Resistance

The endogenous SA content increased in the leaves of drought-stressed Mediterranean plant, *Phillyrea angustifolia*. However, it was revealed that SA level showed a strong negative correlation with the level of leaf hydration (relative water content, RWC) and a positive correlation with α-tocopherol level during drought, which may indicate the possible involvement of SA in the regulation of water balance as well as activation of antioxidative mechanisms (Munné-Bosch and Peñuelas 2003). Water deficit induces leaf senescence, which is sometimes regarded as a negative consequence of stress. However, senescence of mature leaves permit nutrient remobilization to the youngest leaves, allowing plant survival during prolonged periods of drought (Munné-Bosch and Alegre 2004). It has been reported that drought stress induced SA accumulation in leaves of field-grown common sage plant (*Salvia officinalis*) that may regulate leaf senescence and plant survival under water deficit conditions (Abreu and Munné-Bosch 2008). Our results provide the first confirmation that water deficit effects a significant increase of SA content in barley seedlings. We have shown that polyethylene glycol-induced (PEG 6000) water deficit causes a significant increase of SA content in barley roots after 6 and 24 h of stress, where as in leaves SA level did

not change at this time (Bandurska and Stroiński 2005). The results of our recent study revealed that water deficit of the same level but acting longer affected the increase of SA level both in leaves and roots of barley seedlings. However, an increase of SA content was first observed in roots (after 3 days) than in leaves (after 6 days), which indicates the involvement of SA in signal transduction between roots and leaves (Bandurska and Cieślak 2012). A significant increase of SA under the conditions of PEG-induced water deficit was also shown in leaves of soybean. The examined soybean plants had a higher SA level and were more resistant to water deficit at the reproductive than the vegetative stage (Hamayun et al. 2010).

3.2 Effects of Exogenous Application of SA on Plant Resistance to Water Deficit

Exogenous application of SA was found to be effective in modeling of plant metabolic and physiological processes that may enhance resistance to water deficit. SA application at various concentrations through roots, seed soaking and foliar spraying in a concentration-dependent manner alleviated the negative effect of water deficit on tissue water status, stomatal conductance, chlorophyll content, membrane properties and plants physiological activities (Horváth et al. 2007; Hayat et al. 2010). The application of SA in muskmelon, either through seed soaking or foliar application, provided protection against drought. Lower concentrations within the range of 0.1–0.5 mM were more effective in reducing the negative effect of drought than higher concentration (1 mM). The mode of application did not make any significant difference (Korkmaz et al. 2007). In cucumber, the application of SA by seed soaking or foliar spray ameliorated injury caused by water deficit. SA was more effective when applied by soaking the seeds and the best results were obtained using 0.5 mM SA solution (Baninasab 2010). However, application of SA (0.7 mM) in rice was more effective in ensuring better resistance to water deficit when applied by foliar spray than seed treatment (Farooq et al. 2009). Much higher SA concentrations used to the foliage were effective in protecting amaranth, tomato (3 mM) and *Satureja hortensis* (1.0–3.0 mM) plants against water stress (Umebese et al. 2009; Yazdanpanah et al. 2011). In wheat, a beneficial effect on improving resistance to water deficit was shown after leaf spraying with relatively high SA concentrations, i.e. 3.0 and 50 mM (Singh and Usha 2003; Aldesuquy et al. 2012). Foliar spraying with much lower SA concentration (1 μM) alleviated the damaging effect of long term drought stress in *Ctenathe setosa* and maize providing increased resistance to stress (Kadioglu et al. 2011; Saruhan et al. 2012). Increased resistance to drought in bean was obtained similarly as in cucumber by soaking seeds in 0.5 mM SA concentration before sowing (Sadeghipour and Aghaei 2012). Seedlings of four chickpea genotypes

became more resistant to drought when their seeds were soaked in 1.0–1.5 mM SA solution (Patel et al. 2011). Seed soaking in much lower SA concentrations was effective in amelioration the negative impact of drought on soybean (0.6 mM) and wheat (0.01–0.05 mM) (Sakhabutdinova et al. 2003; Khan et al. 2012). Bean and tomato seedlings grown from seeds imbibed in 0.1–0.5 mM solutions of SA or the plants which were soil drenched with 0.5 mM solution of SA did not exhibit signs of wilting (Senaratana et al. 2000). There is some evidence that application of SA through roots by mixing with the nutrient solution improved drought resistance. Increased resistance to water deficit was achieved in tomato at 0.01 mM SA concentration and lower, in wheat 0.3–0.7 mM, barley 0.3–012 μM, potato 0.1 mM and in banana 1.0–3.0 mM (Bandurska and Stroiński 2005; Szepsi et al. 2005; Waseem et al. 2006; Deneshmand et al. 2009; Bidabadi et al. 2012).

3.3 Mode of Action of SA in Increasing Resistance to Water Deficit

SA applied exogenously with various methods and concentrations (0.1–10 mM) activates protective mechanisms enhancing resistance to water deficit. It was found that SA improves leaf water status under water deficit conditions (Raskin 1992; Senaratana et al. 2000; Szepsi et al. 2005; Hussain et al. 2009; Bidabadi 2012; Sadeghipour and Aghaei 2012). Some authors reported that foliar application of SA plays a positive regulatory role in stomatal closure and proposed that SA might support the ABA dependent mechanism of stomatal closure. SA application induces production of reactive oxygen species (ROS) via peroxidase-catalyzed reaction, which may lead to the elevation of cytosolic Ca^{2+} and NO production causing stomatal closure (Manthe et al. 1992; Mori et al. 2001; Acharya and Assman 2009). On the other hand, it was shown that exogenous application of SA by seed soaking diminished the reduction of stomatal conductance induced by water deficit (Sadeghipour and Aghaei 2012). Other researchers have revealed that the decrease of stomatal conductance under drought was lower in SA-treated (applied to roots/leaves) than untreated plants (Waseem et al. 2006; Saruhan et al. 2012). The above results are consistent with the findings of Rai et al. (1986), who found that SA application reversed the stomatal closure induced by ABA. However, it appears that the influence of SA on stomatal conductivity may depend among other things on its concentration, the mode of application, duration of treatment, species and physiological state of the plant.

The results of available studies indicate that better leaf hydration under water deficit conditions as a result of SA pre-treatment is not necessarily the effect of stomatal closure. Indeed, in many cases, application of SA did not lead to reduction of stomatal conductance, under water deficit despite a marked influence on improving leaf water status (Singh and Usha 2003; Sadeghipour and Aghaei

2012; Saruhan et al. 2012). Plant resistance to water deficit may be the result of two strategies responsible for surviving the stress: strategy of avoiding dehydration and strategy of tolerance dehydration (Levitt 1980). In the strategy of avoiding dehydration an important role is played by osmotic adjustment involving the accumulation of osmolytes, which lower cell water potential to prevent dehydration (Farooq et al. 2010). The protective action of SA during water deficit in many plants was demonstrated by the accumulation of different osmolytes such as sugars, sugar alcohol and proline, responsible for osmotic adjustment (Szepsi et al. 2005; Umebese et al. 2009; Farooq et al. 2010, Bidabadi et al. 2012). The improvement of leaf water status under water deficit through preventing dehydration of leaves as a result of osmotic adjustment as well the restriction of reduction stomatal conductance by the application of SA plays a positive role in maintaining photosynthetic activity and reducing damage. Closure of the stomatal aperture prevents water loss, but it also limits the uptake of carbon dioxide and influences on the decrease of photosynthetic rate, which exerts a harmful effect on the growth and productivity of plants (Pinheiro and Chaves 2011). Moreover, the limitation of CO_2 assimilation may affect the accumulation of ROS and H_2O_2 because the reductive power (NADPH) developed in the light phase of photosynthesis is not utilized in the phase, independent of light (Jaspers and Kangasjärvi 2010; Miller et al. 2010; Gill and Tuteja 2010). Plant pre-treatment with 0.5 mM SA, one day before water deficit imposition increased sensitivity to drought because it caused a decrease of stomatal conductance and net rate of photosynthesis and increased damage of cell membranes (Németh et al. 2002). Moreover, Borsani et al. (2001) found that SA application enhanced the generation of ROS in leaves of *Arabidopsis thaliana* and increased development of stress symptoms under water deficit conditions. However, SA reduced the damage of cell membranes in cucumber, rice, wheat, barley, *Satureja hortensis*, banana and maize, exposed to water deficit (Bandurska and Stroiński 2005; Baninasab 2010; Farooq et al. 2010; Bidabadi et al. 2012; Saruhan et al. 2012; Yazdanpanah et al. 2011). SA ameliorates the water deficit induced injuries by increasing proline content which protects cell membranes against the harmful effects of ROS (Bandurska and Stroinski 2005; Baninasab 2010). The alleviating effect of SA on cell membrane functioning under water deficit conditions can also be associated with the activation of the synthesis of soluble phenolics and anthocyanins, which protect leaf tissue from oxidative damage (Farooq et al. 2010). The application of exogenous SA may also alleviate the damaging effect of water deficit by up-regulation the activity of antioxidant system. The increased activity of antioxidant enzymes with simultaneous reduction in H_2O_2 production and lipid peroxidation level was noted under water deficit conditions in plants pre-treated with SA, at various concentrations through root, seed soaking or foliar spray (Horváth et al. 2007; Korkmaz et al. 2007; Daneshmand et al. 2009, Farooq et al. 2010; Kadioglu et al. 2011; Bidabadi et al. 2012; Saruhan et al. 2012).

3.4 Role of SA in Improving Plant (Crop) Performance and Yields Under Drought Conditions

SA-induced activation of the mechanisms responsible for reducing the damage caused by water deficit and maintenance of normal metabolism plays an essential role in countering the damaging effects of stress on growth and yield (Fig. 3). In muskmelon, the moderating influence of SA on cell membrane permeability under water deficit conditions resulted in the reduction of adverse effect of water stress on leaf chlorophyll level, stomatal conductivity and photosynthetic productivity (Korkmaz et al. 2007). The ability of SA to negate adverse effects of water deficit on cell metabolism had a significant implication in improving growth of wheat under drought conditions. High chlorophyll content and photosynthetic rate coupled with higher activity of Rubisco were responsible for SA-induced improvement of dry matter accumulation and yield under drought (Singh and Usha 2003). Azooz and Youssel (2010) revealed that SA-induced resistance to drought in 'Hassawi' wheat resulted from its stimulatory effect on the synthesis of soluble carbohydrates and proteins, which are involved in osmotic adjustment, which causes the reduction of adverse effect of water deficit on plants. As a result, there was observed a marked improvement of water status, enhancement of the biosynthesis of photosynthetically active pigments as well as photosynthetic rate and finally stimulation of growth. In other studies conducted on wheat the protective and growth promoting effects of SA treatment were due to its influence on preventing the decrease in IAA and cytokinin content induced by water deficit (Sakhabutdinova et al. 2003). SA treatment elicited drought resistance in wheat through a mechanism of osmotic adjustment and reduction of membrane injury,
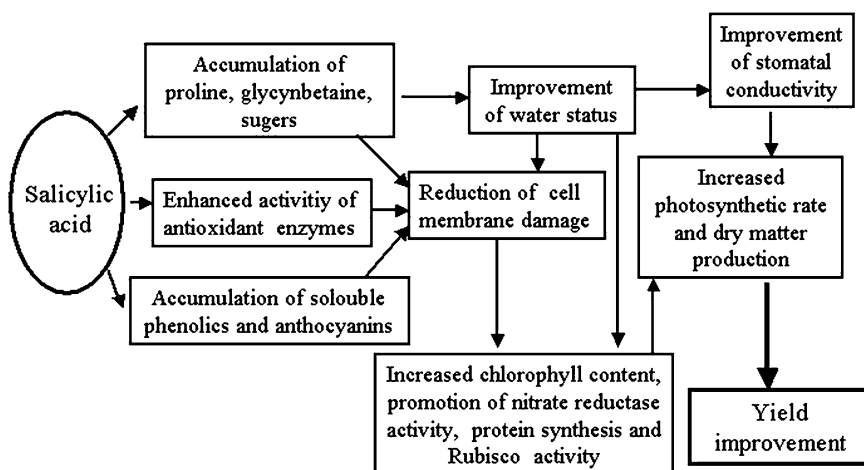
Fig. 3 Schematic model presents the role of SA in the enhancement of plant resistance to water deficit

which improved the yield under stress conditions (Khan et al. 2012). Application of SA enhanced the productivity and yield components of sensitive and tolerant wheat cultivars, grown under water deficit conditions. The effect resulted from the influence of SA on the accumulation of proline, which could act as an osmolyte as well as ROS scavenger (Aldesuqy et al. 2012). SA pre-treatment showed a significant increase on yield and yield components in common bean under water deficit conditions. This impact was associated with the improvement of leaf water status by the activation of osmotic adjustment mechanism, increase of stomatal conductance, and a stimulatory effect on photosynthetic pigment biosynthesis as well as net photosynthetic rate (Sadeghipour et al. 2012). In *Amaranthus* and tomato SA-induced resistance to drought that was caused by increased proline production, which increases the capacity to absorb water from soil and reduces tissue dehydration, enhances nitrate reductase activity and promotes growth of plants under water deficit conditions (Umebese et al. 2009). Exogenous application of SA improved growth and biological yield of sunflower grown under water deficit conditions because of the maintenance of assimilatory surface due to high RWC, which sustained leaf photosynthetic activity (Hussain et al. 2009).

4 Conclusions

From the results presented here it is clear that SA may play a beneficial role in plant response to water deficit stress. Some authors link SA accumulation under water deficit conditions and after pre-treatment with UV-B radiation with amelioration of the damaging effect of water deficit on tissue water status and cell metabolism. Moreover, a body of evidences have revealed that exogenously applied SA effectively protects plants against water deficit induced oxidative stress, membrane injuries, tissue dehydration and metabolic disturbances, which improves plant growth and yield under drought. It seems that SA could be used as a potential growth regulator for improving crop yield under limited soil water availability. The best results were obtained by using a concentration range from 0.5 to 1.0 mM SA and the most convenient method of treatment seems to be pre-sowing seed soaking in the solution of SA. However, further studies are required to fix the most effective SA concentration, the site of application and time of treatment for each crop. In addition, to better understand the specific role of SA in the regulation of plant metabolism under water deficit conditions there are necessary research with mutants producing high levels of SA or mutants with repression of the SA biosynthetic pathway.

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Chapter 2
Salicylic Acid: Physiological Roles in Plants

Mohammad Yusuf, Shamsul Hayat, Mohammed Nasser Alyemeni, Qazi Fariduddin and Aqil Ahmad

Abstract Since ancient times, salicylic acid has been in use by humans because of its therapeutic properties. Salicylic acid, chemically known as 2-hydroxy benzoic acid is one of a diverse group of phenolic compounds, consisting of an aromatic ring bearing a hydroxyl group or its functional derivative, which is synthesized by plants. Salicylic acid biosynthetic pathway in plants has two distinct pathways, the isochorismate (IC) pathway and the phenylalanine ammonia-lyase (PAL) pathway. Moreover, salicylic acid plays exclusive role in plant growth, thermogenesis, flower induction and uptake of ions. It affects ethylene biosynthesis, stomatal movement and also reverses the effects of ABA on leaf abscission. In addition to this, it also enhances the level of photosynthetic pigments, photosynthetic rate and modifies the activity of some of the important enzymes as well. This chapter provides the reader with a comprehensive coverage to above aspects more exclusively with future prospects.

Keywords Growth · Photosynthesis · Salicylic acid · Senescence · Yield

1 Introduction

Since ancient times, plants and their extracts have been used for their therapeutic properties. World Health Organization estimated that approximately 75–80 % of the world's population uses plant medicines either in part or entirely. Ancient

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Egyptians, for example, chewed willow bark to relieve fever and headaches. Thousands of years later, scientists discovered that the bark contains salicylic acid, the active ingredient used to make aspirin. In the year 1928, John Buchner isolated salicyl alcohol glucoside (Salicine) from willow bark that was later name as Salicylic Acid (hereafter SA) by Rafacle Piria in the year 1938.

SA, chemically known as 2-hydroxy benzoic acid is one of a diverse group of phenolic compounds, consisting of an aromatic ring bearing a hydroxyl group or its functional derivative, which is synthesized by plants. Plant phenolics were categorised as secondary metabolites and relatively treated as of low importance in plant metabolism but due to the passage of time this concept changed with the discovery that phenolics play important roles in plant metabolism. For example, phenolics are involved in lignin biosynthesis; others serve as allelopathic compounds, regulate plant responses to abiotic stimuli, or play critical roles in plant disease resistance either by functioning as preformed or inducible antimicrobial defence compounds termed phytoalexins or by signalling defence activation (Humphreys and Chapple 2002; Raskin 1992).

SA influences seed germination, seedling establishment, cell growth, respiration, stomatal closure, senescence-associated gene expression, basal thermotolerance, nodulation in legumes, and fruit yield (Vlot et al. 2009). The reason for some of these processes may be indirect because SA modulates the synthesis and/or signalling of other hormones such as jasmonic acid (hereafter JA), ethylene (hereafter ET), and auxin. It is ubiquitously distributed in the whole plant kingdom (Raskin et al. 1990) and is categorised under group of Plant hormones (Raskin 1992). Here, in this chapter, an effort has been made to insight into the the role of exogenously applied and/or endogenous SA in physiological and biochemical changes that occur in plants under normal conditions.

2 Physiological Responses of SA

Plants have evolved some remarkable chemical substances, often to defend themselves against being eaten. Among various phenolic substances, particularly SA exerts its influences on plant growth and development, photosynthetic machinery, flowering, membrane permeability, and enzyme activities. In this section we will learn about SA mediated physiological processes and try to elucidate the mechanisms behind the action of exogenously applied SA for growth and development.

2.1 Plant Growth and Development

Growth and development of plants, like all organisms, is regulated by various internal external stimuli. In recent years, SA has been in focus of intensive research due to its crucial role in the regulation of physiological and biochemical processes

during the entire life span of the plants and plays key roles in regulating their growth and productivity (Arberg 1981). The role of SA in seed germination has been debatable as there are inconsistent reports suggesting that it can either inhibit germination or increase seed vigour. The reported contradictory effects can be related to the SA concentrations employed. In *Arabidopsis thaliana*, SA concentrations >1 mM delay or even inhibit germination (Rajou et al. 2006). In barley, doses >0.250 mM SA inhibit seed germination (Xie et al. 2007), while in maize germination is completely inhibited by SA doses ranging from 3 to 5 mM (Guan and Scandalios 1995). The effect of SA as a negative regulator of seed germination is probably due to an SA-induced oxidative stress. In *Arabidopsis* plants treated with SA (1–5 mM), hydrogen peroxide (H_2O_2) levels increase up to 3-fold as a result of increased activities of Cu, Zn-superoxide dismutase and inactivation of the H_2O_2-degrading enzymes, catalase and ascorbate peroxidase (Rao et al. 1997). Enhanced germination and seedling growth were recorded in wheat, when the grains were subjected to pre-sowing seed-soaking treatment in SA (Shakirova 2007). Fariduddin et al. (2003) reported that the dry matter accumulation was significantly enhanced in *Brassica juncea*, when lower concentrations of SA were sprayed. However, higher concentrations of SA had an inhibitory effect.

In another study, Hayat et al. (2005) showed that the leaf number, fresh and dry mass per plant of wheat seedlings raised from the grains soaked in lower concentration (10^{-5} M) of SA, increased significantly. Similar growth promoting responses were generated in barley seedlings sprayed with SA (Pancheva et al. 1996). Khodary (2004) observed a significant increase in growth characteristics, pigment contents and photosynthetic rate in maize, sprayed with SA. The exogenous SA application also enhanced the carbohydrate content in maize (Khodary 2004). Hussein et al. (2007) in their pot experiment sprayed salicylic acid to the foliage of wheat plants, irrigated with Mediterranean sea water and reported an enhanced productivity due to an improvement in all growth characteristics including plant height, number and area of green leaves, stem diameter and dry weight of stem, leaves and of the plant as a whole.

In the year 1989, Carswell et al. reported that acetyl SA can promote colony formation in maize protoplasts suggesting a role for SA in the regulation of the cell cycle. Xyloglucan endotransglucosylase/hydrolase (*XTH*) genes encode enzymes that are implicated in cell wall loosening and cell expansion (Rose et al. 2002). Arabidopsis contains 33 *XTH* genes in its genome (Yokoyama and Nishitani 2001). Among 33 *XTH* genes, expression levels of *XTH*8, *XTH*17 and *XTH*31 were strongly down-regulated in both *cpr*5 and *mpk*4 but did not change in *nahG* (Miura et al. 2010). Reverse transcription–PCR (RT–PCR) results indicated that *XTH*8 and *XTH*31, but not*XTH*17, were down-regulated in *siz*1 and expression of *XTH*8and *XTH*31 was recovered in *nahGsiz*1-2 (Miura et al. 2010). Moreover, expression level of *XTH*24 (MERI5), a potential target for ANGUSTIFOLIA (*AN*), which regulates the width of leaves (Kim et al. 2002) and may also play a role in leafmorphogenesis at the early stage (Verica and Medford 1997). Thus, *SIZ*1 regulates SA-dependent *XTH*8 and *XTH*31 expression, but may not be involved in AN-dependent regulation of cell elongation (Miura et al. 2010).

2.2 Photosynthetic Machinery

SA has been established as an important regulator of photosynthesis, water relations and metabolic aspects of plants, depending on its analogues, concentrations, mode of application and plant type. SA is known to affect leaf and chloroplast structure (Uzunova and Popova 2000), stomatal closure (Mateo et al. 2004; Melotto et al. 2006), chlorophyll and carotenoid contents (Chandra and Bhatt 1998; Fariduddin et al. 2003), and the activity of enzymes such as Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and carbonic anhydrase (Slaymaker et al. 2002; Hayat et al. 2012; Yusuf et al. 2008, 2012). However, high SA concentrations (1–5 mM) cause a reduction in the photosynthetic rate and Rubisco activity in barley plants (Pancheva et al. 1996), and reduce chlorophyll contents in cowpea, wheat, and *Arabidopsis* (Rao et al. 1997; Chandra and Bhatt 1998; Moharekar et al. 2003). The decline of Rubisco activity was attributed to a 50 % reduction in protein levels, compared with non-treated plants (Pancheva and Popova 1998), while total soluble protein decreased by 68 %. Exogenously applied SA induces alterations in leaf anatomy that consist of reduced width of the adaxial and abaxial epidermis, and of the mesophyll tissue (Rivas-San and Piasencia 2011). Such changes correlate ultrastructurally with an increase in chloroplast volume, swelling of grana thylakoids, and coagulation of the stroma (Uzunova and Popova 2000). Thus, the diminished photosynthetic activity at high concentrations of SA is due to its effects on the thylakoid membranes and light-induced reactions linked to them. Hayat et al. (2005) reported that the pigment content was significantly enhanced in wheat seedlings, raised from the grains pre-treated with lower concentration (10^{-5} M) of SA, whereas, higher concentrations did not prove to be beneficial. Besides seed-soaking treatment, the foliar application of SA also proved to be equally fruitful in increasing the pigment contents in *Brassica napus* (Ghai et al. 2002). Similar results were obtained when the plants of *Brassica juncea* were sprayed with lower concentration (10^{-5} M) of SA, where, the chlorophyll content was significantly enhanced (Fariduddin et al. 2003). Contrary to these observations, a reduction in chlorophyll content was observed in plants pre-treated with SA (Anandhi and Ramanujam 1997; Pancheva et al. 1996). Moharekar et al. (2003) reported that salicylic acid activated the synthesis of carotenoids and xanthophylls and also enhanced the rate of de-epoxidation with a concomitant decrease in chlorophyll pigments and chlorophyll a/b ratio in wheat and moong. Exogenous application of SA enhanced the net photosynthetic rate, internal CO_2 concentration, water use efficiency, stomatal conductance and transpiration rate in *Brassica juncea* (Fariduddin et al. 2003). Further, Khan et al. (2003) reported an increase in transpiration rate and stomatal conductance in response to foliar application of SA and other salicylates in corn and soybean. In another study carried out on soybean, foliar application of salicylic acid enhanced the water use efficiency, transpiration rate and internal CO_2 concentration (Kumar et al. 2000).

However, contrary to these results, the transpiration rate decreased significantly in *Phaseolus vulgaris* and *Commelina communis* after the foliar application of SA and this decrease in transpiration rate was attributed to SA induced closure of stomata (Larque-Saavedra 1978, 1979; Khokon et al. 2010). Moreover, SA pretreatment alleviated the loss of net photosynthetic rate under heat stress, apparently in part through maintaining a higher Rubisco activation state and greater PSII efficiency (Wang et al. 2010). SA also accelerated the increase of net photosynthetic rate mainly through the more rapid recovery of PSII function after heat stress and may be related to higher levels of HSP21 (Wang et al. 2010). Other mechanisms by which SA mediated protection of photosynthetic machinery are still to be determined.

2.3 Nitrate Metabolism

All the living organisms are basically composed of carbon, hydrogen, oxygen, nitrogen and minor quantities of other elements. These elements contribute to finally organize various biomolecules of the cell. Nitrogen is next to carbon in importance to living organisms. In a living cell, nitrogen is an important constituent of amino acids, proteins, enzymes, vitamins, alkaloids and some growth hormones. Therefore, study of nitrogen metabolism is absolutely essential because the entire life process is dependent on these nitrogen-containing molecules. In this section, we will learn about various effects of SA on nitrogen metabolism including nitrogen fixation in plants. Nitrogen metabolism is an important aspect of legume-Rhizobium symbiosis. The exogenous SA affects the activities of the enzymes of nitrate/nitrogen metabolism as well. The activity of enzyme nitrate reductase (NR) was enhanced in the leaves of wheat following the exogenous application of SA. The treatment also protected the enzyme from the action of proteinases and trypsin (Rane et al. 1995). The total protein content was increased in soybean plants sprayed with SA and this increase might be due to enhanced activity of NR following the SA treatment (Kumar et al. 1999). A significant increase in the activity of nitrate reductase was observed both in roots and leaves of the plants raised from the wheat grains soaked in lower concentration (10^{-5} M) of SA (Hayat et al. 2005). Such a lower concentration of SA when sprayed to the foliage of mustard plants enhanced their NR activity (Fariduddin et al. 2003). However, at higher concentrations (10^{-3} or 10^{-4} M), SA proved to be inhibitory. Mabood and Smith (2007) showed that exogenous SA inhibited the growth of Rhizobia and production of *nod* factors by them and also delayed the nodule formation, thereby decreasing the number of nodules per plant. However, SA level in the roots of *Medicago sativa*, inoculated with specific strain of *Rhizobia*, either decreased or remained close to the basal levels (Martinez-Abarca et al. 1998). Moreover, *Medicago sativa* plants when inoculated with an incompatible strain of *Rhizobia*, resulted in a marked accumulation of SA in the roots of host plant. It was

therefore, concluded that the compatible strains of *Rhizobia* produce certain signals (specific nod factors) which are perceived by the host plant that suppress the accumulation of SA in the roots (Martinez-Abarca et al. 1998).

Shah et al. (2001) reported that certain *Arabidopsis thaliana* mutants produce elevated levels of SA and show constitutive expression of pathogenesis-related genes and in some cases HR lesion formation even in the absence of pathogen challenge. On the other hand, plants that express the bacterial *nahG* gene, encoding salicylate hydroxylase, are unable to accumulate SA and are more susceptible to several pathogens (Gaffney et al. 1993). SA levels can also affect the interaction of plants with symbiotic microorganisms. Medina et al. (2003) found that *Nicotiana tabacum* plants expressing *NahG* had enhanced mycorrhizal fungal infection, while plants constitutive for SA expression exhibited reduced infection.

Martinez-Abarca et al. (1998) showed SA accumulated in alfalfa roots, inoculated with a *nodC* mutant of *Sinorhizobium meliloti* that was unable to synthesize the lipochitin nod signal required for infection. This report also showed that exogenous addition of SA resulted in both reduced and delayed nodule formation on alfalfa roots inoculated with wild-type *Sinorhizobium meliloti*. Subsequently, Bueno et al. (2001) showed a decrease in antioxidant enzyme activities and an increase in H_2O_2 accumulation in alfalfa roots following inoculation with a *Sinorhizobium meliloti nodC* mutant, as well as an increase in lipoxygenase activity after inoculation with the wild-type strain.

Van Spronsen et al. (2003) reported that SA application inhibited indeterminate nodulation of *Vicia sativa* but not determinate nodulation of *Lotus japonicus*. They believed that fatty acid may be active in oxylipin signaling, which is known to be inhibited by SA. Moreover, Rhizobia that form determinate nodules produce *nod* signals lacking poly-unsaturated fatty acids and thus, these signals may act in a different way. However, the theory of van Spronsen et al. (2003) is inconsistent with two reports showing that the addition of exogenous SA to *Glycine max* seedlings inhibited early nodulation (Lian et al. 2000; Sato et al. 2002). *G. max* forms determinate nodules.

2.4 Ethylene Production

The plant hormone, ethylene has been reported to affect a diverse array of plant growth and developmental processes including germination, senescence and abscission of flowers and leaves, fruit ripening as well as the response to a wide variety of stresses such as pathogen attack and drought (Abeles et al. 1992). The induction of ethylene biosynthesis takes place by a wide variety of stimuli, including wounding, pathogen attack, various stresses, mechanical stimulus and by hormones such as salicylic acid (Mattoo and Suttle 1991; Abeles et al. 1992; Raskin 1992). Recently, the survey of literature revealed that SA has been shown to interfere with the biosynthesis and/or action of ethylene in plants (Raskin et al. 1990). SA prevented the accumulation of ACC synthase transcripts induced by

wounding (Li et al. 1992) and inhibited ethylene synthesis in pear suspension cultures by blocking ACC oxidase (Szalai et al. 2000). SA can delay the ripening of banana fruit, probably through the inhibition of ethylene biosynthesis or its action (Srivastava and Dwivedi 2000). Both SA and its derivative acetyl salicylic acid (ASA) have been shown to inhibit ethylene production in cultured pear cells (Leslie and Romani 1986, 1988), mung bean hypocotyls, apple and pear fruit tissue discs (Romani et al. 1989), and carrot cell suspension cultures (Roustan et al. 1990). Fan et al. (1996) demonstrated the inhibitory action of SA on ACC oxidase activity in apple fruit discs. SA has also been shown to suppress lipoxy-genase (LOX) activity in discs of kiwi fruit, with a consequent reduction in the production of free radicals and ethylene biosynthesis (Xu et al. 2000). There is evidence for a positive correlation between LOX activity and ethylene biosynthesis in apple fruit tissue (Marcelle 1991), and free radicals produced by LOX activity have been shown to play a role in regulating the biosynthesis of ethylene (Kacperska and Kubacka-Zebalska 1985, 1989), and in post-harvest ripening and softening of climacteric fruits such as apple (DePooter and Schamp 1989) and tomato (Todd et al. 1990). Moreover, an increase in endogenous ethylene biosynthesis at low concentrations of SA has been reported in suspension cultures of carrot (Nissen 1994) whereas, Srivastava and Dwivedi (2000) observed an inhibition of ethylene production at higher concentration ($>10^{-4}$ M) of salicylic acid. However, it still remains unclear; the mechanism behind the action of SA mediated ethylene biosynthesis. Therefore, much debate is necessary to elucidate and pin point the mechanism associated with SA for ethylene biosynthesis and/or action.

2.5 Mineral Nutrients

Mineral nutrients are essential for growth and development of plants and microorganisms, as they are important factors in the regulation of various physiological and biochemical processes. How each element affects a plant's physiological and biochemical processes, (positively or negatively), is unique to each plant. This section briefly summarizes how different nutrients affect different plant's physiological processes in the presence of exogenous as well as endogenous salicylic acid. SA has an essential function in regulating plant developmental processes that affect nutrient uptake and their status; i.e. vascular differentiation, stem elongation, leaf development, and senescence (Rubio et al. 2009). However, a clear involvement of SA in the control of nutrient assimilation might be expected. Moreover, SA contributes in the control of redox status of plants, most likely by regulating the synthesis of the antioxidant glutathione, which protects plant against oxidative stress that follows many nutritional deficiencies (Freeman et al. 2005; Shao et al. 2007). Although these expectations, till now have no clear cut experimental evidence points to establish a relationship between SA signaling and the control of nutrient homeostasis. However comparisons among genes that respond to N-, K-, or S-limiting growth conditions with those altered by SA treatment

revealed a significant (positive or negative) correlation either for up or down regulated genes. The uptake of phosphate (Glass 1973) and subsequently that of potassium (Glass 1974) by barley roots was reduced by SA. However, the inhibition of the absorption of potassium by oat roots, under the impact of SA, was dependent on the pH and the concentration of the element in the medium. This inhibition was more prominent at lower pH, suggesting higher activity of protonated form of salicylic acid (Harper and Balke 1981; Gordon et al. 2002). SA also caused the collapse of the transmembrane electrochemical potential of mitochondria and the ATP dependent proton gradient of tonoplast enriched vesicles (Macri et al. 1986).

2.6 Heat Production

Heat production, thermogenesis authenticates the discovery of SA as an endogenous plant hormone. Initial findings proved that SA triggers a dramatic increase in the production of metabolic heat and insect-attracting chemicals in the thermogenic inflorescence of *Arum lilies* (Raskin 1992) and possibly other plants also (Raskin et al. 1990). In *Sauromatum guttatum* Schott (voodoo lily), a 100-fold increase in SA precedes with the onset of thermogenesis in the spadix (Vlot et al. 2009). The induction of thermogenesis by SA is very specific: of 33 SA analogues tested, only 2, 6-dihydroxybenzoic acid and aspirin induce this response. SA stimulates thermogenesis primarily by increasing the activity of alternative respiratory pathway in mitochondria. Unlike the cytochrome respiratory pathway, electron flow through the alternative respiratory pathway generates ATP at only one site with the unused potential energy being released as heat (Vlot et al. 2009). Remarkably, SA treatment also induces alternative oxidase expression and increased alternative respiration in tobacco, a nonthermogenic plant (Norman et al. 2004). Exogenously applied SA treatment improved thermotolerance and heat acclimation in mustard seedlings (Dat et al. 1998). A similar response was also observed in potato plantlets, raised from the cultures, supplemented with lower concentrations of acetyl salicylic acid (Lopez-Delgado et al. 1998). Larkindale and Huang (2004) pointed out that the enhanced heat tolerance in plants of *Agrostis stolonifera*, pre-treated with salicylic acid was due to the protection of plants from oxidative damage. These authors further reported that the pre-treatment with salicylic acid had no effect on POX activity, whereas, the CAT activity declined, compared to control. Foliar spray of lower concentrations of salicylic acid conferred heat tolerance to mustard. Further this treatment accompanied with hardening at 45 °C for 1 h enhanced H_2O_2 level and also reduced CAT activity, thereby increasing the potential of plants to withstand the heat stress (Dat et al. 1998).

2.7 Flowering

Plant reproduction relies on the successful flowering at the required season and developmental stage. Studies in *Arabidopsis thaliana* revealed that endogenous factors that affect flowering involved autonomous gibberellin pathways (Blazquez et al. 1998); Koornneef et al. 1998; Wilson et al. 1992). As a result, mechanisms have been evolved that integrate environmental signals with endogenous developmental signals to regulate flowering time (Simpson and Dean 2002). The possibility of SA being an endogenous plant signal was first raised by Cleland and co-workers (Raskin 1992). Moreover, the very first physiological symptom, ever accredited to SA in plants, was its impact on flower induction in tobacco tissue culture, supplemented with kinetin and indole acetic acid (Lee and Skoog 1965; Eberhard et al. 1989). In addition to this, analyzing different fractions of honeydew collected from aphids feeding on vegetative or flowering *Xanthium strumarium*, they identified SA as a phloem mobile activity capable of inducing flowering in *Lemna gibba* (Vlot et al. 2009). The study of Khurana and Cleland (1992) revealed that *Lemna paucicostata* LP6 does not normally flower when grown on basal Bonner-Devirian medium, but substantial flowering is obtained when 10 μM salicylic acid (SA) or benzoic acid is added to the medium. Moreover, Wada et al. (2010) showed that poor-nutrition stress induced flowering was inhibited by amino-oxy acetic acid, a phenylalanine ammonia lyase inhibitor, and this inhibition was almost completely reversed by salicylic acid (SA). However, exogenously applied SA did not induce flowering under non-stress conditions, suggesting that SA may be necessary but not sufficient to induce flowering. Different plant species including ornamental plant *Sinningia speciosa* flowered much earlier as compared to the untreated control, on receiving an exogenous foliar spray of salicylic acid (Martin-Mex et al. 2003, 2005b). Promising results were obtained when plants of *Carica papaya* were treated with salicylic acid which showed a significantly higher fruit set (Herrera-Tuz 2004; Martin-Mex et al. 2005a). In cucumber and tomato, the fruit yield enhanced significantly when the plants were sprayed with lower concentrations of salicylic acid (Larque-Saavedra and Martin-Mex 2007). Moreover, Alaey et al. (2011) reported that SA has the ability to increase the vase-life of cut rose flowers and delay senescence by regulating plant water content and increasing the scavenging capacity of cells. However, the recent demonstrations revealed that (a) SA-deficient Arabidopsis failed to initiate flowering in response to UV-C irradiation and flowered substantially later than wild-type (wt) plants when grown under non-stress conditions and (b) SIZ1, a SUMO E3 ligase, negatively regulates flowering via SA-dependent pathway argue that SA plays some role in this process.

2.8 Senescence

Plant senescence is a phenomenon that resembles age of the plant that closely connects with cell death. It is developmentally well defined that optimizes the growth and reproductive capacity of plants by recycling of resources from senescing leaves into young leaves or seeds. After well documented the importance of role of SA in photosynthesis and flowering, it is not unanticipated that this plant hormone is also involved in regulation of senescence. This process is characterized by yellowing of leaves due to chlorophyll degradation (Vogelmann et al. 2012) and increased ROS levels (Rivas-San and Plasencia 2011). It is believed that these events are due to SA accumulation. In *Arabidopsis* senescent leaves, SA levels increase 4-fold at the mid-senescent stage. Consistent with this observation, Arabidopsis plants affected in SA biosynthesis, such as the transgenic NahG and the mutant pad4, or with a disrupted SA signalling pathway, such as npr1, exhibit altered senescence patterns that include delayed yellowing and reduced necrosis compared with wild-type plants (Morris et al. 2000). Moreover, senescence is escorted by important changes in gene expression, and SA paly pivotal role in successful execution of this process. Transcripts of several SAGs, such as *SAG*12, are considerably reduced or undetectable in SA-deficient *Arabidopsis* plants (Morris et al. 2000). In addition this, SA activates the expression of the *Arabidopsis* senescence-related genes *αVPE, χVPE, WRKY*6, *WRKY*53, and *SEN*1 that encode two vacuolar processing enzymes, two transcription factors, and a protease, respectively (Robatzek and Somssich 2001; Miao et al. 2004; Schenk et al. 2005). The involvement of the SA signalling pathway in senescence was confirmed through a detailed microarray analysis in *Arabidopsis* senescent leaves (Buchanan-Wollaston et al. 2005). Almost 20 % of the up-regulated genes during senescence show at least 2-fold reduced expression in SA-deficient NahG transgenic plants. Most of the senescence enhanced genes that are dependent on the SA pathway encode kinases, transferases, and hydrolases, but their function in senescence progression remains to be elucidated. Although a great deal of effort has been put into identifying the signalling factors required for senescence regulation, further research must determine whether SA is involved in different stages of senescence, and the interconnecting networks with other phytohormones that promote (ABA, JA, an ET) or delay (CKs and GAs) senescence.

3 Effect of SA on Yield

The credibility on any exogenously sourced plant hormones evaluate in terms of biological yield. SA is known to be a natural signal molecule has been shown to play an important role in regulating various physiological processes in plants including yield. Yildirim and Dursan (2009) revealed that foliar application of SA showed positive effect on early yield and total yield and also proposed that highest

yield occurred in 0.50 mM SA treatment and also recommended in order to improve yield. Sharafizad et al. (2012) showed that highest grain yield was obtained with application of 0.07 mmol SA. It is believed that increasing the crop yield might be due to delayed senescence of plant organs (particularly leaves and flowers) in response to exogenous SA (Imran et al. 2007) that will automatically help the plant in extending the duration of photosynthetically active sites and also prevent the premature loss of flowers and fruits. This consequently resulted in the observed increase in the number of crop yield. Moreover, Marschner (2003) that phytohormones increase the degree of sink at the level of seeds, directing the flow of metabolites to the developing seeds consequent to an improvement in the seed mass and seed yield per plant at harvest.

4 Conclusions

Much has been debated during the last decades regarding the applicability of SA as plant hormone by exploring its morpho-physiological responses under exogenous application. This review article showed that much progress has been achieved in the biosynthesis and metabolism of SA, whereas it is the need of time to focus to identify and characterize SA biosynthetic with involved enzymes and also isolate their genes. Better understanding of SA biosynthesis and metabolism may improve the plant resistance to pathogens, in the future by providing the tools necessary to manipulate endogenous as well as exogenous levels of SA. Moreover, biggest concern regarding SA mediated response is that how SA triggers such responses effectively and exactly and the mechanism related to it. In addition to this, it is also necessary to reveal how SA negatively and positively interacts with several other plant hormones and signaling molecules that not only affect defense but also regulate developmental processes. An ongoing challenge is to unravel how these interactions affect different processes that are occurring in parallel.

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Chapter 3
Salicylic Acid and Phospholipid Signaling

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Abstract Salicylic acid (SA) signaling has been associated with phospholipids and the enzymes that metabolize them. However, despite studies conducted by other research groups, the role of SA signaling via phospholipids in plant responses to phytoregulators is not yet fully understood. The signal transduction pathway involves the generation of secondary messengers, through the enzymes such as phospholipase C (PLC) and phospholipase D (PLD). The signaling pathway of SA was evaluated in different models of plants, where it was observed that this compound regulates enzymatic activities to generate a rapid cellular response. In this chapter, we review the important aspects of the relationship of the SA effects with phospholipid signal transduction and cellular responses to this component.

Keywords Salicylic acid · Phospholipases · Phytoregulators · Signal transduction

1 Introduction

Salicylic acid (SA) is an important endogenous signaling molecule in plant defense, which occurs in response to various types of biotic and abiotic stress (Malamy and Klessing 1992; Janda et al. 1999). Studies have shown that SA is a regulator of several plant physiological processes and is essential in the expression of some defense genes (Raskin et al. 1990). Phytoregulators, such as SA and methyl jasmonate (MeJA), are also used as elicitors for the production of some secondary metabolites in different plant species. The term elicitor means a biotic or

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abiotic agent that can trigger a physiological and morphological process in plants (Zhao et al. 2005).

The use of elicitors may facilitate the study of secondary metabolite biosynthesis in cell suspensions and cell lines that produce only trace amounts of these compounds. An elicitor can increase the amount of metabolites through the regulation of the enzymes involved in their biosynthesis (Ramani and Chellilah 2007; Nieto-Pelayo 2006; Babar Ali et al. 2007; DiCosmo and Misawa 1985). The mode of action of the elicitors has been studied, and the results indicate that the elicitors interact with receptors on the plant plasma membrane and thus trigger signal transduction mechanisms to promote plant defense. These compounds will lead to the activation of genes that code for enzymes involved in the biosynthesis of secondary metabolites (Ramani and Chellilah 2007). As part of the response of the plant to the phytoregulator signaling, some signal transduction mechanisms are linked to phospholipids. In the transduction of signals that are generated through the phospholipids, a key class of enzymes involved are phospholipases (phospholipase A_2, C and D, Fig. 1), which catalyze the hydrolysis of phospholipids of the plasma membrane to generate secondary messengers. The activation of phospholipase A_2, C and D has been associated with various signaling processes in plants, such as hormonal and stress responses (Chapman 1998; Munnik et al. 1998; Wang 2001).

Currently, there is interest in studying the correlation between the signal transduction pathways and secondary metabolism in different plant models. In this context, there are a few reports of signaling processes in relation to SA induction in secondary metabolism. Our group is interested in studying this relationship using in vitro cultures of cell suspensions of *Capsicum chinense* Jacq., which is a crop with high commercial potential that generates high economic value metabolites, such as the capsaicinoids.
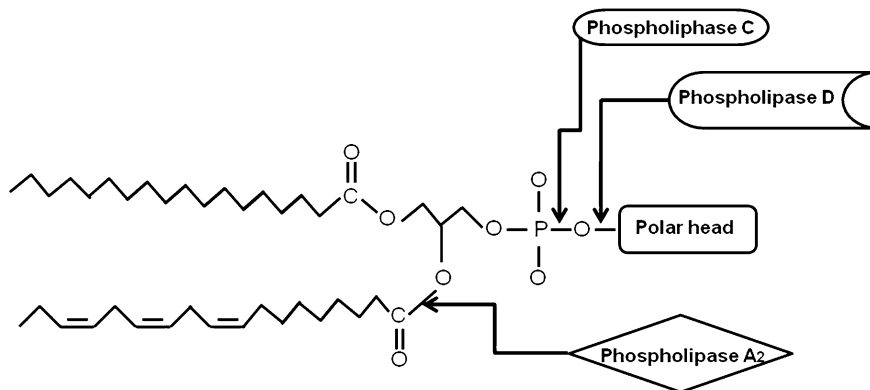

Fig. 1 The site of phospholipid hydrolysis by phospholipase enzymes

2 Salicylic Acid

Salicylic acid, or 2-hydroxybenzoic acid, is a derivative of benzoic acid. This molecule belongs to the group of phenolic compounds that are defined as substances having an aromatic ring and a hydroxyl group or a functional derivative. SA is found in various plant species to regulate biological processes, such as thermogenesis, flowering or defense against pathogens (Nawrath et al. 2005). SA is usually found in plants at basal amounts ranging between 1 and 50 μM, and its concentration increases when the cells, organs or plants are under stress conditions (Chen et al. 1997; Vlot et al. 2009). SA is also a key molecule in various signal transduction pathways in plants. An important signaling mechanism that involves SA is the regulation of mitogen-activated protein kinases (MAPK). In 1997, Zhang and Klessing identified a 48 kDa kinase, which is activated transiently by SA in cell suspensions of tobacum. According to sequence analysis, this enzyme was a new member of the MAPK family and could be related to the defense system against the tobacco mosaic virus. Other studies also indicate that SA is involved in signaling caused by abiotic stress (Horváth et al. 2007), which can be produced by toxic metals such as copper in cucumber (*Cucumis sativus* L.) and tobacco (*Nicotiana tabacum* L.) cultures (Strobel and Kuc 1995), heat stress in mustard plants (*Lepidium campestre*) (Dat et al. 1998) and in response to oxidative damage (Strobel and Kuc 1995).

3 Signal Transduction System

The process by which cells detect external signals and then transmit them into the cell to activate responses is known as signal transduction. These molecular circuits detect, amplify and integrate diverse external signals to generate responses, such as changes in enzyme activity, gene expression or ion channel activity (Hong et al. 2010). Likewise, plant cells have a sensitive perception system for a variety of external signals that are generated by biochemical, physiological and molecular responses and are considered to promote cell survival as a result of stimulus perception and signal transduction within the plant cells (Munnik and Verneer 2010). In plants, there is little information available about how SA mediates the formation of secondary messengers. However, it has been found that some components of phospholipid-mediated signaling are modified in the presence of plant growth regulators, such as SA and MeJA (Altúzar-Molina et al. 2011). In the following sections, we describe some important aspects of signaling via phospholipids to understand the function or role of the components involved in this signaling cascade and how they are possibly involved in the regulatory processes that are activated by SA.

3.1 Phospholipid Signal

Phospholipids provide the structural basis of the cell membrane, but they can also be cell regulators (Xue and Chen 2009). The hydrolysis of phospholipids by phospholipases is often the first step in the generation of messengers. Additionally, the phospholipid transduction pathway involves the generation of secondary messengers from the hydrolysis or changes in the phosphorylation state of phospholipids present in the plasma membrane (Xue and Chen 2009). The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the cell membrane by the action of phospholipase C (PLC) results in the production of two important secondary messengers: the cytosolic inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG), which remains associated with the plasma membrane (Fig. 2) (Xue and Chen 2009). The compound IP_3 is soluble in water therefore diffuses quickly throughout the cytosol, where it can be phosphorylated by inositide kinases to form inositol hexaphosphate (IP_6). The main function of IP_3/IP_6 is to mobilize Ca^{2+} from the intracellular stores. As a result of this mobilization, a transient increase in cytosolic Ca^{2+} occurs that can mediate a cellular response indirectly through binding to calmodulin as well as other calcium-binding proteins, and activation or directly through binding to specific enzymes, such as calcium-dependent kinases (CDPK) or other proteins that bind Ca^{2+}. DAG, in turn, is an insoluble lipid and remains in the membrane (Gross et al. 1992) and can activate a class of proteins called protein kinase C (PKC) (Legendre et al. 1993). However, in plants, PKC has not been identified with any member of this family of kinases. There is evidence that DAG quickly becomes phosphorylated by DAG kinase (DGK) to produce phosphatidic acid (PA) (Munnik and Testerink 2008). Thus, two signaling pathways are initiated by PLC: the elevation of cytosolic Ca^{2+}, which results in the modulation of response elements sensitive to Ca^{2+}, and the effect of PA and other lipids on the activity, relocation or binding of different proteins (Xue and Chen 2009; Munnik and Testerink 2008). It should be noted that PA could also be formed by the hydrolysis of membrane lipids, mainly phosphatidylcholine (PC), by the action of phospholipase D (PLD) (Fig. 2) (Munnik and Testerink 2008).

Studies of physiological function involving the enzyme PLD have revealed that it can be rapidly activated. The stimuli that regulate PLD include hormones, growth factors, cytokinins, extracellular matrix components, antigens and stimuli, such as freezing, wounds, plant–pathogen interactions, dehydration and salt stress (Wang 2002; Bargmann and Munnik 2006). The secondary messengers generated from these stimuli are important in the plant cell responses to environmental changes or extreme conditions (Xue and Chen 2009). The activation of PLC and PLD phospholipases has been linked to several signaling processes in plants, such as hormonal and stress responses (Chapman 1998; Munnik et al. 1998; Wang 2001).
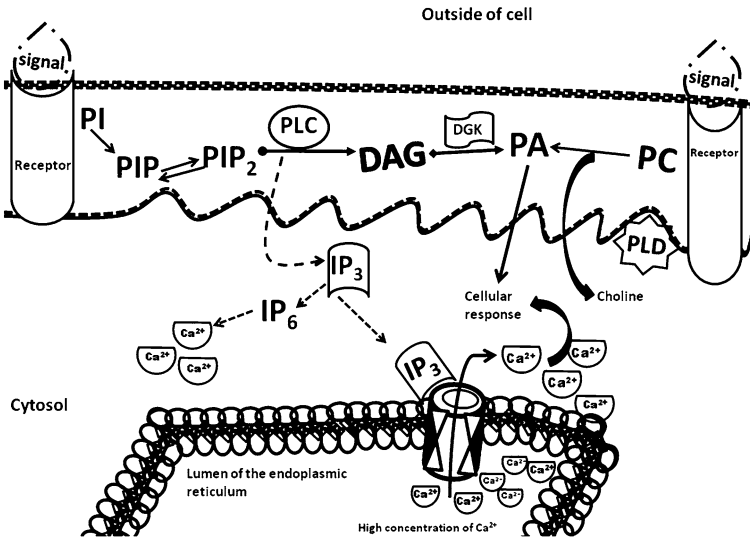
Fig. 2 The phospholipid signaling pathway in plants. *PLC* phospholipase C, *PLD* phospholipase D, *PIP₂* phosphatidylinositol 4,5-bisphosphate, *DAG* diacylglycerol, *IP₃* inositol 1,4,5-triphosphate, *InsP₆* inositol hexaphosphate, *PA* phosphatidic acid, *DGPP* diacylglycerol pyrophosphate, *PIP* phosphatidylinositol 4-phosphate, *Ca²⁺* calcium, *DAGK* diacylglycerol kinase, *PC* phosphatidylcholine

3.2 Salicylic Acid and Phospholipid Signaling Cascade

SA signaling has been linked to phospholipids and the enzymes involved in their metabolism. However, despite studies of the signaling in response to plant growth regulators, there is little information about the relationship of SA with phospholipid signaling.

The involvement of PLC and PLD in the response to SA has been evaluated in other genera, such as the *Brassica napus* plant, where the behavior of these enzymes was investigated in response to treatment with various inducers of systemic resistance. The authors demonstrated that both PLC and PLD (isoforms, and) were activated rapidly within the first 6 h of treatment with SA. They concluded that these enzymes have differential regulation of their enzymatic activities, thus generating a fast cellular response to SA (Profotová et al. 2006). In a study by Krinke et al. (2007) the treatment of cell suspensions of *Arabidopsis thaliana* labeled with orthophosphate (^{32}Pi) with SA was accompanied by increased levels of PIP and PIP₂.

In other studies, it has been used as a strategy to establish the contribution of phospholipid enzymes to the cellular responses of several substances that have inhibitory effect (Toyoda et al. 2000). Pharmacological studies using the inhibitors neomycin and U73122 (PLC inhibitors) in models of plants, such as alfalfa, rice, seeds, peas and carrot cell suspensions (Yamaguchi et al. 2005; Legendre et al. 1993;

Staxen et al. 1999), have established the role of PLC in the cellular response. The identification of the in vivo activity of PLD in various cellular responses can be evaluated by the ability of this enzyme to use primary alcohols, such as n-butanol, instead of water in a reaction known as transphosphatidylation (Chen et al. 2011). In this reaction, PLD transfers the phosphatidyl group to primary alcohols, resulting in the formation of phosphatidylbutanol (Pbut). This lipid is not normally present in cells, but is easy to synthesize in vivo when the cells are preincubated with low concentrations (0.1–0.5 %) of 1-butanol (Munnik et al. 1998; Chen et al. 2011). The involvement of PLD in various plant cellular processes has been evaluated using a strategy similar to the one conducted by Krinke et al. (2009). The SA signaling pathway was evaluated through the activation of PLD by Pbut formation in cell suspensions of *A. thaliana*. The SA activation of PLD was dose-dependent, but none of the existing PLD isoenzymes in the plant were significantly induced by SA. These results suggest that the PLD isoenzyme involved in the SA response can be activated at the protein level by an increase in translation or by the activation of existing protein.

Liu et al. (2006) conducted a study of the relationship of SA and PIP_2-PLC in response to heat stress in *Pisum sativum*. The results obtained in this study indicate that SA levels were significantly increased at the start of acclimation to heat stress and that there was also an increase in PLC activity and protein level, which can be explained due to post-translational modification. Furthermore, in experiments of PLC inhibition by neomycin, a detectable loss of heat acclimation-induced thermotolerance was observed. All of this research indicates that there is a relationship between SA and the enzymes of phospholipid signaling system, which may be a plant pathway response.

As mentioned previously, various studies have yielded evidence of the possible interaction between the phytoregulators and the enzymes involved in phospholipid signaling in various plant models. However, little is known about the effect of SA on the activity of these enzymes in vitro cultures and cell suspensions. Our research group is interested in the signal transduction pathway that is mediated by phospholipids and SA in cell suspensions of *Capsicum chinense*, which is a model that has been used to evaluate the effect of SA in secondary metabolism.

In a preview study in 2006, Canché-Chay evaluated the in vitro enzymatic activity of PLC in two days of a culture cycle (6 and 14) in cell suspensions of *C. chinense* treated with SA (250 μM). The results indicated that the PLC activity was stimulated by SA at 72 and 48 h after adding the plant regulator to the cell suspensions on both days of the culture cycle. Altúzar-Molina (2008) investigated the effect of SA on the in vitro enzymatic activities of PLC and PLD in cell suspensions of *C. chinense* treated with 200 μM of SA. The results indicate that SA inhibits the activity of both phospholipases 30 min after treatment; whereas, the cells treated with different concentrations of SA had a variable response that depended on the concentration. These results suggest that SA affects the phospholipid signaling in cell suspensions of *C. chinense*, providing evidence that implicates this signaling pathway in response to SA (Altúzar-Molina 2008). The results obtained in the work described above indicates that there is most likely a

relationship between the key enzymes of phospholipid signaling to the presence of SA in vitro cell suspensions of *C. chinense*.

Another important function of SA in plants is as an enhancer the production of secondary metabolites. Studies have indicated that the exogenous addition of this molecule enhances the production of alkaloids (Idrees et al. 2010; Pitta-Alvarez et al. 2000), anthraquinone (Bulgakov et al. 2002), glucosinolates (Kiddle et al. 1994) and capsaicinoids (Gutiérrez-Carbajal et al. 2011; Sudha and Ravishankar et al. 2003). Recently, other studies indicate that the SA signaling pathway is involved in the biosynthesis of terpenoids, including triterpenoids (Shabani et al. 2009), diterpenoids (Wang et al. 2007) and sesquiterpenoids (Aftab et al. 2010). Some authors report that the addition of exogenous SA stimulates the endogenous biosynthesis of secondary metabolites in plants (Zhou and Zhon 2011; Profotová et al. 2006) and therefore activates signaling pathways that regulate genes coding for enzymes involved in secondary metabolite biosynthesis (Ramani and Chellilah 2007; Zhao et al. 2005). In many studies, the flow of calcium and transient changes of Ca^{2+} into the cytosol have exposed that events are necessary for the activation of defense genes related to secondary metabolism (Zhao et al. 2005; Vasconsuelo et al. 2003).

It is therefore of interest to investigate the signaling processes that follow the perception of the phytoregulator in plant cells during the synthesis and transport of secondary metabolites. In this context, studies suggest that the products of the phospholipid signaling cascade could be involved as secondary messengers during the stimulation of secondary metabolism in plants (Yamaguchi et al. 2003; Munnik and Nielsen 2011). Yamaguchi et al. (2004) suggest that phytoalexins generated by the elicitor in rice cell suspensions is due to the participation of the enzyme PLD because the use of 1-butanol slightly suppresses the accumulation of the metabolite momilactone A. It has also been suggested that the activation of PLD and the production of PA can act as positive regulator of taxol biosynthesis in cells of *Taxus chinensis* var. *mairei* (Yang et al. 2007).

Since the commercial importance of the genus *Capsicum* is its ability to bio-synthesize metabolites called capsaicinoids, which are responsible for pungency in chilies. The accumulation of capsaicinoids in cell suspension cultures has been studied in species such as *C. frutescens* and *C. annum*, and low production amounts of these compounds were found in comparison with fruits (Ochoa-Alejo and Gómez-Peralta 1993). Therefore, attempts have been made to increase the production of capsaicinoids in in vitro cultures of *Capsicum* by manipulation of the culture medium composition (Ochoa-Alejo and Salgado 1992; Leslie and Romani 1986) by the addition of precursors and inducers of secondary metabolism, such as SA and/or MeJA (Sudha and Ravishankar 2003; Nieto-Pelayo 2006; Canché-Chay 2006; Gutiérrez-Carbajal 2006; Altúzar-Molina 2008).

For these reasons, we propose that phospholipid signaling and the accumulation of vanillin, which is an intermediate in the biosynthesis of capsaicinoids, are two events that are closely related to response of SA induction. Our results have indicated that the addition of SA, when used at concentrations of 100 and 200 μM, causes an increase in the content of vanillin (Altúzar-Molina et al. 2011).

However, when the content of vanillin was evaluated in the presence of neomycin, there was attenuation of the SA-stimulated vanillin production. These results suggest that the secondary messengers generated by PLC are involved in the stimulation of the vanillin production.

This fact led us to investigate how the phospholipid signaling pathway regulates the production of vanillin in response to SA. We evaluated the activity of phenylalanine ammonia lyase (PAL), the first enzyme of the phenylpropanoid pathway that leads to the production of capsaicinoids, phenylpropanoids and lignin (Chen et al. 2001). This enzyme is a biochemical marker of secondary metabolism in response to different types of stress or to plant growth regulators such as SA (Maldonado et al. 2007).

Using a suspension of *C. chinense* cells as a model, the effect of SA on PAL activity was studied. We found stimulation of PAL activity. However, this increase is diminished when neomycin or U73122 was also present in the media. This result might suggest that the secondary messengers generated by PLC may be modulating the SA-stimulated vanillin production through the activation of key enzymes of the biosynthetic pathway.

We therefore propose a signaling model in which SA is perceived by receptors in the plasma membrane (Fig. 3). The perception of this signal results in a stimulation of the enzymatic activity of PAL and the content of vanillin in the cell suspensions of *C. chinense*. However, in the presence of PLC inhibitors (neomycin

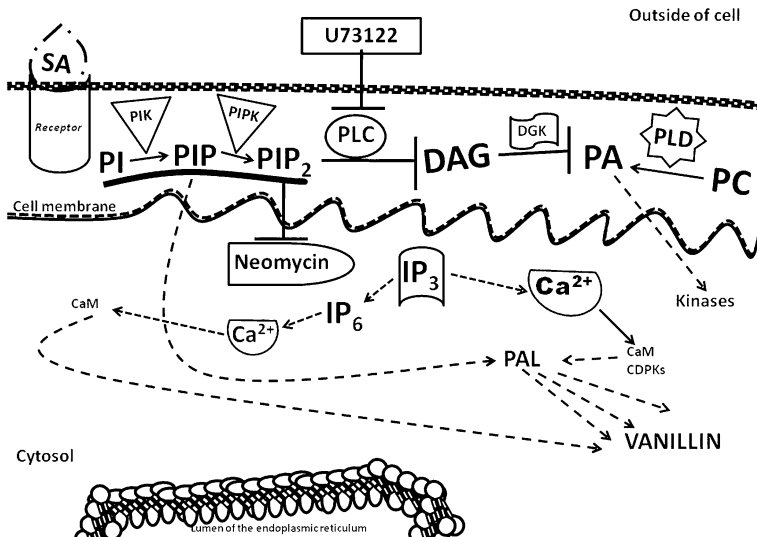**Fig. 3** A hypothetical model of phospholipid signaling in response to SA. Our model proposes that, in cell suspensions of *C. chinense*, secondary messengers regulate the production of vanillin. *SA* salicylic acid, *PIP₂* phosphatidylinositol 4,5-bisphosphate, *IP₃* Inositol 1,4,5 triphosphate, *DAG* diacylglycerol, *PAL* phenylalanine ammonia lyase, *DGK* Diacylglycerol kinase, *PA* Phenylalanine, *PC* Phosphatidylcholine

and U73122) in conjunction with modifications produced by SA, both the PAL activity and the vanillin content are altered; therefore, phospholipids such as PI, PIP and PIP_2 (substrate for PLC) may be involved in the regulation of PAL activity as they are important signaling molecules in regulating diverse cellular processes in plants. Another hypothesis is that by blocking one of the processes coupled to IP_3 or its derivates, sensors are affected, such as Ca^{2+} or calmodulin failing to activate kinases or the regulation of PAL activity.

4 Concluding Remarks

SA functions as an endogenous signal molecule and has been established in different types of stress in plants. The most important functions of SA are the activation of the defense response, the prevention of pathogen attack (Malamy and Klessing 1992) and the induction of secondary metabolism. However, this response may also be linked to a signaling cascade that regulates various physiological responses.

It has been observed that there is a response to SA by phospholipid signaling and that SA differentially regulates the enzymatic activities of phospholipases C and D. As it has been suggested that the activity of PLC and PLD are modified in response to SA in other plant species, we have been studying the effect of SA on different components of this route of signaling in cell suspensions of *C. chinense*.

SA has also been widely studied as an elicitor of secondary metabolism in plants, including the genus *Capsicum*. However, there is not much research correlating the signaling pathways and secondary metabolism. This lack of information sparked our interest in establishing the involvement of phospholipid signaling in the increase of vanillin content in response to SA in cell suspensions of *C. chinense*. The results suggest that SA regulates the activity of phospholipases, and the secondary messengers generated by this route might be modulating, through the activation of key enzymes of the biosynthetic pathway, the production of vanillin that is stimulated by the PLC pathway.

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Chapter 4
Transport of Salicylic Acid and Related Compounds

J.-L. Bonnemain, J.-F. Chollet and F. Rocher

Abstract Various stresses promote SA accumulation. SA is in part conjugated in the cytoplasm to inactive compounds such as salicylic acid O-β-glucoside (SAG) or modified to active compounds such as methylsalicylate (MeSA). SAG is sequestered in the vacuole by an ATP-binding cassette transporter mechanism or an H^+-antiporter mechanism. Free SA is mobile and can be transported within the plant, mainly via the phloem. SA molecules found in the phloem sap may come from the synthesis area via the symplastic route in symplastic loaders or may be taken up from the phloem apoplast (apoplastic loaders). In this latter case, SA must cross the plasma membrane of the companion cell-sieve cell complex. Similarly, synthetic derivatives or analogs applied to the foliage to enhance plant defence must cross at least once the plasma membrane before reaching the sieve tubes. The ability of molecules to diffuse through the plasma membrane is dependent on their chemical properties (size of the molecule, Log D, polar surface area, number of hydrogen bond donors). On these bases, the discrepancies between the computed predictions of phloem mobility of SA and various analogs and the actual results, as well as the effect of pCMBS on uptake suggest that SA transport involves a pH-dependent carrier system in addition to the ion trap mechanism, at least in the cotyledons of *Ricinus communis*. Although SA levels increase in both the phloem and systemic leaves after mature leaf infection, this salicylate is clearly not the primary systemic signal which contributes to SAR. Several data strongly suggest

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that MeSA as well as azelaic acid and small lipids are earlier signals. As MeSA is predicted to be very poorly phloem mobile, the mechanism of long distance transport of this volatile compound remains to be elucidated.

Keywords Salicylic acid · Salicylic acid metabolites · Salicylic acid analogs · Cell compartmentation · Long distance transport · Ricinus model · Diffusion predictors

1 Introduction

Salicylic acid (SA) or 2-hydroxybenzoic acid belongs to the very large group of plant phenolics which play an essential role in plant growth, development and interaction with other organisms (Harborne 1980). It has been known for a long time that SA is the natural inducer of thermogenesis in several aroid species (Raskin et al. 1987; Raskin 1992a, b) and can be involved in the regulation of flowering in some species, especially in combination with gibberellins (Raskin 1992a, b). A new chapter in SA research started from the discovery that SA applied to leaf tissues induces resistance to tobacco mosaic virus (TMV) in tobacco as well as the formation of PR proteins (White 1979).

Two decades ago, SA was classified under the group of plant hormones (Raskin 1992a, b). According to the well admitted definition, a hormone is an organic molecule that acts at very low concentrations locally and/or at distance from the site of its synthesis. SA meets these criteria taking into account its small amounts in plant tissues, its presence in the phloem sap and the variation of its concentration in response to pathogen attack (Malamy et al. 1990; Metraux et al. 1990). Since these early findings, many aspects of SA properties have been studied in detail (see the other chapters in this book). It can be added that SA is also highly involved in the plant-phloem feeding insect relations (Bostock 2005; Zarate et al. 2007; Giordanengo et al. 2010).

2 Transport Between Cell Compartments

Pathogen infection and abiotic stress such as UV light and ozone promote SA accumulation. SA synthesis occurs via the phenylalanine pathway in the cytosol or the isochorismate pathway in the chloroplast (Wildermuth et al. 2001). SA can be converted in the cytoplasm to several metabolites such as salicylic acid 2-*O*-ß-D-glucoside (SAG), methyl salicylate (MeSA), methyl salicylate 2-*O*-ß-D-glucoside (Me SAG), salicylic acid glucose ester (SGE) and dihydroxyderivatives. In most cases, SAG appears to be the major metabolite. Like glucose conjugates of other hormones, it is stored in the vacuole (Dean and Mills 2004).

Due to its physicochemical properties (Table 1 and see below), SAG diffusion through phospholipidic layers must be very low and this suggests that another mechanism is involved in SAG compartmentation within the cell. In fact, two active mechanisms control SAG accumulation in the vacuole (Fig. 1). In soybean cells, the vacuolar uptake of the conjugate occurs through an ATP-binding cassette transporter mechanism (Dean and Mills 2004) whereas in tobacco cells it occurs through an H^+-antiport mechanism energized by the proton gradient resulting from the activity of the tonoplast proton pumps (Dean et al. 2005).

Free endogenous SA can enter the neighboring cells either via the plasmodesmata (symplastic pathway) or via the cell wall (apoplastic pathway) (Fig. 1). In the latter case, the hormone must cross the plasma membrane of the donor cell and then the plasma membrane of the receiving cell.

It is well known that the physicochemical properties of SA make it well suitable for rapid diffusion through the plasma membrane of animal and plant cells, more especially under its undissociated form (Table 1). From the apoplastic compartment (pH 4.5–5.5), SA can accumulate in the cytosol (pH $\simeq$ 7.3) under its anionic form via the ion trap mechanism (Yalpani et al. 1991). Nevertheless, a slow SA efflux also occurs (Chen et al. 2001). SA at 20 or 200 μM added to tobacco cells in suspension is rapidly taken up (within 5 min). With time (5 h) 50 and 85 % of the absorbed SA are excreted from the cells treated with 20 and 200 μM respectively. SA excretion is significantly inhibited by EGTA and the inhibition can be reversed by Ca^{2+} addition in the 200 μM but not in the 20 μM treatment. Similarly, cycloheximide blocks SA excretion only in the 200 μM treatment. According the authors, this may suggest, among other hypotheses, a possible involvement of an inducible SA efflux carrier under the latter experimental conditions in addition to an efflux carrier constitutively present (Chen et al. 2001; Kawano et al. 2004) (Fig. 1).
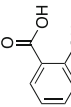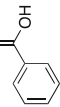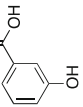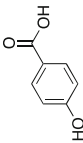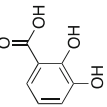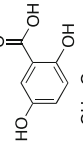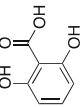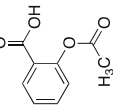
3 Long Distance Transport of SA and Related Compounds

3.1 Phloem Transport

3.1.1 Phloem Loading Strategies
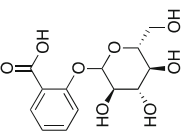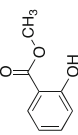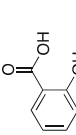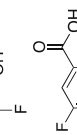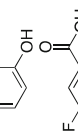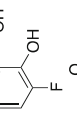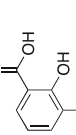
The phloem is a central actor in plant growth and development, allocating organic nutrients, ions, water, hormones and other signals from the leaves to the sink organs. Analyses of phloem sap indicate that sugars, potassium and amino acids are the main osmotic components (Dinant et al. 2010 and references therein). There are two main phloem loading mechanisms (Van Bel 1993; Turgeon 2010). The first one called apoplastic loading provides the driving force for nutrient transport by generating turgor pressure in the sieve-tubes. In many herbaceous species, phloem loading involves an apoplastic step. Sucrose enters the cell wall space in the vicinity of the companion cell-sieve element complex and the mechanism of its accumulation in this complex is a cotransport with protons

Table 1 Structure and physicochemical properties of salicylic acid and derivatives

Name	Structure	MW	Log D			Undissociated form (%)			Log K_{ow}	pKa	PSA (Å^2)	HBD
			pH 5.0	pH 6.5	pH 8.0	pH 5.0	pH 6.5	pH 8.0				
Salicylic acid (**a**)		138.12	−0.65	−1.11	−1.14	12.6	5.0	0.0	2.01	3.01	57.53	2
Benzoic acid (**b**)		122.12	0.70	−0.68	−1.50	85.3	15.5	0.6	1.56	4.20	37.30	1
3-Hydroxybenzoic acid (**c**)		138.12	0.15	−1.22	−1.87	61.7	4.8	0.2	1.23	4.08	57.53	2
4-Hydroxybenzoic acid (**d**)		138.12	0.74	−0.63	−1.64	87.7	18.4	0.7	1.40	4.57	57.53	2
2,3-Dihydroxybenzoic acid (**e**)		154.12	−1.17	−1.51	−1.53	3.6	0.1	0.0	1.62	2.96	77.76	3
2,5-Dihydroxybenzoic acid (**f**)		154.12	−1.34	−1.73	−1.75	2.8	0.1	0.0	1.40	3.01	77.76	3
2,6-Dihydroxybenzoic acid (**g**)		154.12	−0.77	−0.77	−0.77	0.1	0.0	0.0	2.38	1.30	77.76	3
Acetylsalicylic acid (**h**)		180.16	−0.12	−1.38	−1.73	43.7	2.4	0.1	1.40	3.48	63.60	1
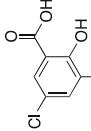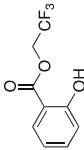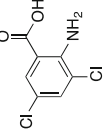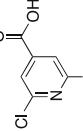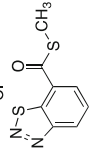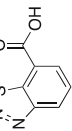
(continued)

Table 1 (continued)

Name	Structure	MW	Log D			Undissociated form (%)			Log K_{ow}	pKa	PSA (Å^2)	HBD
			pH 5.0	pH 6.5	pH 8.0	pH 5.0	pH 6.5	pH 8.0				
Salicylic acid glucoside (**i**)		300.26	−2.95	−4.12	−4.39	2.0	0.1	0.0	−1.25	3.34	136.7	5
Methyl salicylate (**j**)		152.15	2.52	2.52	2.52	100	100	100	2.52	9.76	46.53	1
3-Fluorosalicylic acid (**k**)		156.11	−0.28	−0.49	−0.50	13.2	0.5	0.0	2.65	2.45	57.53	2
5-Fluorosalicylic acid (**l**)		156.11	−0.19	−0.47	−0.48	18.9	0.7	0.0	2.67	2.68	57.53	2
3,5-Difluorosalicylic acid (**m**)		174.1	0.21	0.12	0.11	12.7	0.5	0.0	3.26	2.06	57.53	2
3-Chlorosalicylic acid (**n**)		172.57	0.05	−0.15	−0.15	19.6	0.8	0.0	3.00	2.43	57.53	2
5-Chlorosalicylic acid (**o**)		172.57	0.31	0.06	0.05	30.9	1.4	0.0	3.20	2.64	57.53	2

(continued)

Table 1 (continued)

Name	Structure	MW	Log D			Undissociated form (%)			Log K_{ow}	pKa	PSA (Å²)	HBD
			pH 5.0	pH 6.5	pH 8.0	pH 5.0	pH 6.5	pH 8.0				
3,5-Dichlorosalicylic acid (**p**)		207.01	1.11	1.03	1.03	16.7	0.6	0.0	4.18	1.99	57.53	2
Trifluoroethyl salicylate (**q**)		220.15	3.89	3.87	3.46	100	100	99.9	3.89	7.77	46.53	1
3,5-Dichloroanthranilic acid (**r**)		206.03	2.34	0.99	0.45	98.4	66.6	5.9	3.57	4.20	63.32	3
2,6-Dichloroisonicotinic acid (**s**)		192	−0.06	−0.83	−0.91	40.1	2.1	0.1	2.24	2.63	50.19	1
Acibenzolar-S-methyl (**t**)		210.28	2.74	2.74	2.74	NA	NA	NA	2.74	NA	96.39	0
1,2,3-Benzothiadiazole-7-carboxylic acid (**u**)		180.18	−1.94	−2.04	−2.04	0.3	0.0	0.0	1.11	1.35	91.32	1

All predictions were computed with ACD Log D Sol Suite v.12.02 software. *MW* Molecular Weight; *PSA* Polar Surface Area; *HBD* Hydrogen Bond Donors; *NA* Non Available
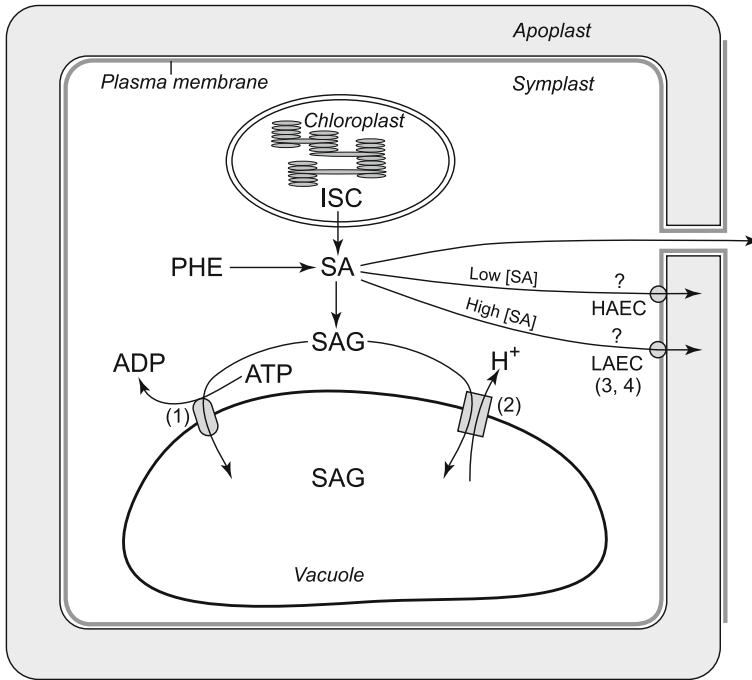
Fig. 1 SA biosynthesis and transport between cell compartments. *ISC* isochorismate pathway; *PHE* phenylalanine pathway; *SAG* salicylic acid glucoside; *HAEC* high affinity efflux carrier; *LAEC* low affinity efflux carrier. (1) From Dean and Mills (2004); (2) from Dean et al. (2005); (3) and (4) from Chen et al. (2001) and Kawano et al. (2004)

energized by the protonmotrice force (Giaquinta 1977; Delrot and Bonnemain 1981). These species are characterized by a low or very low plasmodesmatal density between the companion cell—sieve element complex and the neighboring cells, a high expression of the plasma membrane H^+-ATPase in the companion cells and a loading inhibition by pCMBS (Bourquin et al. 1990; Bouchepillon et al. 1994; DeWitt and Sussman 1995; Turgeon 2010). On the other hand, especially in many tree species, phloem loading is passive, driven by a downhill concentration of nutrients from the mesophyll to the companion cell—sieve element complex. These species are characterized by a high symplastic connectivity between mesophyll and conducting cells (symplastic loading) and non-inhibition of phloem loading by pCMBS (Gamalei 1989; Van Bel 1993; Turgeon 2010).

Consequently, phloem loading of endogenous hormones depends on the leaf anatomy. It may be apoplastic or symplastic according to the characteristics of the symplastic continuity between the companion cell—sieve element complex and the neighboring cells. However, when hormone biosynthesis occurs in companion cells as it is the case for jasmonic acid, the hormone may move directly into the sieve-tubes via the branched plasmodesmatal connections for long distance transport (Champigny and Cameron 2009). By contrast, synthetic hormones

applied to the foliage must cross at least once the plasma membrane whatever the phloem loading mechanism, symplastic or apoplastic.

3.1.2 Phloem Transport of SA and Related Compounds

SA Phloem Transport in Relation to Biotic and Abiotic Stress

The first experiments on SA phloem transport were conducted with labeled molecules three decades ago. They failed to demonstrate an efficient long distance transport of the label, probably because ^{14}C-SA is quickly metabolized and sequestered in vacuoles (Bental and Cleland 1982).

A few years later, an increasing interest on SA phloem transport arose from the demonstration that development of SAR in a cultivar of tobacco resistant to tobacco mosaic virus (TMV) is accompanied by a dramatic increase in the level of endogenous SA in infected leaves after TMV inoculation and also, to a lesser extent, in uninfected upper leaves (Malamy et al. 1990). Furthermore, inoculation of mature cucumber leaves with either the tobacco necrosis virus (TNV) or *Colletotrichum lagenarium* leads to a clear rise in SA in the phloem sap and the development of SAR (Metraux et al. 1990). TMV infection of nearly fully expanded leaves of tobacco plants also induces an increase in SA concentration in the phloem sap (Yalpani et al. 1991). These data strongly suggest that SA, previously known as an exogenous inducer of resistance (White 1979) can also function as a mobile signal that triggers local and systemic induction of PR-1 proteins and, possibly, some components of systemic acquired resistance (Metraux et al. 1990; Yalpani et al. 1991).

Then SA phloem transport from inoculated mature leaves to systemically protected apical organs was demonstrated. The first evidence came from in vivo labeling with $^{18}O_2$ of the SA synthesized in TMV-inoculated lower leaves of tobacco (Shulaev et al. 1995). Spatial and temporal distribution of ^{18}O-SA indicates that most of the SA molecules detected in the upper healthy tissues are ^{18}O-labeled and have therefore been transported from the inoculated leaves. The second evidence came from transport studies of ^{14}C-SA after injection of ^{14}C-labeled benzoic acid into cucumber cotyledons inoculated with TNV (Molders et al. 1996). Labeled SA moves in the phloem and is clearly detected in the upper uninoculated leaf before the development of SAR. However, the specific activity of ^{14}C-SA decreases indicating that, in addition to transport from the inoculated cotyledons, the upper leaf produces SA in response to a primary signal. In this regard, previous data from leaf removing and grafting experiments show that the primary SAR inducing signal is not SA (Rasmussen et al. 1991; Vernooij et al. 1994). Studies are now focused on the mechanism of SAR activation. Several data suggest that multiple signals are required among which methyl salicylate (Liu et al. 2011).

By contrast, studies on long distance transport of SA in response to abiotic stress are scarce. In *Vitis vinifera* plants ^{14}C-SA molecules move in part from the non-stressed leaves to leaves exposed to high temperatures (Wang et al. 2004).

Predicting Phloem Mobility of SA and Related Compounds

Phloem mobility of endogenous compounds, synthetic derivatives and various xenobiotics is often predicted using the model of Kleier (Kleier 1988; Hsu and Kleier 1996) (Fig. 2) and Bromilow and coworkers (Bromilow et al. 1991). Although these models are based on only two physicochemical properties of the molecules, namely their lipophilicity (assessed as the 1-octanol/water partition coefficient, Log K_{ow}) and their pKa values, the experimental data often fit rather well with the predictions. Discrepancies between predictions and experimental data may indicate the involvement of a carrier system instead of—or in addition to—diffusion through the plasma membrane (Oparka 1991; Rocher et al. 2009).

Other diffusion predictors must also be taken into consideration, especially Log D (i.e., the pH dependent Log K_{ow}) which indicate the true behavior of ionizable compounds in the various cell compartments (Bhal et al. 2007), the polar surface
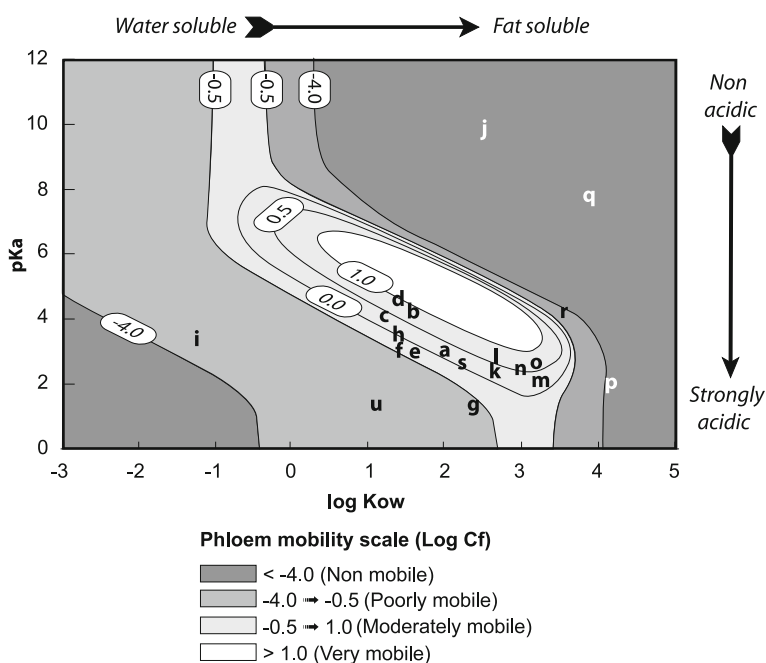
Fig. 2 Prediction of phloem mobility of salicylic acid and derivatives using Kleier map (Log Cf as a function of Log K_{ow} and pKa) according to Kleier et al. (1996). Plant parameters are for a short plant. Compounds are identified with letters listed in Table 1

area (PSA) which is defined as the sum of surfaces of polar atoms in a molecule, usually oxygen and nitrogen and the hydrogens bonded to these atoms (Ertl et al. 2000), and the number of hydrogen bond donors (HBD), i.e., a hydrogen atom attached to a relatively electronegative atom (Winiwarter et al. 2003). To diffuse significantly through the animal tissue barrier, the drugs must meat several criteria: MW < 500 Da, Log $D < 5$, PSA < 60 Å^2 (blood–brain barrier) or <140 Å^2 (intestinal barrier) and HBD < 5 (Lipinski et al. 1997; Palm et al. 1997, 1998; Winiwarter et al. 2003; Bhal et al. 2007). The first two parameters (MW and Log D) are approximately the same for plant membranes. No data are available about the PSA and HBD values limits but it may be suggested that PSA $\geqq 140$ Å^2 and HBD > 5 are poorly consistent with diffusion through cell plant membranes. Then, according the ACD Log D Sol Suite software predictions, all the molecules of Table 1 meet these criteria except SAG (PSA $= 137$ Å^2, high HBD) which is exclusively under its hydrophilic anionic form in the cytosol. This is why SAG accumulation in vacuole requires the involvement of intrinsic proteins of the tonoplast (Fig. 1).

Most of the molecules of Table 1 are predicted to be more or less phloem mobile according to the Kleier's model (Fig. 2) except methyl salicylate (**j**), trifluoroethylsalicylate (**q**) a salicylate derivative recently synthesized (Qian et al. 2006) and 3,5-dichlorosalicylic acid due to its Log K_{ow} value. SAG (**i**) which is near the boundary between poorly mobile and non mobile molecule areas (Fig. 2) is not present in detectable amounts in the phloem sap of tobacco infected with TMV (Enyedi et al. 1992). The ability of the dihydroxyderivatives of benzoic acid to diffuse through the plasma membrane to accumulate in the phloem is probably very low taking into account their presence almost exclusively under the anionic form in the apoplast and a Δ Log D $_{pH = 5.0–8.0}$ from 0 (2,6-dihydroxybenzoic acid, **g**) to about 0.4 (2,3-dihydroxybenzoic acid, **e**, and 2,5-dihydroxybenzoic acid, **f**) (Table 1). Similarly, according to ACD Log D software predictions, acibenzolar-S-methyl (**t**) and its metabolite 1,2,3-benzothiadiazole-7-carboxylic acid (**u**) cannot diffuse easily from apoplast to symplast (Table 1).

While jasmonic acid and azelaic acid, two putative primary signals in SAR although the former is a matter of debate (Truman et al. 2007; Jung et al. 2009) exhibit physicochemical properties perfectly suitable for long distance transport within the sieve tubes (Chollet et al. unpublished data), MeSA (Vapor Pressure $= 0.0700$ Torr at 25 °C) does not meet the required criteria for such translocation. MeSA can diffuse through the plasma membrane taking into account its MW, PSA and HBD values but similarly in both directions because it is not ionizable at biological pH values (Δ Log D $_{pH = 5.0–8.0} = 0$) (Table 1). Its concentration in the phloem sap is extremely low (Park et al. 2007). Therefore, this rises an intriguing problem to solve about the pathway and mechanisms of MeSA transport because SAR clearly requires both MeSA biosynthesis in the primary infected leaf and MeSA esterase activity which converts MeSA to SA within the uninoculated systemic tissues (Park et al. 2007; Liu et al. 2011).

Phloem Transport Mechanisms of SA and Related Compounds

The ability of phloem to load salicylic acid is in general not easy to evaluate for several reasons: the plant cuticles function as barrier, the injection of SA solution in the mesophyll can induce callose formation in the sieve plates in the vicinity of injured tissues, SA may be more or less rapidly converted to several metabolites, especially SAG, and finally phloem sap is usually difficult to collect for analysis.

The structural and physiological particularities of *Ricinus communis* seedlings make the evaluation of the potential ability of such loading possible. Firstly, the cuticle is very thin and permeable to nutrients excreted from endosperm, secondly castor bean is one of the plant species that exude phloem sap spontaneously (Orlich and Komor 1992). SA phloem loading is pH-dependent. The accumulation is the highest (concentration factor near 10) at the most acidic values tested (pH 4.6) (Fig. 3a) but a residual loading still occurs (concentration factor from 0.5 to 1) at pH 7.0 and 8.0, i.e., when SA is exclusively under its anionic form in the incubation medium (Rocher et al. 2006). A similar residual loading of the anionic form of the molecule can also be observed with monohalogenated SA derivatives. It cannot be explained by the ion trap mechanism, especially when an accumulation in the phloem sap (concentration factor near 2) is noted at neutral and slightly alkaline pH (Fig. 3c) (Rocher et al. 2009). On the other hand, 3-hydroxybenzoic acid (Fig. 3b) and 4-hydroxybenzoic acid which are predicted to have a phloem mobility similar to that of SA (Fig. 2) and to exhibit a better profile for the ion trap mechanism in terms of Log D values and percentage of their permeant undissociated form in the apoplast (Table 1), are poorly translocated in the phloem (Fig. 3b). This indicates that the efficiency of phloem loading of the hydroxylated benzoic acid derivatives is tightly dependent on the position of the hydroxyl group on the aromatic ring. These data suggest that, as in intestinal cells (Tamai et al. 1995), SA transport in *Ricinus* cotyledons involves a pH-dependent carrier system in addition to the ion trap mechanism (Rocher et al. 2009).

A further support for the involvement of such mechanisms comes from the effect of pCMBS on SA uptake and phloem loading in *Ricinus communis* cotyledons (Rocher et al. 2009). Due to its physicochemical properties, it is considered as a very poorly or non permeant tool which reacts with Cys residues of the external part of several nutrient carriers (Delrot et al. 1980; Bush 1993; Lemoine 2000). SA uptake by cotyledon tissues is significantly inhibited by pCMBS, especially at pH 7.0 (Table 2), i.e., under an experimental condition which abolishes the contribution of the ion trap mechanism. Finally, autoradiographs show that SA phloem loading itself is completely or almost completely inhibited by pCMBS (Rocher et al. 2009). In intestinal epithelial cells, SA uptake involves at least two carriers, MCT1 (the first member of the monocarboxylic acid transporter family) and OATP-B (a member of the organic anionic transporting polypeptide family) (Koljonen et al. 2008). pCMBS is known to block MCT1 which functions as a proton-coupled transporter (Enerson and Drewes 2003) and OATs (Tanaka et al. 2004).
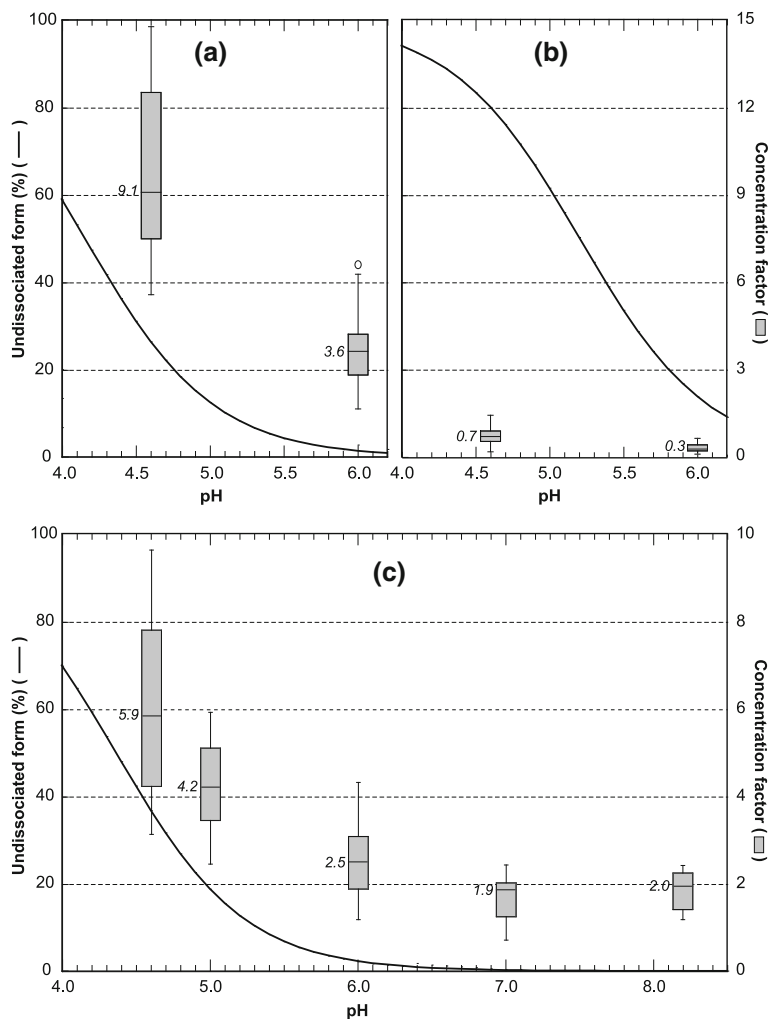
Fig. 3 Percentage of undissociated form of salicylic acid (SA; **a**), 3-hydroxybenzoic acid (3-OHBA; **b**) and 5-fluorosalicylic acid (5-FSA; **c**) in an octanol–water mixture and phloem mobility as a function of pH. Predictions were computed with ACD Log D suite v 12.02 software. Concentration factors in *Ricinus* phloem sap were determined using SA, 3-OHBA or 5-FSA added to the incubation medium at 10 µM final concentration. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio [SA or 3-OHBA or 5-FSA]sap/[SA or 3-OHBA or 5-FSA]medium. For box plots, 12 < n < 24. (From Rocher et al. 2006, 2009)

Table 2 Effect of 1 mM pCMBS on 10 μM salicylic acid (SA) uptake by discs from *Ricinus* cotyledons for 10 min

	SA uptake					
	pH 4.6		pH 5.0		pH 7.0	
	nmol.cm^2 10 min^{-1}	Inhibition (%)	nmol.cm^2 10 min^{-1}	Inhibition (%)	nmol.cm^2 10 min^{-1}	Inhibition (%)
Control	0.828		0.671		0.06	
pCMBS 1 mM	0.606	−27 (**)	0.325	−52 (***)	0.016	−74 (***)

Discs were preincubated in a buffered standard solution for 30 min and then on the same medium without or with 1 mM pCMBS for 15 min. After the pretreatment, tissues were transferred to the incubation medium (the same solution containing 10 μM [^{14}C]SA without (control) or with pCMBS for 10 min. The Mann–Whitney U test was used to assess statistically significant differences (*** $P < 0.001$, ** $P < 0.01$), $12 < n < 16$. pCMBS did not affect the uptake of the internal pH probe 5,5'-dimethyloxazolidine-2-^{14}C,4-dione (from Rocher et al. 2009)

3.2 Xylem Transport

There are very few data about SA transport in the vessels. The vascular sap is usually acidic (pH from 5.5 to 6.5) in herbeacous plants during the growing season and in trees from late winter to the end of spring (Ferguson et al. 1983; Fromard et al. 1995; Thomas and Eamus 2002). Consequently, the ion trap mechanism cannot be taken into consideration to explain its presence in this compartment. SA is translocated in the vessels but at low amounts. In *Ricinus communis* seedlings a very small part of SA transported to the root system via the phloem is redistributed upward via the xylem (Rocher et al. 2006). However these data may probably contribute to explain why labeled SA distribution within the *Arabidopsis* rosette is not limited to young leaves situated directly above the donor leaf as should be the case according to the phloem allocation pattern (Kiefer and Slusarenko 2003).

Endogenous SA and SAG have been recently reported in xylem sap of *Brassica napus*. Their concentrations increase after infection with the vascular pathogen *Verticillium longisporum* (Ratzinger et al. 2009). Due to the lack of symplastic connection between vessel elements and the neighbouring cells, SAG must cross the plasma membrane at least once to reach the vascular sap. By contrast, in other plant materials, SA and not SAG is excreted from symplast to apoplast as mentioned before (Chen et al. 2001). SAG can be hydrolyzed in the cell wall by an extracellular salicylic acid ß-glucosidase which regulates the level of free SA in the apoplastic compartment (Seo et al. 1995).

It is well known that various stresses such as drought, flooding and fungal infection lead to an increase of the xylem sap pH (Wilkinson 1999). The bacterial elicitor harpin also induces a similar pH change in the incubation medium of *Arabidopsis* cells (Clarke et al. 2005). These pH increases, sometimes up to 7.5–8.0 (Wilkinson 1999), must logically modulate SA compartmentation within the tissues and the whole plant.

4 Conclusion

Membrane transport mechanism and long distance transport of SA and related compounds in plant tissues are still poorly documented probably because it was believed for a long time that SA is taken up by plant cells solely via the ion trap mechanism. By contrast, as aspirin is one of the most popular drug, the membrane transport of SA (and other monocarboxylic acids) has been intensively investigated in animal tissues from the beginning of the nineties, mainly using brush-border vesicles and the human colon adenocarcinoma cell line Caco-2. These works lead to the identification of two carriers involved in SA uptake, firstly MCT1 and more recently OATP-B, both localized at the apical membrane of intestinal epithelial cells in humans.

Nevertheless significant advances in the understanding of plant cell compartmentation and long distance transport of SA and related compounds have been made over the last decade using vacuoles and tonoplast vesicles, plant cell suspension cultures and the *Ricinus* model. The diffusion predictors usually used to study drug compartmentation in animal tissues appear to be useful tools for plant cell compartmentation and long distance transport studies. However several data, hypotheses and speculations need further investigations, especially on efflux mechanisms of SA and metabolites from symplast to apoplast and long distance transport mechanisms of SA and metabolites, especially MeSA. To our knowledge, the advances in the understanding of SA and related compound transport in plant tissues do not still reach the molecular level.

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Chapter 5
Interplay Between Environmental Signals and Endogenous Salicylic Acid Concentration

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Abstract Salicylic acid (SA), a naturally occurring plant hormone, is primarily associated with the induction or activation of defence mechanism responses by higher plants when they are attacked by pathogens. Attack of these plants by pathogens rapidly triggers changes in a wide range of the plant's metabolic pathways which in turn are followed by modifications in the plant's growth and development. There are a number of references in the recent literature where SA was applied to plants that are being subjected to changes in environmental signaling without the involvement of pathogens. In these examples, SA appears to be functioning as a hormone. Significant changes (usually positive) in shoot growth and photosynthesis occur when SA is applied at low concentrations to plants subjected to environmental stresses. In this review we focused on the interplay between changes in endogenous SA concentrations and key environmental signals, i.e. light intensity and quality, temperature, soil water availability and carbon dioxide levels. In doing so, we evaluated the concept that endogenous SA functions as an important signaling hormone in the plant's growth response to a changing environment, even in the absence of pathogen attack.

Keywords Salicylic acid · Environmental signals · Light · Temperature · Water stress · Carbon dioxide · Growth · Photosynthesis

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1 Introduction

Salicylic acid (SA) has only recently been recognized to have a role in the plant's ability to mount a defence against pathogen attack. In contrast, the human therapeutic properties of SA (2-hydroxy-benzoic acid) have been known for over 25 centuries. In traditional medicine pieces of bark from willow trees (*Salix* spp.) were chewed to provide relief from pain and inflammation, as described in writings by the Greek physician, Hippocrates (Fifth Century B.C.) and the physician and botanist Pedanius Dioscorides (First Century A.D.). The therapeutic component in willow bark, SA, was first identified and isolated during the 19th century. Toward the end of the 19th century it was chemically modified into what is known today as aspirin or acetylsalicylic acid (Delaney 2010). In plants, SA is biosynthesized by the shikimic acid pathway, which can produce SA in two ways: chorismic acid → phenylalanine → trans-cinnamic acid → benzoic acid → SA, or chorismic acid → isochorismic acid → SA (Delaney 2010).

The role of SA as an endogenous signaling molecule in the plant's defence mechanism against pathogens has now been extensively characterized (Raskin 1992). In a similar manner, the effect of SA, when applied exogenously, on the physiology, metabolism and reproductive development of some higher plants was also reported (Raskin 1995). Exogenously applied SA can promote fresh and dry biomass accumulation at low (near-physiological) concentrations (Hayat et al. 2010). However, at higher exogenous concentrations, SA often shows an inhibition of dry mass accumulation (Schettel and Balke 1983). Applications of SA can also induce stomatal closure (Larque-Saavedra 1979) and influence ion uptake and transport (Harper and Balke 1981), as well as regulate chlorophyll accumulation and the rate of photosynthesis (Hayat et al. 2010). Applications of low concentrations of SA have thus been shown to increase chlorophyll content, though higher concentrations were inhibitory (Ghai et al. 2002; Fariduddin et al. 2003; Hayat et al. 2005). Low concentrations of exogenously applied SA also gave a slightly elevated photosynthetic efficiency of photosystem (PS) II in both *Arabidopsis thaliana* (Heynh.) wild type (WT) plants and *A. thaliana* SA non-responsive mutant plants, although higher concentrations of SA reduced PS II photosynthetic efficiency (Chen et al. 2009). Consistent with these results, an *A. thaliana* SA-overproducing mutant exhibited decreased photosynthetic efficiency of PS II when SA was applied across wide range (low and high) of concentrations (Chen et al. 2009).

Responses by barley (*Hordeum vulgare* L.) leaves to applied SA at relatively high concentrations included a decreased rate of leaf expansion as well as a decrease in overall leaf area growth and decreased thickness of all leaf tissue components (Pancheva et al. 1996; Pancheva and Popova 1998; Uzunova and Popova 2000). Further, these morphological responses were associated with decreases in both the rate of photosynthesis and chlorophyll content (Pancheva et al. 1996; Pancheva and Popova 1998; Uzunova and Popova 2000). Thus, as appears to be the case with many of the plant hormone classes, exogenously applied SA may promote photosynthesis and growth at lower concentrations, but

be inhibitory when applied at high concentrations. Further, the growth-promotive or inhibitory effects of exogenous SA application appear to depend on the environmental conditions at the time of application (Hayat et al. 2010). Based on the above literature, we think it is time to evaluate the effects of environmental signaling on endogenous SA levels in the context of a range of plant growth mechanisms. Such an approach should allow us to better understand how exogenously applied SA alleviates stress symptoms in many higher plants.

2 Assessing Endogenous Salicylic Acid Levels

Endogenous SA levels in plant tissues can be precisely identified and accurately quantified using the stable isotope dilution method (Gaskin and MacMillan 1991). This is accomplished by selected ion monitoring (SIM) using capillary gas chromatography (GC)—mass spectrometry (MS) or liquid chromatography (LC)—MS (Scott et al. 2004; Kurepin et al. 2010a). The first stage, consisting of extraction, addition of stable isotope internal standards and purification is the same for both SIM-GC–MS and LC–MS methods. Plant tissue is collected and immediately frozen in liquid N_2, then freeze-dried or stored at $-80\ ^\circ C$ until extraction. Extraction consists of grinding in a mortar and pestle with liquid N_2, followed by use of 80 % aqueous methanol (H_2O–MeOH = 20:80, v/v) as an extraction solvent. A known amount of high specific activity deuterated SA (usually $^2H_6$-SA) is added to the 80 % MeOH extract as a quantitative internal standard. The 80 % MeOH extract is then purified with a C_{18} preparative column (using reversed phase C_{18} material) and dried *in vacuo* at 35 $^\circ C$. For the LC–MS method, the dried residue is reconstituted in 0.05 % HOAc in H_2O–MeCN (85:15, v/v) and then filtered with a 0.45 μm filter prior to the injection into the LC–MS. The characteristic *m/z* ions that are usually used for quantification are (*m/z* 141 for d$_6$SA and *m/z* 137 for the endogenous protio SA). The quantification of endogenous SA is then accomplished based on the relative intensities (peak areas) of these characteristic *m/z* ions, the known amount of d$_6$-labeled SA internal standard added and the recorded fresh or dry tissue weight (Scott et al. 2004). For the SIM-GC–MS method, the dried residue is reconstituted in 1 % HOAc in H_2O–MeOH (90:10, v/v) and injected into the high performance LC (HPLC). In one example (Kurepin et al. 2010a) the HPLC used a manually implemented 10–73 % linear gradient program for separation of SA from other plant metabolites. The eluted fractions that are expected to contain SA (based on previous HPLC runs with a SA standard) are dried *in vacuo* at 35 $^\circ C$ and then methylated using ethereal CH_2N_2 at room temperature as described in Kurepin et al. (2010a). The methylated SA sample is then injected into the GC–MS and the SIM program is set to monitor characteristic *m/z* ions for both the deuterated SA-Me standard (124, 96 and 156 for d$_6$SA) and endogenous protio SA-Me (120, 92 and 152). For identification of endogenous SA a comparison of the relative intensities of the three *m/z* ion pairs, i.e. 124/120, 96/92 and 156/152, is accomplished. The amount of endogenous (protio) SA is then

quantified by the reference to the stable isotope-labeled internal standard using equations for isotope dilution analysis using the 124/120 ion pair (see Gaskin and MacMillan 1991; Jacobsen et al. 2002).

As with other classes of plant hormones, large variations in endogenous SA levels between species, ecotypes, tissues, and, more importantly in response to various environmental signals are not uncommon. Species-related difference in endogenous SA levels can be very significant. In two-week old *A. thaliana* and sunflower (*Helianthus annuus* L.) shoots grown under exactly the same conditions, they can vary from a few ng per gDW in *A. thaliana* to several thousand ng per gDW in sunflower (Kurepin et al. 2010a). Ecotype-related differences can also be large. For example, alpine plants *of Stellaria longipes* [(L.) Goldie] have ca. half the amount of endogenous SA, relative to plants of a prairie ecotype of the same species, both ecotypes being grown under the same conditions and also being of the same age (Kurepin et al. 2012a). Tissue-related difference in SA can be highlighted by comparing sunflower internodes (which contain several hundred ng per gDW) with leaves (which have several thousand ng per gDW) above that internode. Although species-, ecotype- and tissue-related differences are "interesting", environment-related differences in endogenous SA will have the most substantial impact on plant performance and, even survival (Scott et al. 2004; Abreu and Munne-Bosch 2008; Kurepin et al. 2010a)—see also evidence from numerous SA application studies (Hayat et al. 2010).

3 Light Signaling and Endogenous Salicylic Acid Levels

Among the many environmental factors, light plays a key role in plant growth and development. The irradiance levels of "visible" light (ca. 400–800 nm) received by a plant can control both photosynthesis and growth, including etiolation and de-etiolation. In contrast, it is the quality or specific wavelengths of visible light that regulates many growth and developmental events (Smith 2000). Additionally, invisible light, such as ultraviolet (UV) light (ca. 100–400 nm), can both influence growth and cause damage to plants (Jenkins 2009). For example, UV-C irradiance of tobacco (*Nicotiana tabacum* L.) plants caused a significant accumulation of SA (Yalpani et al. 1994). Silencing the isochorismate (a SA biosynthetic precursor) synthase (ICS) gene using a virus-induced technique prevented an accumulation in endogenous SA in tobacco plants inoculated with a pathogen (Catinot et al. 2008). Exposure of these transgenic plants to UV-C light also significantly decreased the accumulation of endogenous SA (Catinot et al. 2008). This implies that a *de novo* synthesis of SA may occurr in response to UV light stress. Additionally, exogenously applied SA is reported to alleviate the UV-B irradiance-related stress in swards of both Kentuky bluegrass (*Poa pratensis* L.) and tall fescue (*Festuca arundinacea* Schreb.) (Ervin et al. 2004).

In *A. thaliana*, a plant that is often grown for experimental use at light irradiance levels well below those of natural sun light, endogenous SA levels were

higher by ca. 50 % at low light irradiance relative to dark (Genoud et al. 2002). However, a further increase in light irradiance had no significant effect on endogenous SA levels (Zeier et al. 2004). For sunflower (*Helianthus annuus* L.), a plant that is adapted to grow best at full sun light, endogenous SA levels in hypocotyl tissue showed consistent and appreciable (ca. 10-fold) increases as light irradiance levels increased from very low to low and then to full sunlight (Kurepin et al. 2010a). These increases in endogenous SA levels of sunflower shoot tissue at the increased light irradiance levels were associated with significant decreases in hypocotyl elongation and biomass accumulation (Kurepin et al. 2010a). Thus, the difference in the endogenous SA response and its magnitude in response to changes in light irradiance levels, vary between *A. thaliana* and sunflower. The changes in growth and endogenous SA levels seen in the plant's response to light irradiance level can also be ecotype-specific. The *Stellaria longipes* sun ecotype grows in an alpine habitat characterized by very short plants and distant surrounding vegetation. In contrast, the *S. longipes* shade ecotype grows in nearby low elevation prairie grasslands, where the habitat is characterized by tall neighbouring vegetation which causes canopy shading and/or shading by neighbours. These two ecotypes grow just a few kilometres apart, but the habitat differences caused by elevation make them an excellent model for light signaling research (Emery et al. 1994). Plants of both ecotypes decrease their shoot growth in response to increase in light irradiance levels and endogenous SA levels increase coincidentally (Kurepin et al. 2012a). This decrease in shoot growth in response to an increase in light irradiance was proportionally similar for both sun and shade ecotypes of *S. longipes*. However, the magnitude of changes in endogenous SA levels varied between the two ecotypes. The sun ecotype plants showed less than a 2-fold increase in endogenous SA levels. Yet shade ecotype plants had more than a 3-fold increase in endogenous SA levels (Kurepin et al. 2012a). Thus, the magnitude of the SA concentration in response to increasing irradiance levels can vary depending on species and ecotype. Even so, the high light irradiance-mediated increase in endogenous SA levels may have ecological significance. For example, it is a well-known fact that there is a higher susceptibility of younger trees to pathogen attack in understory environments. This may be because the fungal activity in understory environments is greater than in forest openings (Kitajima and Augspurger 1989). Thus, when seeds of *Betula papyrifera* Marsh., (a species of birch native to North America) were planted in a range of habitats differing in light availability (from understory to open forest), the application of a fungicide reduced losses in a habitat-dependent manner (O'Hanlon-Manners and Kotanen 2004). Thus, the fungicide was more effective in understory than in open habitats. One possible explanation is that low light irradiance in the understory forest habitat prevents the establishment of *B. papyrifera* plants because their ability to modify endogenous SA levels in response to pathogen attack is depressed.

In both sunflower and *S. longipes* plants the high light irradiance-mediated increase in endogenous SA levels was correlated with a decrease in shoot growth. Simplistically, then, the role of SA could be classified as growth inhibitory for light irradiance-mediated responses. However, *A. thaliana* mutants with

constitutively high endogenous SA levels (*cpr1-1, cpr5-1* and *cpr6-1*; Bowling et al. 1994, 1997; Clarke et al. 1998) exhibit the dwarf growth phenotype at low, but not at higher light irradiances, relative to wild type line *Col-0* (Mateo et al. 2006). Further, these *A. thaliana* mutants with high SA levels, when grown at a lower light irradiance, had lower maximum efficiency and quantum yield of PSII, as well as having reduced stomatal conductance and a lower accumulation of photo-assimilates (Mateo et al. 2006). Thus, the negative effects of possessing high SA levels are only expressed, in these mutant lines, under low irradiance levels. Further, a mutant with constitutively reduced endogenous SA levels (*sid2-2*; Nawrath and Metraux 1999) did not show a dwarfing response, nor did it exhibit reduced metabolic responses to low light irradiance levels (Mateo et al. 2006). Therefore, low light irradiance-induced growth promotion is associated with low SA levels and plants with high endogenous SA levels fail to etiolate under low light irradiances, yet they have the same phenotype as wild type plants under high irradiance light.

In tobacco (*Nicotiana benthamiana* L.) plants inoculated with *Turnip mosaic virus* (TuMV), both low light irradiance and photosystem impairment can increase the susceptibility of the host to TuMV infection (Manfre et al. 2011). Although endogenous SA levels were not measured, exogenous SA application had no effect on TuMV infection. Further, the expression of *pathogen response-1* (*pr-1*) gene was not affected by lower light irradiances or photosystem impairment (Manfre et al. 2011). By implication, the reduction in light irradiance and associated increased susceptibility of host to pathogen may not be mediated by lowered endogenous SA levels. Such a conclusion is also supported by an earlier observation where the inoculation of *A. thaliana* with *Pseudomonas syringae pv. maculicola* resulted in systemic acquired resistance (SAR) for plants grown at both low and high light irradiances (Zeier et al. 2004). However, the higher light irradiance did not result in an accumulation of endogenous SA or PR-1 protein (Zeier et al. 2004).

In addition to light irradiance regulation of endogenous SA levels in plant tissues, the quality of light perceived by a plant, via plant photoreceptors such as phytochromes and cryptochromes, can also influence endogenous SA levels (Kurepin et al. 2010a, 2012a). For example, narrow-band far red (FR) light [supplied by light emitting diodes (LED) as the dark period began] yielded up to a 5-fold increase in endogenous SA levels of sunflower hypocotyls, relative to white (W) light or dark (D) treatments (Kurepin et al. 2010a). Here, the FR-induced increase in endogenous SA levels was positively associated with increased hypocotyl elongation. In contrast, LED-produced red (R) light and blue (B) light inhibited hypocotyl elongation relative to W or D treatments (Kurepin et al. 2010a). That inhibition was associated with a decrease in endogenous SA levels, relative to a W light treatment (but not a D treatment). Thus, it appears that the endogenous SA content can also be regulated by light quality. That conclusion is further supported by experiments where the effect of a change in R/FR ratio was tested on endogenous SA accumulation. There, sunflower plants were grown at both low and high light irradiances, each with varying R/FR ratios, and effects on

hypocotyl growth and endogenous SA concentrations were measured. A decrease in the R/FR ratio (i.e. an increase in FR radiation relative to R irradiance) from a high R/FR ratio to a normal R/FR ratio, and then to a low R/FR ratio yielded a gradual increase in endogenous SA levels. This, in turn, was positively associated with increased hypocotyl elongation (Kurepin et al. 2010a). For another species, *S. longipes*, using both sun and shade ecotypes, decreasing the R/FR ratio from high to normal had no effect on endogenous SA levels. However, the further decrease in R/FR ratio from normal to a low R/FR ratio significantly increased endogenous SA levels in the shade ecotype plants (Kurepin et al. 2012a). Again, there was a clear positive association between the changes in endogenous SA levels and shoot growth, as only shade (but not sun) ecotype plants increased their shoot growth in response to decreases in the R/FR ratio (Kurepin et al. 2012a).

Therefore, light irradiance (a sum of total light that a plant can absorb, i.e. photosynthetically active radiation) and light quality (a manipulation of individual light wavelengths, especially R and FR) have different effects on endogenous SA levels (and also on growth). Further, SA levels are associated with growth changes in a different manner, i.e. a decrease in light irradiance causes an increase in shoot elongation, but that increased growth is associated with decreased endogenous SA levels. However, when shoot elongation is induced by a decrease in R/FR ratio, endogenous SA levels rise. Does endogenous SA, then, play any role in these light-induced growth responses? There are reports that exogenously applied SA promotes stem elongation in bean (*Phaseolus vulgaris* L.; Hegazi and El-Shraiy 2007) and applied SA also promotes the growth of isolated stem segments of *Ullucus tuberosus* (Caldas) plants (Handro et al. 1997). In contrast, there are numerous examples where high concentrations of exogenously applied SA have inhibited shoot growth, while lower doses of SA promoted it (Hayat et al. 2010). For sunflower hypocotyls, exogenous SA applied at higher concentrations inhibited growth at lower light irradiances, whereas lower concentrations had no effect on growth (Kurepin et al. 2010a). Similar results (different responses from low versus high SA concentrations) were obtained when sunflower hypocotyl growth was measured under different R/FR ratios across a range of exogenously applied SA doses (Kurepin et al. 2010a).

To summarize, the light irradiance and light quality effects on endogenous SA levels are generally quite different than is seen for other classes of plant hormones that have a long and proven history of causally regulating plant shoot growth. In fact, for each of sunflower and *A. thaliana*, both light irradiance and light quality signaling are known to modify endogenous hormone levels. More specifically, they increase gibberellin, auxin and cytokinin levels, decrease ethylene evolution and generally have a nil effect on abscisic acid and brassinosteroid levels (Kurepin et al. 2007a, b, c, 2010b, 2011a, b, 2012b, c). Additionally, only gibberellins, auxin and ethylene have been shown to directly regulate stem elongation growth increases in response to low light irradiance and low R/FR ratio signals for both sunflower and *A. thaliana* (Kurepin et al. 2007a, b, c, 2011a, b, 2012b, c). Since a low R/FR ratio increases endogenous SA levels in sunflower hypocotyls, while low light irradiance has the exact opposite effect, it does not seem reasonable to

assign a direct role to SA in light signaling. Rather, we conclude that the observed changes in endogenous SA levels are more likely to be an indirect result of changes in endogenous levels of other hormones.

4 Temperature and Endogenous Salicylic Acid Levels

Plants can experience a significant stress from exposure to temperatures that are either lower or higher than optimal. Freezing or heat stress administered over a brief period of time will usually result in irreversible damage to plant tissues, and thus to growth and metabolism. However, cold stress, when the temperature does not drop below freezing, is often a reversible event in terms of both plant growth and metabolism. There are many examples in the literature where pre-treatment applications with SA increased cold stress tolerance across a range of plant species. In hydroponically-grown corn (*Zea mays* L.) plants, pre-treatments with exogenous SA or acetyl-SA decreased net photosynthesis, stomatal conductivity and transpiration prior to cold stress, but had no effect on Fv/Fm chlorophyll fluorescence ratio (Janda et al. 1999, 2000). However, upon transfer of these plants to cold temperature conditions, the SA-treated plants fared significantly better than the control, untreated corn plants, in terms of growth and metabolism (Janda et al. 1999, 2000). Pre-treatment of tomato (*Lycopersicon esculentum* L.) fruits with lower concentrations of exogenous methyl salicylate (MeSA) increased their resistance to cold stress and there was also a decreased incidence of decay in low-temperature storage and an increase in the synthesis of PR proteins (Ding et al. 2002). However, pre-treatment of tomato fruits with high concentrations of MeSA had just the opposite effect (Ding et al. 2002). Pre-treatment of germinating radicals of corn, cucumber (*Cucumis sativus* L.) and rice (*Oryza sativa* L.) plants with exogenously applied SA improved leaf and hypocotyl tolerance to cold stress, but had no effect on the radicles tolerance to the stress (Kang and Saltveit 2002). Further, the cold stress-induced electrolyte leakage was lower in SA-treated leaves and hypocotyls, but there was no change in electrolyte leakage from the radicles for all three species (Kang and Saltveit 2002). Pre-treatment of banana (*Musa acuminate* L.) seedlings with exogenous SA (as a foliar spray or via soil irrigation) increased seedling tolerance to subsequent cold stress treatments (Kang et al. 2003). Pre-treatment of potato (*Solanum tuberosum* L.) plants with exogenous SA applied at low doses increased tolerance to cold stress in cultivars with a wide range of cold stress-susceptibility, whereas high doses of SA were not as effective in doing this (Mora-Herrera et al. 2005). Pre-treatment of mature peach [*Prunus persica* (L.) Batch.] fruits with exogenous SA, followed by storage at 0 °C for a four week-period, resulted in higher fruit firmness, lower decay and reduced chilling stress injury than was seen for untreated fruits (Wang et al. 2006). Pre-treatment of hybrid corn seeds with exogenous SA considerably improved seedling emergence, root and shoot length, and seedling fresh and dry weight, all compared with the controls, both at optimal temperatures and under cold stress temperatures

(Farooq et al. 2008). Finally, exogenously applied SA alleviated the cold stress effect on growth of cucumber seedlings (Lei et al. 2010). Thus, there is a plethora of examples where a pre-treatment with exogenous SA has reduced the negative effects of cold stress on plant growth and metabolism.

A. thaliana Col-0 plants grown at 23 °C and then transferred to 5 °C for several weeks, had significantly increased endogenous SA levels when compared to plants that remained at 23 °C for the same time period (Scott et al. 2004). *A. thaliana* mutant lines with reduced SA levels (*NahG*; Larkindale and Knight 2002), deficient in SA levels (*eds5*; Nawrath and Metraux 1999) and possessing elevated SA levels (*cpr1*; Bowling et al. 1994, 1997) were compared to wild type *Col-0* plants at both 23 and 5 °C. Under cold stress conditions, the plants of *NahG* line showed no increase in endogenous SA levels and accumulated considerably more dry weight than did *Col-0* plants (though at 23 °C the *NahG* plants accumulate less dry weight than *Col-0* plants) (Scott et al. 2004). Further, the cold-stressed *NahG* plants also had larger leaves as a result of increases in cell size, but there was no change to leaf number. Although there was no cold stress-related damage in photosystem II in either *Col-0* or *NahG* plants, net assimilation rate after the cold stress treatment was higher for *NahG* plants (Scott et al. 2004). Cold-stressed *eds5* plants had a phenotype similar to *NahG* plants, but possessed even lower endogenous SA levels. Another mutant, *cpr1*, whose plants have a dwarf phenotype at 23 °C relative to *Col-0*, showed even higher dwarfism at low temperatures, and this dwarfism change was associated with ca. 2-fold higher levels of SA, relative to cold-stressed *Col-0* plants (Scott et al. 2004). Finally, canola (*Brassica napus* L.) plants grown at 5 °C for four weeks after germination had significantly higher endogenous SA levels than plants grown at 20 °C.

The application of low concentrations of SA can also influence (increase) the tolerance of plant tissues to short-term heat stress, whereas at higher concentrations SA had either the opposite or nil effect. There are numerous examples of this phenomenon, i.e. mustard (*Sinapis alba* L.; Dat et al. 1998a), tobacco (*Nicotiana tabacum* L.; Dat et al. 2000), *A. thaliana* (Larkindale and Knight 2002; Clarke et al. 2004), Kentucky bluegrass (*Poa pratensis* L.; He et al. 2005), creeping bentgrass (*Agrostis stolonifera* L.; Larkindale and Huang 2005) and pea (*Pisum sativum* L.; Pan et al. 2006). Exogenous SA pre-treatment also improves net photosynthetic rate of leaves of heat-stressed grape (*Vitis vinifera* L.) plants, apparently by maintaining a higher Rubisco activation state and accelerating the recovery of PSII (Wang et al. 2010). Short-term heat stress caused increases in endogenous SA levels, likely as a result of its *de novo* synthesis (Pan et al. 2006), in mustard (Dat et al. 1998b), *A. thaliana* (Kaplan et al. 2004) and pea (Liu et al. 2006) plants during the first 30 min of the stress. The heat-stressed pea leaves also had parallel (with SA) increases in the activities of phenylalanine ammonia lyase (PAL) and benzoic acid 2-hydroxylase (BA2H) (Pan et al. 2006). However, as occurred with grape plants, the initial and substantial increase in endogenous SA following the administration of short-term heat stress diminishes over time. Thus, after 24 h the endogenous SA levels in control and heat-stressed plants were essentially the same (Wang et al. 2004, 2005). Interestingly, it appears that the source of this increased SA in heat-stressed grape

stem and leaf tissue is the roots, as progressive increases in labelled SA transported in xylem from the roots to the shoot occurred coincidentally with the time of the heat treatment (Liu et al. 2008). Other support for high temperature induction of SA synthesis comes from studies with *A. thaliana* mutant lines which have low (*NahG*) or high (*cpr5*) endogenous SA levels. Thus, *cpr5* plants (high SA) were more tolerant of heat stress, whereas *NahG* (low SA) were less tolerant, both relative to *Col-0* plants (Clarke et al. 2004). Also, unlike *Col-0*, the *NahG* plants did not show a short-term spike in endogenous SA levels following the administration of heat stress (Clarke et al. 2004).

Heat stress also causes an accumulation of endogenous abscisic acid (ABA) levels within the first hour in grape (Abass and Rajashekar 1993) and also other plant species (Chandler and Robertson 1994). It was postulated that this increase in endogenous ABA may help plants cope with the excessive transpiration caused by heat stress, e.g., by closing the stomata (Assmann 2010). Also, endogenous ABA levels are reported to increase in plants grown at low temperatures (Assmann 2010). Young grape leaves subjected to short-term heat stress showed rapid increases in endogenous SA and ABA accumulation during the first hour, with a rapid decline over the next 24 h (Wang et al. 2005). Pre-treatment of the grape plants with exogenous SA caused the same increase (in terms of both absolute levels and trend over time) of endogenous ABA (Wang et al. 2005). In a similar manner, heat-stressed young pea leaves showed rapid increases in endogenous SA and ABA (with a peak occurring at about the 25 min mark). Then, levels of both of these hormones declined before the end of the first hour to levels found in unstressed plants (Liu et al. 2006). Pre-treatment of pea leaves with abamine, an ABA-specific biosynthesis inhibitor (Han et al. 2004), gave similar (to control) low baseline endogenous SA and ABA levels in heat-stressed plants, i.e. there was no heat-induced increase in ABA or SA levels (Liu et al. 2006).

Paclobutrazol is a known gibberellin biosynthesis inhibitor (Rademacher 2000) which has also been shown to inhibit the activity of benzoic acid 2-hydroxylase (BA2H), a key enzyme in the final step of SA biosynthesis (Leon et al. 1995). Pre-treatment of pea leaves with Paclobutrazol did not, however, influence the increase in endogenous SA and ABA levels seen when the pea plants were heat-stressed (Liu et al. 2006). Heat-stressed pea plants also showed increased SAG (SA 2-*O*-β-D-glucose, a major conjugated form of SA) levels, peaking around the 50 min mark of the heat stress treatment. However, SAG levels showed no change in Paclobutrazol-treated heat stressed pea plants (Liu et al. 2006). Finally, *A. thaliana* mutants with lower endogenous ABA (*aba1*) (Robertson et al. 1994), or reduced SA (*NahG*) levels were more susceptible to heat stress than wild type lines (Larkindale et al. 2005).

Numerous studies show that there are optimal concentrations for exogenously applied SA, concentrations which can alleviate the symptoms (growth inhibition and reduced metabolism) of both low temperature and short-term heat stress. Additionally, there is evidence that both low temperature and heat stress can increase endogenous SA levels in several plant species. However, studies with SA biosynthesis mutant lines of *A. thaliana* indicate that while endogenous SA levels

are important for increasing tolerance to short-term heat stress, this same conclusion is not applicable to gaining tolerance to low temperature stress. Rather, SA accumulation was associated with increased susceptibility to low temperature stress. Further, based on studies with ABA and SA biosynthesis inhibitors, it seems that the heat-induced increases in endogenous SA levels are not the result of a direct interaction. Rather, there is an indirect interaction, one where heat stress increases ABA accumulation, which then acts to protect the plant from heat shock. Coincidental with elevated ABA, an accumulation of SA also occurs, cause unknown. A similar scenario also seems likely for the apparent ABA-SA interaction in plants subjected to low temperature stress.

5 Water Stress and Endogenous Salicylic Acid Levels

There is good evidence that exogenously applied SA can improve the growth and productivity of plants subjected to drought stress. For example, pre-treatment of bean (*Phaseolus vulgaris* L.; Senaratna et al. 2000), chickpea (*Cicer arietinum* L.; Kumar Patel et al. 2011), rice (Farooq et al. 2010), tomato (Senaratna et al. 2000; Hayat et al. 2008) and wheat (*Triticum aestivum* L.; Singh and Usha 2003) plants with SA by seed imbibition, soil drenching or foliar spray improved the ability of these plants to tolerate drought stress. These SA-treated plants accumulated increased biomass and exhibited a higher photosynthesis rate relative to drought-stressed plants which did not receive SA pre-treatment. Currently, the decreases in plant growth and photosynthesis that occur in response to drought stress are generally attributed to increases in stress-induced biosynthesis of ABA which then functions to reduce water stress by enhancing stomatal closure (Assmann 2010). However, Rai et al. (1986) demonstrated that subsequent application of SA actually antagonizes the stomatal closure that is induced by applied ABA. While this may be correct when both of ABA and SA are applied exogenously, we would postulate that an antagonism between endogenous ABA and endogenous SA, in the regulation of stomatal closure, is unlikely. This conclusion is supported by studies where application of SA at low (optimal) concentrations increased endogenous ABA levels (Shakirova et al. 2003; Bandurska and Stroinski 2005). Further, as presented below for SA (Munne-Bosch and Penuelas 2003; Abreu and Munne-Bosch 2008; Bechtold et al. 2010; Fig. 1) and from a vast literature on ABA (Assmann 2010), moderate drought stress increases endogenous levels of both ABA and SA. Finally, numerous studies using a range of plant species have observed a closure of stomata in response to the exogenous application of SA (Manthe et al. 1992; Chen et al. 1993; Rao et al. 1997; Shirasu et al. 1997; Mateo et al. 2004).

   Munne-Bosch and Penuelas (2003) have worked with field-grown *Phillyrea angustifolia* (L.), a member of the Oleaceae, which is an evergreen bush or a small tree and native to the Mediterranean region. They reported that drought stress of these plants in their natural habitat resulted in an increased accumulation of endogenous SA in leaves, relative to SA levels seen for well-irrigated plants.

However, if drought-stressed *P. angustifolia* plants were allowed to recover by providing irrigation, their endogenous SA levels quickly decreased to the levels of unstressed plants. Further, they demonstrated a significant linear negative correlation between endogenous SA levels of the leaves and the leaf relative water content. That is, as leaf water content increased the endogenous SA levels (per gram DW) decreased. Thus, a direct link between plant water balance and endogenous SA levels was established. Munne-Bosch and Penuelas (2003) also tested the association of photosynthesis with endogenous SA levels. They took this approach since previous work (Singh and Usha 2003) suggested an increase in photosynthetic activity by drought-stressed plants that had been pre-treated with SA. However, Munne-Bosch and Penuelas (2003) found that the drought-induced increases in endogenous SA of *P. angustifolia* plants were associated with decreases in the maximum efficiency of PSII (Fv/Fm), though leaf chlorophyll levels were not affected. Also, irrigating the drought-stressed *P. angustifolia* plants restored the maximum photochemical efficiency of PSII to levels seen for non-stressed, irrigated plants. A later study by Abreu and Munne-Bosch (2008), this time with field-grown plants of common sage (*Salvia officinalis* L.), demonstrated that drought stress increased endogenous SA levels, though coincidentally decreases were seen for total chlorophyll content. Finally, the same study showed that MeSA applied to the drought-stressed sage plants caused decreases in total chlorophyll content. Thus, while pre-treatment with SA can improve growth and photosynthesis of plants subjected to drought stress, plants that are not treated with SA show increased endogenous SA levels that coincide with a decreased growth and reduced photosynthesis.

In experiments with canola (*Brassica napus* L.) plants, a one week drought stress given to two-week old seedlings caused a ca. 3-fold increase in endogenous SA levels (Fig. 1). Interestingly, flooding also increased SA levels, up to 2-fold, relative to well-watered control plants (Fig. 1). It should be noted that leaf biomass growth of the canola seedlings was unaffected by the drought stress (though the plants did wilt). In contrast, flooding caused a ca. 2-fold increase in leaf biomass accumulation relative to the well-watered control plants. The fact that both drought and flooding stresses increased endogenous SA concentrations suggest that the SA increases are essentially a general "stress-related" phenomenon. Therefore, SA may not be causally or directly involved in many of the plant's physiological responses to water stress. Finally, as expected, endogenous ABA levels in these canola leaves increased for drought-stressed seedlings relative to well-watered control. However, there was no increase in endogenous ABA levels in leaves of canola seedlings subjected to flooding relative to well-watered control (data not shown).

An examination of drought stress responses was accomplished for two *A. thaliana* genotypes (*cpr6-1* and C24) which possess inherently elevated endogenous SA levels (Bechtold et al. 2010). The *A. thaliana* genotype *cpr6-1* is a mutant line with constitutive expression of PR1-6 and both of genotypes *cpr6-1* and C24 are known to be highly resistant to biotrophic pathogens (Clarke et al. 1998; Ton
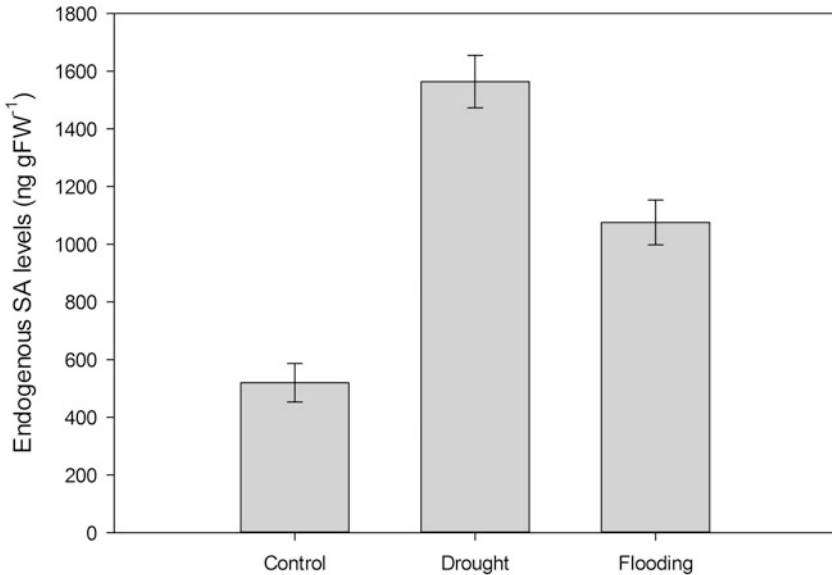
Fig. 1 Leaf endogenous SA levels (ng gFW^{-1}) in three week-old canola seedlings subjected to "normal" watering conditions (watered bi-daily for all growth period), drought stress (watered bi-daily for the initial two weeks of growth) and flooding (watered bi-daily for the initial two weeks of growth and then moved to a tray with water cover above soil level). The harvested leaf tissue was analyzed on LC-MS using stable isotope dilution technique (see above). The error bars indicate one $\pm$SE of the mean and the mean was calculated from three independent biological experiments

et al. 1999). Here, a biotrophic pathogen is defined as a fungus which establishes a long-term feeding relationship with the living cells of a host, rather than killing the host (Zeier et al. 2004). The endogenous SA levels of these two mutant genotypes are ca. 6-fold higher than wild type lines such as *Col-0* and *Ws-0* lines (Bechtold et al. 2010). Finally, plants of the C24 genotype have the same seed yield as *Col-0* and *Ws-0*. In contrast, seed yield of *cpr6-1* plants is ca. 6-fold lower than seed yield of *Col-0* and *Ws-0* plants. Further, when *cpr6-1* and C24 plants were subjected to drought stress, both lines retained their respective seed yield characteristics, whereas the seed yield of drought-stressed *Col-0* and *Ws-0* plants was appreciably reduced (Bechtold et al. 2010). Therefore, under drought stress conditions, the seed yield of C24 genotype remained high, unlike *Col-0* and *Ws-0* plants (and *cpr6-1* plants), all of which had very low seed yield under drought stress. Thus, *cpr6-1* plants fit a "working hypothesis" whereby enhanced drought resistance and enhanced biotrophic pathogen resistance is a result of higher endogenous SA accumulation. In contrast, C24 plants have a "disconnect", where elevated SA levels ensure high seed yields, as well as higher drought and biotrophic fungal resistance.
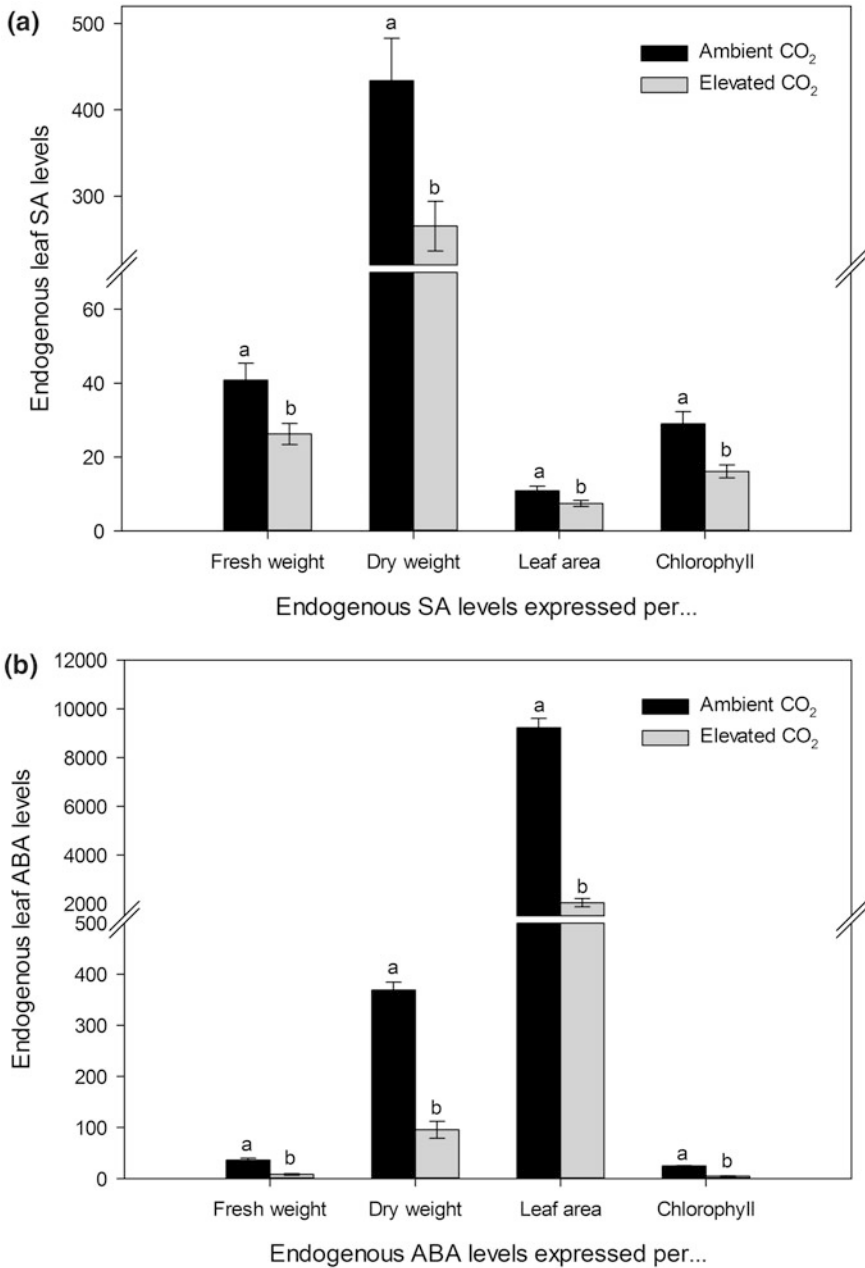
Fig. 2 Leaf endogenous SA and ABA levels in three week-old canola seedlings grown at ambient (340 ppm) or elevated (700 ppm) CO_2 levels from the germination stage. The endogenous SA levels are expressed per fresh weights (ng gFW^{-1}), dry weights (ng gDW^{-1}), leaf area (μg gFW^{-1}) and total chlorophyll content (ng mg^{-1}). The harvested leaf tissue was analyzed on LC-MS using stable isotope dilution technique (see above). The error bars indicate one $\pm$SE of the mean and the mean was calculated from three independent biological experiments

6 Carbon Dioxide and Endogenous Salicylic Acid Levels

Based on Intergovernmental Panel on Climate Change (IPCC 2007) predictions, the atmospheric carbon dioxide (CO_2) concentration is expected to be double (to ca. 700 ppm) by the end of 21st century. Thus, it is important to understand what impact this change will have on plant growth and survival. At present, the effect of elevating CO_2 levels on endogenous SA biosynthesis is poorly understood. In plants of tomato that were grown at each of ambient and elevated CO_2 levels, an infection with root rot (*Phytophthora parasitica*) gave significant increases in endogenous SA levels (Jwa and Walling 2001). However, there was no difference detected in SA levels between infected plants grown at ambient or elevated CO_2 levels. Even so, plants grown at elevated CO_2 showed an increased degree of tolerance to inoculation with the pathogen (Jwa and Walling 2001). In another system elevated CO_2 levels increased the total (but not free) endogenous SA levels in tobacco plants and also led to increased resistance against infection by *potato virus* Y (Matros et al. 2006). Elevated CO_2 levels significantly decreased the endogenous levels of compounds related to SA metabolism, i.e. *myo*-Inositol and two hydroxybenzoic acids (*m*-hydroxybenzoate and *p*-hydroxybenzoate) in leaves of rice (Prins et al. 2011). However, elevating CO_2 had no effect on photosynthesis, photorespiration, leaf C/N ratios or anthocyanin contents (Prins et al. 2011). Finally, growing canola plants at elevated levels of CO_2 significantly decreased the endogenous SA concentrations, relative to canola plants grown at ambient CO_2 levels (Fig. 2). Furthermore, the decrease in SA levels of the high CO_2-grown plants was associated with increased photosynthetic efficiency as well as photosynthetic capacity, both relative to plants grown at ambient CO_2 levels (Dahal et al. 2012). Elevated ambient CO_2 levels also decreased endogenous ABA levels in canola leaves (Fig. 2), though a flush with very high (ca. 50-fold relative to ambient) CO_2 levels had no effect on endogenous ABA levels in grape leaves (Loveys et al. 1973). Growth of Chinese red pine (*Pinus tabuliformis* Carr.) plants for prolonged period under elevated CO_2 levels (ca. 2-fold higher than ambient) significantly decreased endogenous ABA levels in needle tissue (Li et al. 2011). Although decreases in endogenous ABA of plants growing at elevated CO_2 may be beneficial for growth, the decrease in endogenous SA levels could potentially make plants more susceptible to pathogen attack.

7 Conclusion

To conclude, fluctuations in environmental signals such as light, temperature, water availability and carbon dioxide concentrations can all influence endogenous SA levels (Table 1). However, it does not seem likely, based on the literature we have reviewed, that environmentally induced changes in plant SA content are a direct (*per se*) effect of most environmental fluctuations. Nor does it seem likely

Table 1 Summary of the effects of environmental stresses on the plant endogenous SA levels and the plant response to exogenous SA concentrations

Environmental stress	Endogenous SA levels	Effect of exogenous SA
Light irradiance	Concentrations increase as light irradiance increases	Although mutant studies point toward a positive role for high endogenous SA concentrations at high light irradiance levels, exogenously applied SA does not appear to regulate light irradiance-induced changes in growth
R/FR ratio (light quality changes)	SA concentrations increase as R/FR ratio decreases	No definitive evidence that exogenously applied SA regulates R/FR ratio-induced changes in growth
UV light	Increase when UV-C irradiation is administered	Exogenously applied SA alleviates UV-B stress in grasses
Low temperature	Endogenous SA concentrations increase at low temperatures but this is not associated with increased plant tolerance to cold stress	Exogenously applied SA improves plant tolerance to cold stress in many species
High temperature	Endogenous SA concentrations increase at high temperatures and increased tolerance to high temperatures parallels these SA increases	Exogenously applied SA improves plant tolerance to high temperature stress in many species
Drought (water stress)	Endogenous SA concentrations increase as shoot tissue water content decreases	Exogenously applied SA improves the plant's tolerance to drought stress in many species
Flooding	Endogenous SA concentrations increase	Unknown
CO_2 levels	Endogenous SA concentrations decrease under elevated CO_2 levels	Unknown

that the observed changes in endogenous SA levels play a direct role in adjusting plant growth in response to fluctuating environmental signals. Rather, as is evident from application studies with exogenous SA, it appears more likely that changes in endogenous SA levels result from cross-talk with other endogenous plant hormones. Thus, it will be changes in concentration of the other hormones (not SA) that are responsible for adjusting the plant's growth in response to the fluctuating environmental signal. For example, another class of plant hormones, brassinosteroids, when applied exogenously, can alleviate many plant abiotic stress responses (Khripach et al. 1999; Kang and Guo 2011). However, this stress alleviation is likely a result of the brassinosteroid influencing endogenous levels of other plant hormones, which then play a direct role in the alleviation of symptoms of plant stress (Kurepin et al. 2008, 2012b).

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Chapter 6
Impact of Salicylic Acid on the Transport and Distribution of Sugars in Plants

M. S. Krasavina and N. A. Burmistrova

Abstract The article discusses the ways of salicylic acid influence on transport of sucrose and its distribution in plants. The intercellular and long-distance transport along phloem depends on the presence or absence of SA. As a result of sucrose influx in heterotrophic tissues the content of sucrose in the sink organs may increase. Complex interactions between SA, sucrose, Ca^{2+}, ROS and transmembrane electrical potential that occur in the apoplast and at the level of plasma membrane are discussed.

Keywords Salicylic acid · Sucrose · Cell-to-cell transport · Phloem transport · Electric potential

1 Introduction

Carbohydrates play a complex role in plant life. Sugars are the main products of photosynthesis and serve as a source of energy and material for growth and development of heterotrophic organs. Numerous factors are known to control the contents of sugar and their metabolites. Salicylic acid (SA) is a hormone-like signaling molecule which has a direct impact on diverse processes of plant growth and development and transmits messages to all organs about changes in any one of them. On the other hand, SA also affects the biosyntheses and activities of other hormones—ethylene, abscisic acid, and cytokinins.

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2 Effect of Salicylic Acid on the Content of Sugars in Plants

In recent years, a series of observations show the dependence of carbohydrate accumulation in plant cells and tissues on SA. In most of these experiments, spraying the leaves of seedlings and even of adult plants with a SA-containing solution altered the sugar content in the above— and underground organs. The total carbohydrate content in the plant increased, especially that of soluble sugars—sucrose, glucose, and fructose (Khan et al. 2012; Mathur and Vyas 2007; Shaaban et al. 2011; Kaveh et al. 2004; Ghasemzadeh and Jaafar 2012; Khodary 2004; Amin et al. 2007, 2008; Sahar et al. 2011; Dong et al. 2011; Mostajeran and Rahimi-Eichi 2009). This increase in sugar content in various plant organs was positively correlated with accelerated growth in wheat (Shakirova et al. 2003), pea, cucumber, (Farouk et al. 2008), common bean (Gharib and Hegazi 2010), and groundnut (Jayalakshmi et al. 2010). In other experiments, SA also improved germination and vigor of seeds (Gharib and Hegazi 2010), stem diameter, leaf and stem dry weight, leaf area, and leaf index (Bayat et al. 2012). The cause for such activation of growth processes might be due an increase in the turgor pressure in the cells because of the accumulation of soluble sugars and other osmotically active compounds, including proline and soluble proteins (Baghizadeh et al. 2009; Khan et al. 2012). An increase in turgor pressure leads to the enhancement of cell expansion (Morgan 1994). Healthy growth lead to higher biological yield with bold grains with an increase in their length and diameter, their number and weight per plant (Klessig and Malamy 1994; Mathur and Vyas 2007; Farouk and Osman 2011; Shaaban et al. 2011).

It should be noted that SA affects many of the physiological processes that dependents on SA concentration, plant species, developmental stage, and functional state, as well as environmental conditions. The data concerning SA impact on plants are rather contradictory. For example, low SA concentrations enhanced growth of soyabean (Gutierrez-Coronado et al. 1998), maize (Shehata et al. 2001), and wheat plants (Shakirova et al. 2003; Iqbal and Ashraf 2005, 2006), whereas high concentrations caused an inhibitory effect on growth of tomato, lupine, wheat, and maize plants (Haroun et al. 1998; Singh and Usha 2003; Abdel-Wahed et al. 2006). SA effects on sugar content are contradictory as well. Low SA concentrations (below 2.5 mM) increased markedly total sugar content in seeds; the higher concentrations (above 5 mM) reduced it (Haroun et al. 1998; Shehata et al. 2001; Abdel-Wahed et al. 2006). High SA concentrations suppressed not only sugar accumulation but also other processes—growth, yield and photosynthetic rate (Amin et al. 2007).

Beneficial SA impact on plants is significantly manifested especially under stress conditions (Hayat et al. 2010). It is known that, under most abiotic stress, the accumulation of osmotically active compounds occurs, mainly soluble sugars, proline, and soluble proteins (Maria et al. 2000; Al-Hakimi and Alghalibis 2007). For example, at salinity (physiological drought) the content of these osmotica in the cells is usually increased and the content of polysaccharides in the leaves

(Khodary 2004; Sahar et al. 2011) and roots (Maria et al. 2000; Zahra et al. 2010) is reduced. SA treatment of plants subjected to salinity can induce further sugar accumulation (see, for example, Aldesuquy et al. 2012; Khan et al. 2012). Similar accumulation of soluble sugars under the influence of SA was observed also under drought conditions (Loutfy et al. 2012).

In experiments where SA influence on growth and carbohydrate content under unfavorable conditions was studied, the results were ambiguous as well. Along with the enhancement of defensive responses, an increase in the content of osmotica in the cells, induced by water deficit, was further enhanced by SA. However, in some experiments under salinity leaf spraying with SA, several days or even several weeks before the analysis normalized the composition of carbohydrates. When salinity increased marked, after SA treatment the content of soluble sugars and proline, decreased, relative control (salinity without SA treatment), whereas the content of polysaccharides increased (Hussain et al. 2011; Khodary 2004). When the salinity declines the content of reducing sugars, SA treatment stimulated their accumulation (Barakat 2011).

Differences in the direction of SA action on sugar content may be evidently explained by the stress severity. In particular, the degree of salinity determined the response of plant carbohydrate status to SA treatment. For example, experiments performed on tomato plants, a relatively small increase in the salt content in the root medium (25–50 mM NaCl) induced sugar accumulation but leaf treatment with SA (0.5–1.5 mM) reduced their content. In contrast, the higher salt concentration (100 mM) suppressed sugar accumulation, whereas SA increased their content. These experiments once again emphasize the ability of the SA to normalize plant carbohydrate status (Zahra et al. 2010).

SA can exert no direct action but has an impact on the general plant preparation to subsequent stress conditions (priming). Many workers have shown that seed soaking for several hours in the solution with low SA concentrations (10^{-6}–10^{-5} M) modified seedling growth and that of the adult plants even after months (see for example Hayat et al. 2005; Al-Hakimi and Alghalibis 2007). In our experiments, treatment of apical segments of maize roots with 10^{-10}–10^{-4} M SA for several hours did not affect growth during the first day. However, on the second day we observed growth activation at low SA concentrations (Burmistrova et al. 2009). The SA influence during the seed imbibition and at the early seedling growth turned out to be a key factor for subsequent plant growth and yield. In such cases, growth of both above— and underground organs is said to be accelerated (Hayat et al. 2007). For example, two-month-old wheat plants, exposed to water stress, exhibited a decrease in root and shoot dry mass. However, short-term seed soaking in SA solution improved water content, growth and pigment contents in the leaves (Aldesuquy et al. 2012). The seedlings developed from the seeds pretreated with SA were more tolerant to stressful conditions, in particular to infection with pathogens (Khodary 2004; Yarullina et al. 2011). On infection with pathogens SA lead to defense gene expression and hypersensitive cell death in soybean cells (Kawano 2003; Kawano and Furuichi 2007).

Two main processes primarily affect sugar accumulation: (1) the rate at which they are formed in the process of photosynthesis and (2) shift in their consumption at the site of their metabolism. The data on SA influence on these processes are very scarce. There are some reports about SA-induced activation of photosynthesis (Khan et al. 2003; Aldesuquy et al. 2012), chlorophyll and carotenoid synthesis in soybean (Zhao et al. 1995), maize (Khodary 2004), and wheat (Singh and Usha 2003; Arfan et al. 2007). One of the reasons for photosynthesis activation may be decrease in the stomatal resistance which facilitated CO_2 transport to the meso-phyll cells. The effect of SA on the stomatal apparatus is well known (Rai et al. 1986; Khodary 2004). Several researchers reported about the increase in the content of chlorophylls in common bean and wheat leaves (Zhao et al. 1995; Sinha et al. 1993; Khodary 2004; Farouk and Osman 2011; Amin et al. 2008; Uzunova and Popova 2000). El Tayeb and Ahmed (2010) presented the results of Hamade and Al-Hakimi experiments where seed treatment with 100 μM SA enhanced photosynthesis in the plants and suppressed dark respiration under drought con-ditions. Some authors observed SA-induced changes in leaf anatomy: an increase in the thickness of the leaf blade, the thickness of palisade and spongy paren-chyma, changes in the vascular bundle density, the diameter of the midrib (Fa-roukand and Osman 2011; Maslenkova et al. 2009). However, Najafian et al. (2009) reported that spraying of thyme (*Thymus vulgaris*) leaves with SA at the concentrations of 100, 300, and 400 ppm resulted a decline in the transpiration rate (i.e. increase in stomatal resistance).

Under stress conditions, the direction of SA action got a shift. For example, Najafian et al. (2009) reported that SA suppressed photosynthesis in thyme plants, not subjected to stress; however, under salinity SA increased the rate of photo-synthesis. Salinity reduced the content of pigments in wheat leaves, but short-term seed soaking treatment with SA solution prevented this reduction (Aldesuquy et al. 2012). SA affected photosynthetic activity: Rubisco activity, Hill reaction, and quantum yield of photosynthesis. In addition, changes are also observed in the functioning of the stomatal apparatus, the content of chlorophylls, and gas exchange during photosynthesis. The tolerance of photosynthetic apparatus to salinity increased (Khodary 2004). According to Maslenkova et al. (2009), the SA influence on photosynthesis occurs on the membrane level. Since SA is well soluble in lipids, its action may be unspecific, via change in the lipid—protein interactions in membranes. In fact, Uzunova and Popova (2000) observed SA-induced changes in both the composition of the chloroplast envelope and the granum membrane structure. The authors assume that change in the photosystem II functioning, they observed, may be explained just by SA impact on chloroplast membranes.

Photosynthesis activation may be one of the reasons for increased sugar pro-duction in the presence of SA (Barakat 2011). Another reason may be SA-induced activation of enzymes synthesizing sucrose, e.g., sucrose phosphate synthase and sucrose synthase (Dong et al. 2011; Gadi and Laxmi 2012). The additional factor affecting the contents of soluble sugars in plants is their metabolization. This aspect of SA action has not been studied in depth. It is observed that SA improved

the contents of polysaccharides and starch (Maria et al. 2000). In this case, the ratio between soluble sugars and starch changed. According to Dong et al. (2011), treatment of arabidopsis plant with SA resulted in an increase in the contents of glucose, fructose, raffinose, and stachyose in both leaves and roots but the contents of sucrose and starch in the leaves decreased. The hydrolysis of these compounds was possibly a cause for soluble sugar accumulation in leaves. Activation of invertase and amylase after SA treatments is argued as a reason for this assumption (Kaveh et al. 2004; Bernard et al. 2012). An increase in monosaccharides is important for plant growth not only to increase the turgor pressure in the cells but the metabolization of glucose and fructose is used as a source of energy and structural elements. In addition, hexoses participate in plant defense responses via activation of PR-genes (Herbers et al. 1996).

3 Sugar Transport in Plants

Plant growth activation may be a consequence of directed assimilate transport to growing apices. Sugar distribution over the plant is dependent on many complexly regulated processes. Primarily, sugar transport through membranes of intracellular organelles has an important significance. Initially, trioses produced in photosynthetic reactions are transported through the chloroplast envelope into the cytoplasm where sucrose is synthesized. The regulation of this early step of transport is poorly studied. There are few data on SA-induced changes in the chloroplast membrane permeability (Uzunova and Popova 2000; Maslenkova et al. 2009).

Further carbohydrate transport is carried out mainly in the form of sucrose. The need for the delivery of sugar to each cell of a large multicellular organism requires the development of a variety of ways and mechanisms of transport. There are two main categories of this process: (1) the intercellular transport on short distances, so-called short-distance transport and (2) long-distance transport, i.e., assimilate delivery to distant organs.

3.1 SA Impact on Sucrose Transport to the Root

Various mechanisms and various ways of regulation are involved in cell-to-cell and long-distance transport, and they are still far from being fully disclosed. Short-distance transport (cell-to-cell transport) functions in sites of sucrose distribution predominantly between parenchymal cells: between mesophyll cells, between parenchymal cells of vascular bundles and phloem conducting complex (sieve elements plus companion cells), and between the cells in the site of phloem unloading, including those in the root apex.

Along the entire pathway of transport, the exchange between cells occurs. Such an exchange can take place through intercellular bridges—plasmodesmata—or

through the apoplast. Transport through plasmodesmata occurs without crossing cell membranes within the continuous intracellular compartments uniting the specific group of cells in the symplast. The apoplast pathway includes transmembrane exit of substances from the cells into the intercellular space and their subsequent uptake by neighboring cells. The latter way is more energetically costly because it involves active mechanisms of absorption against the concentration (or electrochemical for charged molecules) gradients.

Both the routes operate in plants; but as a rule one of them prevails over the other at a specific time/stage (Patrick 1997; Lalonde et al. 2003; Zhang et al. 2007). Most clearly the way of intercellular transport can be detected by
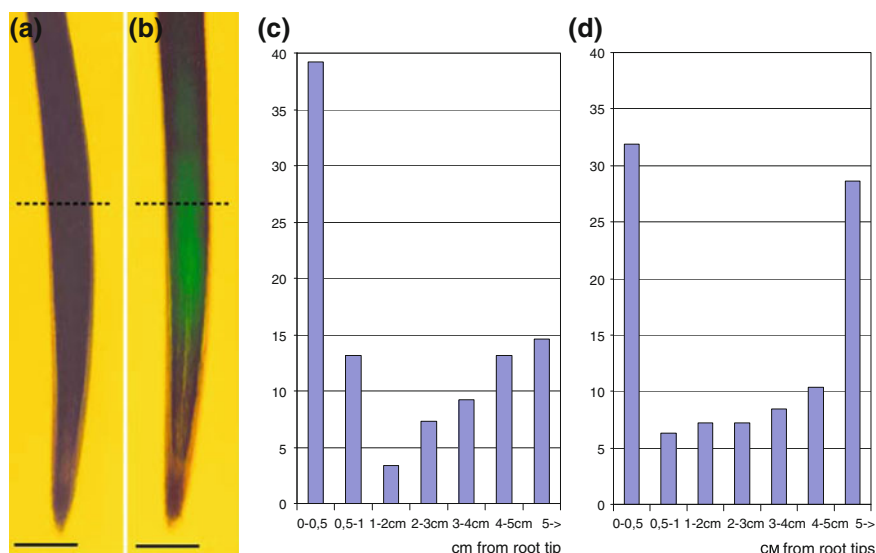
Fig. 1 Distribution of carboxyfluorescein and labeled sucrose coming from the scutellum into the maize seedling root. *Notes* Experiments were performed on the model seedlings with simplified source–sink system: all seedling parts, excluding the scutellum (source) and main root (sink) were removed. Transported compounds (carboxyfluorescein diacetate, ^{14}C-sucrose, and 2-deoxyglucose) were loaded on the scutellum (cotyledon). The scutellum cell absorbed these compounds. Its intracellular esterases cleaved carboxyfluorescein diacetate penetrating through membranes into carboxyfluorescein, which is devoid of this capacity to cross membranes; it can be transported only in the symplast. Loaded 2-deoxyglucose is a substrate for 2-deoxysucrose synthesis in the scutellum cells; it is capable of crossing the plasmalemma, but its further metabolization is limited. At the same time, ^{14}C-sucrose is actively involved in metabolism. Carboxyfluorescein distribution was assessed after fluorescence of the whole root or its thick longitudinal sections. To study sucrose distribution, the root was subdivided into 1-cm segments, which were used for determination of radioactivity or the content of deoxysucrose. **a** control (water was placed on the scutellum); **b** carboxyfluorescein distribution along the root length; **c** deoxysucrose distribution along the root length; **d** ^{14}C-sucrose distribution along the root length. *Y-axis* sucrose in root segments, % from total outflow from cotyledons. *X-axis* cm from root tips. *Dashed line* on Figs. 1a and b point out to transition zone between meristem and elongation zones

comparing the distribution of compounds transported along the symplast or capable of membrane crossing. For an example is transport of carboxyfluorescein, which can not cross membranes and is transported solely via plasmodesmata, and sucrose which can use both the pathways.

The main assimilate sink in young seedlings is the actively growing root apex. Until the development of photosynthesizing leaves, the nutrients come to it from cotyledons. The plant root is a convenient model for studying the transport system, because it permits separation of physiologically and anatomically differing zones with the cells at different developmental stages and manifesting different requirements in nutrients. The root tip comprises of mainly meristematic, actively dividing cells united by a number of plasmodesmatic connections. These cells strongly need structural components for intense synthetic processes and energy for their occurrence. The next stage of cell development, elongation, also requires energy and plastic compounds. These root zones are the strongest sinks of assimilates in the root of seedlings. In the root zone of differentiation with developed functionally different tissues, there is no united symplast. Differences in the structural organization suggest different pathways of compounds entry into the cells.

To study sucrose transport to different root zones, we used a simplified model: maize seedlings with removed endosperm, coleoptile with leaflets inside, and adventitious roots. Only the scutellum (cotyledon) and main root were retained. Transportable compound was placed on the scutellum, which served as a source organ. The root, especially its actively growing apex, served as a sink. A distribution along the root length of compounds loaded on the scutellum was compared. These compounds were the marker of symplast transport, carboxyfluorescein; sucrose evenly labeled with ^{14}C, and deoxysucrose. In the last case, the scutellum was loaded with the non-natural glucose analog, 2-deoxy-D-glucose (2-DG); 2-DG is absorbed by the cells like natural glucose (Zippel and Ehwald 1980).

In the scutellum cells, 2-DG is used for the synthesis of sucrose retaining a deoxy group. Deoxysucrose is transported in the plant similarly as the natural sucrose, but its metabolization is limited. Deoxysucrose is not used in respiration and in the tricarboxylic acid cycle and only is rarely used in the synthesis of polysaccharides. Therefore, it is only poorly consumed in the transport pathway, therefore may be a good marker for the study of sucrose transport.

When observing a distribution of carboxyfluorescein coming from the cotyledon to the root of young seedling, we noted that dye fluorescence was present only in the root tip (Fig. 1a, b). The simultaneously performed cytological analysis showed that dye accumulation occurred in the intermediate zone, e.g., in between the distal region of the meristem and the beginning of the elongation zone whereas in more distal root region fluorescence was absent. These data are in agreement with results performed earlier on other plant material. In these experiments, the accumulation of fluorochromes transported in the symplast was observed in the root tips also (Oparka et al. 1994). In the root apices, sucrose content was also higher than in more distal regions. Thus, the accumulation of the symplast transport marker, carboxyfluorescein, and sucrose occurred in this root region.

Fig. 2 The effect of maize seedling root treatment with 0.1 mM salicylic acid (pH 5.5) on the distribution of ^{14}C-sucrose coming from the scutellum. *Notes* Seedlings were fasted vertically so that the 1-cm root tips were immersed in the SA solution. ^{14}C-Sucrose was loaded on the scutellum. After experiment the root was cut into 1-cm segments, and they were used for radioactivity assay. Exposure duration: **a** 1 h; **b** 5 h. *Y-axis* ^{14}C-sucrose in different root segments, μmol/10 tips. *X-axis* segments, cm from root tip Control—Mes-buffer pH 5.5, Experiment—SA in Mes-buffer
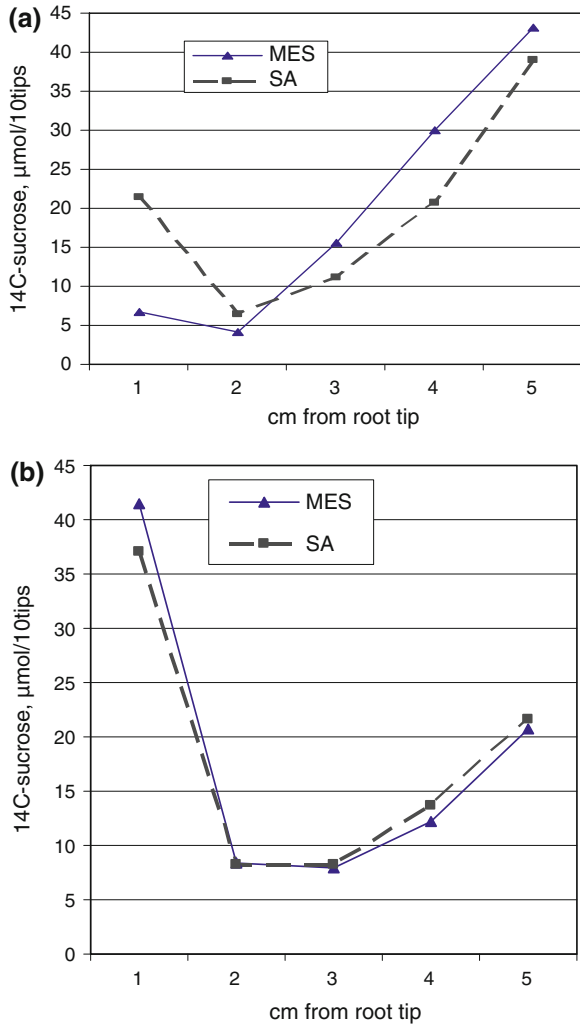
A possibility of symplastic substance distribution in the root apex is in agreement with anatomical data showing that the cells in the tissues of the root apex are connected to each other through a number of plasmodesmata (see Schulz 2005; Patrick 1997; Lalonde et al. 2003). Biochemical data confirm predominantly symplastic sucrose transport in the root apex: transported sucrose was not detected in the apoplast; exogenous sucrose did not suppress sugar exit from the phloem and its distribution between root cells. When unevenly labeled ^{14}C-sucrose was transported, a ^{14}C distribution between hexose components of sucrose molecule was retained (Patrick 1997). Free GFP, for example, moves readily through the root tip when unloaded from the phloem, whereas larger part of the proteins is restricted to a narrow region surrounding the phloem (Stadler et al. 2005b).
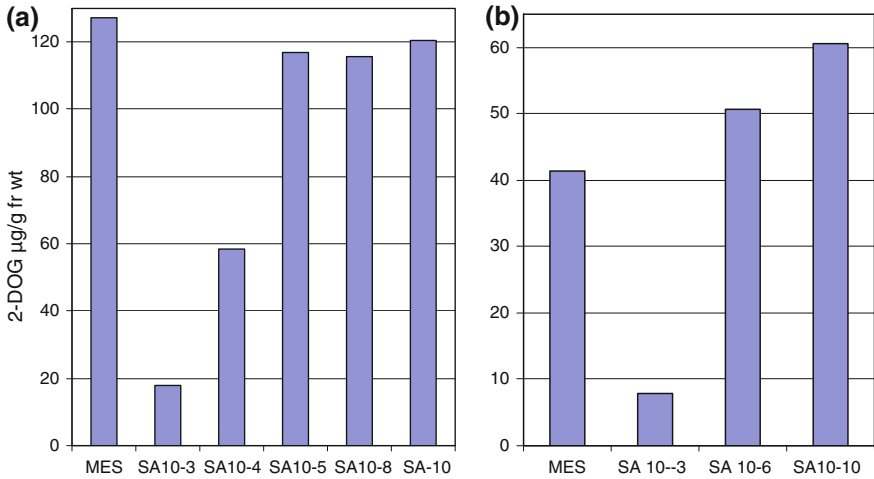
Fig. 3 Effects of different SA concentrations on the DOG-sucrose transport to the root tip. *Notes* **a** at high transport intensity; **b** at low transport intensity. Exposure duration was 5 h. *Y-axis* 2-DOG-sucrose content in 1-cm root tips. *X-axis* control (Mes buffer) and different SA-concentrations

Symplastic unloading dominates in strong sinks, such as root tips, developing fruits, integuments around developing seeds, and in young sink leaves.

In distal differentiated root part, carboxyfluorescein fluorescence was not observed either at the analysis of the whole root (even in the case of the more easily viewed arabidopsis root) or on the maize root cut longitudinally through the middle. However, ^{14}C-sucrose and deoxysucrose were found in all root parts (Fig. 1c, d). ^{14}C-sucrose, which is easily metabolized, was unloaded in the distal root parts more intensely than deoxysucrose. A comparison of these data allows a conclusion that the site of symplast phloem unloading is limited by the intermediate zone between mature meristem and the zone of elongation. Apoplastic unloading evidently occurs along the entire root length.

When seedling root tips were immersed in SA-containing buffer solution, sucrose distribution in the root changed. The effects of SA on ^{14}C-sucrose and deoxysucrose transport into the root tip depended on the exposure duration. Treatment with 0.1 mM SA for 1 h significantly increased compound accumulation, whereas 5-h exposure in this solution did not exert such an effect (Fig. 2).

In the beginning of experiment (in 1 h after sucrose loading on the scutellum) the rate of sucrose influx was slow [about 1 μg/(g fr wt h)]; with time the influx was gradually accelerated; and after 5 h it could attain 20 μg/(g fr wt h). It may be that SA impact on the sucrose accumulation in the root tip depends on the rate of its influx. This is confirmed by experiments with 5-h exposure to SA of plants with the slow or high rate of influx (Fig. 3). The high SA concentrations (10^{-4} M and especially 10^{-3} M) inhibited sucrose incoming from the scutellum independently of the rate of influx. The lower concentration of SA did not induce any change on

the intense influx (Fig. 3a). However, when the influx was weak, SA significantly enhanced sucrose transport even at the concentration of 10^{-10} M (Fig. 3b).

It might be that the enhancement of sucrose transport to the growing part of the root of weakened plants, poorly supplied with nutrients, has an adaptive nature. Similarly, under stress conditions, SA-induced improvement of plant tolerance can be facilitated by improved plant supply with nutrients. Thus, SA affects sucrose symplast transport in the root tip. The fact that SA shifts sucrose symplastic transport depending on its concentration, duration of action, plant physiological state, this allows us to assume that SA involvement in the control of intercellular sucrose transport in the plant.

3.2 Symplastic Ways of Transport, Under SA Regulation

All cells of symplastic domain are connected through plasmodesmata, fine tunnels surrounded by the membrane, which pass across the cell wall.

Through plasmodesmata neighboring cells are directly connected via continuous plasmalemma, the endoplasmic reticulum modified and pressed into the desmotubule, and cytoplasmic annulus (see reviews by Lucas and Lee 2004; Scholthof 2005; Burch-Smith and Zambryski 2012). Via plasmodesmata, cells exchange nutrients and signaling molecules, to coordinate the biochemical and physiological processes in the tissues (Oparka and Roberts 2001; Lucas and Lee 2004; Benitez-Alfonso et al. 2010; Lee et al. 2011; Burch-Smith et al. 2011; Heinlein and Epel 2004; Lucas et al. 2009; Ruiz-Medrano et al. 2004). Transport of specific proteins, transcription factors, and small RNAs through plasmodesmata determines cell morphogenesis and fate. Plasmodesmata can be also used by the pathogens for their spreading over the plant (Khan et al. 2012), although due to the existence of the fine regulatory mechanisms of plasmodesma they can limit the infection spreading (Lee and Lu 2011).

Plasmodesmata open to have the maximum permeability or they can be closed, isolating the cells and creating symplast boundaries and physiological gradients in

Table 1 SA effect on ^{14}C-DMO uptake by the root tip, % of control

SA concentration (M)	Exposure (h)	
	2	5
10^{-5}	91.0 ± 1.1	97.9 ± 5.7
10^{-4}	88.9 ± 1.4	84.8 ± 2.5
10^{-3}	62.0 ± 1.5	58.0 ± 5.6

Notes 1-cm root tips were incubated for 1–5 h in 15 mM Mes containing various SA concentrations. One hour before the end of the exposure, root tips were transferred on similar solutions containing ^{14}C-DMO. Radioactivity was measured in the methanolic extracts. Means and their standard errors are presented

Table 2 SA effect on CO_2 evolution by root segments

Treatment	CO_2 evolution [µl/(h 10 segments)]
Control (MES, pH 5.5)	30.5 ± 1.7
SA 10—5	32.0 ± 3.4
SA 10—4	28.7 ± 1.1
SA 10—3	22.8 ± 1.6

Notes Exposure duration was 1 h. Only SA 10^{-3} M significantly inhibited respiration

tissues at some stages plant developmental. Changes in plasmodesmata permeability in the developing cotton fiber is an excellent example. At the stage of active growth, callose is deposited at the fiber base, breaking the symplastic connections between the fiber and boll cells. This fiber isolation creates turgor pressure required for fiber elongation. The interruption of symplastic connections occurs also in plants entering dormancy. For example, in beach apical meristems plasmodesmata close in autumn when the light period is shortened, whereas, in spring when the bud dormancy is to break, symplastic connections for the sap to move are restored. Isolated symplastic fields arise during the development of shoot apex, embryos and hametophytes (see references in reviews Burch-Smith et al. 2011; Ueki et al. 2010). Compound transport through plasmodesmata depends on environmental conditions: oxygen supply, osmotic balance, light quality (see review by Ueki and Citovsky 2011). The mechanisms of changes in the plasmodesmal conductivity are not still completely disclosed, only some pathways depending on SA are described.

Intracellular pH. One of the causes for SA influence on sucrose transport may be changes in the intracellular pH in its presence. Such changes will affect both symplastic and apoplastic sugar transport. It is well known that transmembrane translocation of organic compounds is determined not only by the gradient of compound concentrations in compartments separated by the membrane but also by a proton-driving force generated in the membrane. Changes in these parameters affect transmembrane absorption of compound by the cells from the apoplast. Medium acidity affects also the plasmodesmal conductivity (Lyalin et al. 1986) and as a consequence symplastic transport. Being a weak acid, SA penetrates into the cell in a non-dissociated form and then dissociates in neutral medium that may change the cytoplasmic pH. The assessment of pH changes under the influence of SA using another weak acid—^{14}C-5,5-dimethyl-2,4-oxazolidindion (^{14}C-DMO)—showed that in fact SA can reduce cytosolic pH but only at its high concentrations. In the presence of 1 mM SA, DMO absorption by the cell was markedly suppressed. Cytosol acidification was also observed at the SA concentration of 0.1 mM but only at 5-h interval (Table 1). The assessment of cytosolic pH using DMO can fail in the detection of small changes occurring in the layer near the membrane. Therefore, it is not excluded that, under the influence of SA, local and may be short-term pH changes occur around the membrane only.

Respiration. Assimilates flow to sinks depending on the sink strength, which is determined among other factors by the intensity of metabolism in the tissue. Since most part of sucrose coming to the root is used in respiration, this process can characterize the degree of sucrose metabolization. Measurement of CO_2 evolution by root segments showed that a significant inhibition of respiration rate occurred only under the influence of 1 mM SA; the lower concentrations were inefficient. Therefore, the effects of most commonly used SA concentrations are not evidently determined by changes in the rate of metabolism (Table 2).

Callose. Callose deposition is well known and is widely applied way for understanding plasmodesma permeability. Callose is a β-glucan polysaccharide, which is deposited in the neck region of plasmodesmata, narrowing their holes and retarding transport. Callose can accumulate around the plasmodesma and function as a sphincter, pressing the hole. It is known since long that callose deposition is a rapid and sensitive plant response to every external stress factor (Currier and Webster 1964; Shimoura and Dijkstra 1975). Callose appears very rapidly, with in minutes after stimulation (Furch et al. 2010; Radford et al. 1998). Mechanical pressure, ultra sound, cooling, or heating, osmotic stress, and also cell damage induce callose deposition (Currier and Webster 1964; McNairn and Currier 1968; Samardakiewicz et al. 2012). Callose plays an important role during pathogenesis, isolating the infected region and hindering pathogen spreading (Van Bel 2003a, b). At normal cell development and functioning, callose is required during cell division, during the formation of cell plate or sieve pores, at protonema differentiation, seed germination, pollen formation and maturation.

The level of callose is determined by the balance between its synthesis and degradation. The synthesis of callose is catalyzed by callose synthase (ß-1,3-glucan synthase), the transmembrane protein localized in plasmalemma. Callose synthase transfer glucose from uridine diphosphate glucose to oligosaccharide chain on the outer side of the plasmalemma (Simpson et al. 2009; Zavaliev et al. 2011). Callose degradation occurs with the involvement of ß-1,3-glucanase localized on plasmodesmata (Levy et al. 2007). The intracellular content of callose can be changed by detergents, polycations, agents interacting with phospholipids (polymyxin B, phospholipase), i.e., by affecting membrane fluidity and permeability (Köhle et al. 1985). The effects on callose synthesis and breakdown are one of the ways of SA action on the plasmodesma capacity for transport.

Tobacco plant leaves or epidermis strip treatment with SA resulted in the appearance of points of callose fluorescence in the cell walls (Krasavina et al. 2002). Callose deposition can be noted as early as with in 1 h, after treatment with SA; the reaction is reversible, and fluorescence disappeared after SA removal.

The cause for callose accumulation under the influence of SA treatment may be its action on activities of both enzymes determining callose accumulation in the cell, callose synthase and ß-1,3-glucanase. Arabidopsis plant treatment with SA activated expression of *AtGSL*5 as early as within 2 h; this gene encodes a protein homologous to the catalytic subunit of ß-1,3-glucan synthase (Østergaard et al. 2002). Such activation was transient and in 16 h the content of mRNA reduced to the initial level

even in the presence of SA. The *mpk* 4 mutant accumulating much SA concentrations is characterized by the accumulation of *AtGSL*5 transcript (Petersen et al. 2000). In this case, activity of ß-1,3-glucan synthase was increased and callose was accumulated (Østergaard et al. 2002). When the *mpk* 4 mutant contained the bacterial gene *Nah*G preventing SA accumulation, expression of *AtGSL*5 gene was suppressed. This argues for SA requirement for *GSL*5 gene functioning.

The content of a protein degrading callose, ß-1,3-glucanase, depends on SA as well. In our experiments, tobacco leaf treatment with SA (3–20 h) resulted in the suppression of ß-1,3-glucanase activity (Serova et al. 2006). Such suppression was correlated with callose accumulation. However, ß-1,3-glucanase not only degrades callose and in such a way is involved in the control of plasmodesma permeability, it is one of the main defensive proteins. May be therefore, at longer SA treatment, the synthesis of this enzymes was induced simultaneously with the development of defensive processes (Shah and Klessig 1996; Kang et al. 1998; Zhen and Li 2004).

Callose deposition around the plasmodesma is an important element of defense against the pathogen. However, callose deposition around the site of infection is not a universal way of plant defense. Thus, at fungal infection, callose synthesized by the plant supplies to the fungus an additional nutrition, stimulating its growth (Jacobs et al. 2003). After identification in *Arabidopsis thaliana* glucan synthase-like gene *AtGSL*5 (Østergaard et al. 2002), plants with silenced callose synthase gene *GSL*5 (Jacobs et al. 2003) were produced. In such plants, wound callose and callose forming papillae at infection with powdery mildew and other fungal pathogens were absent. In spite of the absence of callose, plant resistance did not reduce and papillae were formed but using other compounds. Similar data were obtained on the arabidopsis *pmr*4 mutant, which did not synthesize callose at infection with powdery mildew; such plants were even more resistant than wild-type plants. It turned out that the cause for improved resistance in plants deficient in callose was the activation of SA-depending responses (Nishimura et al. 2003). The authors concluded that callose formation was on the one hand a rapid plant response to fungal infection and on the other hand results finally in the inhibition of SA-dependent defense responses.

Cytoplasmic calcium. The callose content depends on cytoplasmic calcium which is essential for the synthesis of callose, most likely due to its direct effect on the Ca-sensitive callose synthase (Köhle et al. 1985; Kauss 1985). In the presence of chelator (EDTA), the deposition of callose is sharply reduced (Eschrich 1965; Köhle et al. 1985). The inhibitors of Ca^{2+}-channels, nifedipin and gadolinium, also block the synthesis of callose (Kartusch 2003). Stress exposure, induces the entry of calcium into cells that causes an increase in its concentration near membrane, activating callose synthesis (Bhuja et al. 2004). SA can act similarly.

Cytoskeleton. The actomyosin complex may be involved in the regulation of intercellular transport through plasmodesmata. Both actin and myosin were immunolocalized to these transcellular channels (Blackman and Overall 1998; Radford et al. 1998; Aaziz et al. 2001; Baluška et al. 1999, 2001, 2004). The inhibitors of actin cytoskeleton, cytochalasin and latrunculin B were interrupted with callose

deposition (Kobayashi and Hakuno 2003). As a consequence, plasmodesmal conductivity in tobacco leaves increased (Ding et al. 1996) and the electric resistance of plasmodesmata in the root of the aqueous plant *Trianea bogotensis* and in trichomes of fern *Salvinia* decreased (Krasavina et al. 2001). Therefore, the movement of compounds along plasmodesmata, including macromolecules, is activated. The stabilizer of actin filaments, phalloidin, in contrast blocked the intercellular transport (Ding et al. 1996). According to some views, actin filaments may form "rails" within plasmodesmata, along which some substances may move driven by the myosin-based motor (Roberts and Oparka 2003). There is evidence that modifiers of cytoskeletal elements behave differently in different types of plasmodesmata: some of the plasmodesmata increase their conductivity, but in others the inhibitors and stabilizers have no effect or even inhibit transport. Since cytoskeleton functions within the plasmodesmata change with cell development, such variability may be explained by a difference in the stage of cell development and even at the level of plasmodesmata within a single cell. Nevertheless, it is clear that the cytoskeleton and associated proteins play an important role in the regulation of intercellular transport, primarily by interacting with a transportable substance and directing it to plasmodesma (White and Barton 2011).

Calcium controls both callose synthesis and cytoskeleton functioning. Some calcium-dependent cytoskeletal elements were found in plasmodesmata, for example centrin, a calcium-binding contractile protein (Baluška et al. 1999), whereas, in the cortical endoplasmic reticulum associated with plasmodesmata, the calcium-sequestering protein calreticulin was detected (Baluška et al. 1999, 2001). It might be that the elevation of calcium content in the cells affects just these plasmodesmal components (Holdaway-Clarke et al. 2000). SA can act by the regulation of cytoplasmic calcium content; transiently increasing its concentration in the near-membrane layer, SA may change the content of callose and activity of the cytoskeleton.

Reactive oxygen species (ROS). ROS take an important place among factors affecting callose deposition around the plasmodesmata: higher be the level of ROS more is the callose produced (Benitez-Alfonso et al. 2010). Localization of some redox compounds, peroxidases and thioredoxins near plasmodesmata or inside them contribute to the dependence of callose synthesis on ROS (Ehlers and van Bel 2010; Fernandez-Calvino et al. 2011).

Chloroplasts and peroxisomes are known to be the main ROS producers in plants whereas mitochondria generate less ROS (Foyer and Noctor 2003). It turned unexpected that ROS influence on plasmodesmata depends on the site of their generation. Using agents enhancing ROS formation in chloroplasts (paraquat) or mitochondria (salicylhydroxamic acid, SHAM), it was observed that these ROS exerted different effects on plasmodesma conductivity. Thus, ROS produced in mitochondria activated intercellular transport of green fluorescent protein, whereas ROS generated in chloroplasts retarded. Difference in the impact of these organelles on symplastic compound transport was also evident from experiments

performed on *ISE*1-silenced *Nicotiana benthamiana.* In these plants, mitochondria are in more oxidized state. It was found that, under conditions of highly oxidized mitochondria and reduced chloroplasts, plasmodesmata enhance their transport ability (Stonebloom et al. 2009, 2012). In such experiments, the creation of different ROS concentrations in the region of plasmodesmata is possible. ROS effects on plasmodesmata are known to depend on their concentration: low H_2O_2 concentrations enhanced plasmodesma transport capacity, whereas high concentrations inhibited it. Since more ROS is generated during photosynthesis than at mitochondrial respiration, the higher ROS concentrations may be attained in chloroplasts, and this might be a reason for transport inhibition.

Plasmodesmal proteins involved in the control of intercellular transport.

- Along with ß-1,3-glucanase (Levy et al. 2007), other proteins localized on plasmodesmata are also involved in the regulation of Class1 reversibly glycosylated polypeptides (**C1RGP**s), whose over-expression suppresses intercellular transport via plasmodesmata (Burch-Smith and Zambryski 2012).
- Receptor-like kinases (**RLK**s) and receptor-like proteins (RLPs) play an important role in the initiation of the signaling chain during development, hormone action, and defense responses. PD-callose binding proteins (**PDCB**s) (Simpson et al. 2009); their overexpression leads to increased callose deposition at plasmodesmata and inhibits symplastic GFP cell-to-cell diffusion (Simpson et al. 2009).
- Serine/threonine kinases (**CRINKLY4**, CR4) are localized mainly on the plasmodesmal membranes connecting the cells of the aleurone layer and surrounding cells. Such plasmodesmata are characterized by a greater diameter as compared with plasmodesmata in other tissues, producing subdomain allowing transport of larger proteins than those transported between the endosperm cells.
- Plasmodesmata Localized Proteins 1–8 (**PDLP1–8**). *PDLP1*-8 is a small family of RLP proteins with domains exposed to the apoplast. Therefore, these proteins served as receptors of extracellular signals (Bayer et al. 2008). Being accumulated in the central region of plasmodesmal channel, the protein primarily creates a physical barrier for transport. In addition, PDLP5 can otherwise interfere plasmodesmal transport functions, e.g., via the enhancement of callose synthesis. It was shown that an increase in the protein content led to callose synthesis activation, whereas a decrease in PDLP5 synthesis interfered with callose deposition and promoted plasmodesmal trafficking activity. It is assumed that PDLP5 affects the membrane and surrounding apoplast, inducing generation of second messengers, which directly affect callose synthase activity (Zavaliev et al. 2011). Like PDLP5, SA also upregulates many isoforms of callose synthase (Dong et al. 2008). Combined presence of these components enhances action of each of them. Callose deposition at the plasmodesma neck induced by SA and PDLP suppresses pathogen spreading. For example, PDLP5 expression retards TMV movement directly or via stimulation of SA accumulation and then SA retards virus transport (Murphy and Carr 2002). The acceleration of cell death under the influence of PDLP5 may be also explained by SA

overproduction. There is another possible scenario: infection induces the SA synthesis which induces the synthesis of callose and PDLP5. The effect is enhanced by PDLP5, which, in turn, induces the formation of additional amounts of SA. Callose and PDLP5 together clog plasmodesmata and isolate the infected cell. That is, PDLP5 plays an important role in controlling the plasmodesma conductivity during the salicylic acid-mediated cell death responses

Cytoplasmic proteins. Recently, more and more cytoplasmic protein factors involved in the regulation of plasmodesma conductivity are opened. Some of them function in coordination with callose. Among such proteins is ankyrin repeat-containing protein (**ANK**), a cellular factor facilitating transport of nucleoprotein complex between the cells. It is known that, in order to facilitate the passage through the plasmodesmata, TMV-type viruses produce a movement protein, which forms a complex with viral RNA. This nucleoprotein complex binds to the plasmodesmata and increases their conductivity, ensuring the transfer of the complex in a neighboring cell. ANK interferes with this interaction. In mutants overexpressing ANK, virus spreading is enhanced. Conversely, a weak expression of this protein inhibited the spread of viral infection (Ueki et al. 2010). It is possible that ANK interaction with the movement protein explains to a certain extent the long known capacity of the latter to improve the conductivity of the plasmodesmata. It is suggested that ANK binds to the viral protein on plasmodesmata and to the cytoplasmic domain of some subunits of transmembrane callose synthase. As a result, the callose synthesis is suppressed; its content in the vicinity of plasmodesmata decreases, plasmodesma conductance increases, and virus transport is enhanced. Such a possibility of cytoplasmic protein ANK to facilitate the penetration of viral ribonucleic complex through the plasmodesmata may be in use for symplastic transport of their own molecules. Some endogenous plant proteins are known, which, like viral movement protein, can improve plasmodesmal conductivity. It is possible that they also involve ANK in the interaction with plasmodesmata.

Comparing these methods of regulation of plasmodesmal conductivity, we can see that in most cases the end result is changing the content of callose. Protein factors, salicylic acid, and calcium combine their efforts in the stimulation or inhibition of callose deposition at plasmodesmata. In the case of the stimulatory effect, not only the isolation of damaged areas of tissue is achieved, but also isolated symplastic domains are formed, which is a step in the plant development. The inhibitory effect of these compounds on callose synthesis contributes to the intense exchange between cells and tissues of assimilates, hormonal substances, and other signaling molecules that needs to coordinate their functions at intence growth.

3.3 Membrane Transport

Apoplastic movement is an alternative to compound transport through plasmodesmata. In the system of intercellular transport, of apoplastic transport usually occurs only on short distances. Such transport comprises of two steps: compound released from one cell into the apoplast and its absorption by another, usually closely located cell. Both processes include the crossing of cell membranes and also require the maintenance of their integrity.

Each stress (heat shock, drought, salinity, heavy metals and infection) disturbs membrane integrity, which is easily detected after electrolyte or UV-absorbing compound leakage from the cell (Gunes et al. 2005; Al-Hakimi and Alghalibis 2007; Liu et al. 2008). Many researchers showed that leaf spraying with SA or its addition to soil restores membrane semi-permeability and reduces compound leakage (Mishra and Choudhuri 1999; Alpaslan and Gunes 2001; Gunes et al. 2005; Al-Hakimi and Alghalibis 2007; Misra and Saxena 2009; Khan et al. 2012). In this case, absorption of toxic heavy metals, sodium, and chlorine is retarded. SA affects absorption of necessary inorganic cations: potassium, calcium, magnesium, and phosphorus also. Like in the case of other effects of SA, it changes cation absorption in different directions: in some experiments SA activated absorption (Gunes et al. 2005) and in others suppressed it (Hayat et al. 2007, 2010). SA impact on membrane stability can be related to its action on the sugar content. It was noted that, under the influence of SA, the cells of roots under drought conditions accumulate soluble sugars along with of inorganic ions (Ca^{2+}, K^+, Mg^{2+}) and all these components maintain required turgor pressure. Under unfavorable conditions, sugar can exert not only osmoregulation but also osmoprotection, thus stabilizing cell membranes and preventing protein denaturation (Loutfy et al. 2012).

It has been shown a long ago that transmembrane sucrose uptake is determined by the sucrose concentration in the apoplast (Giaquinta 1977, 1979; Guan and Janes 1989; Chapleo and Hall 1989; Turgeon and Gowan 1990). At low concentrations (5–10 mM), the dependence on sucrose is described by the saturating curve whereas at high concentrations, the dependence is linear.

In the saturating phase of the concentration curve, sugar absorption occurs against a concentration gradient and requires energy. The source of energy is a transmembrane proton gradient generated by membrane H^+-ATPase. Therefore, one of the ways for SA action on sucrose uptake by the cells may be changes in H^+-ATPase activity. Since ATPase is a membrane protein, its activity depends on the state of the plasma membrane. SA stabilizing action on the membrane is correlated with changes in membrane-localized ATPase activities. The evidence concerning dependence of this enzyme activity on SA is very scarce. Scattered information in most cases shows that an increase of SA activities ATPases localized in the cell membrane, thus maintains the activity of H^+-ATPase and Ca^{2+}-ATPase, under stress conditions (Sondergaard et al. 2004; Gunes et al. 2005; Liu et al. 2008, 2009a). For plasma membrane vesicles of potato tuber parenchyma cells, studied the dependence of H^+-ATPase activity on SA concentration: 10^{-8}–

Table 3 ^{14}C-sucrose absorption by apical segments of maize seedling roots

Treatment	Uptake (μmoles/g fr.wt 10 tips)	% from MES
MES, pH 5.5	48.8 ± 1.2	100
SA 0.5 mM	20.0 ± 3.8	40.9
SA 0.1 mM	35.1 ± 3.3	71.9
SA 0.01 mM	39.0 ± 3.0	80.0
SA 0.001 mM	55.2 ± 2.4	113.1
PCMBS 0.5 mM	34.8 ± 1.5	71.4
PCMBS 1 mM	33.7 ± 4.3	69.0
PCMBS 5 mM	32.3 ± 3.2	66.2
CCCP 0.01 mM	19.0 ± 0.8	38.9

Notes 1-cm root tips were incubated for 1 h in 1 mM ^{14}C-sucrose dissolved in 15 mM MES, pH 5.5. Inhibitors and SA were added to the incubation medium

10^{-10} M stimulated activity and 10^{-4}–10^{-5} M inhibited it (Ladyzhenskaya and Korablyova 2011). Leaf spraying with SA increased the content of H^{+}-ATPase protein, i.e., enzyme activation occurred on transcriptional and translational level (Liu et al. 2009a). SA action on membrane functional activity may be related to stimulation of ROS generation, which will modify membrane structure by membrane lipid peroxidation (Kawano et al. 1998).

It was established that transmembrane sucrose transport occurs in symport with protons by protein transporters. The genes encoding Suc/H^{+} symport-Suc transporters (SUT) are known as Suc carriers (SUC) (see reviews Lalonde et al. 2004; Sauer 2007; Slewinski and Braun 2010; Kuhn and Grof 2010). Transporters were detected along the entire route of sucrose transport: at sucrose entry into the conducting phloem complex, in phloem cells involved in long-distance transport and in cells participating in phloem unloading (Truernit and Sauer 1995; Kuhn et al. 2003). It was shown that SUT expression depends on light and some hormones (ethylene, gibberellin, ABA) (see references in Ayre 2011). It is quite unclear how SA affects transporter expression and functioning.

At high sucrose concentration in the external solution, its absorption occurs along the gradient of concentration and the rate of absorption (i.e. facilitated diffusion) is linear. Passive transport occurs with the help of recently found proteins Suc-facilitators (SUF) which are member of the class of sucrose transporters SUT. SUF proteins are poorly characterized. They participate predominantly in the passive exit from the cell independent of energy supply and pH gradient. They are not inhibited by para-chloromercuribenzene sulfonic acid (PCMBS) (Zhou et al. 2007). It is assumed that SUF proteins are involved, in sucrose escape into the apoplast. Such a process occurs along the entire phloem route in leaf petiole, stem, and roots. Efflux to the apoplast determines sucrose exit from the mesophyll cells before their uptake into the sieve elements and companion cell during apoplastic phloem loading and exit from the phloem in sinks at apoplastic unloading. Passive transport turned out to be not completely independent of energy supply because it is inhibited at anoxia (Anderson 1983). Recently, one more subfamily of plasma membrane-localized

transporters, SWEET sucrose efflux transporters, AtSWEET11 and AtSWEET12, required for phloem unloading was described (Chen et al. 2012).

The mechanisms of sugar exit from the root cells and their retrieval into the cells, however, are far from elucidation. According to recent data, sugar exit from root cells into the apoplast may be mediated by the same transporters that provide for their entry. Use of FRET nanosensors shows the presence of very rapid exchange on membranes of the external cell layers of root tips. Sucrose accumulation occurred for 10 s; it was completely reversible during 10–180 s (Chaudhuri et al. 2008). It was suggested that one and the same transporter is responsible for both fluxes, into the cell and from the cell. At least one SUT can move sucrose in either direction based on prevailing conditions (Carpaneto et al. 2005). Moreover, it is not excluded that at modest changes in the structure of transmembrane transporter SUT, transferring sucrose against the gradient of its concentration, it can be transformed into SUF operating along the gradient; with its help, sucrose escapes the cell passively (Reinders et al. 2002; Kuhn et al. 2003; Ayre 2011).

In the root tip, the main way for sucrose exit from the phloem is symplastic one; its transmembrane transfer does not play a great role in this process. However, some proportion of sucrose coming to the root evidently leaches into the apoplast and should be absorbed by adjoining cells via transmembrane mechanisms. In experiments with the absorption of exogenous ^{14}C-sucrose by 1-cm segments of the maize seedling root tips, the inhibitor of H^+/sucrose SUT symporters, PCMBS, which can not penetrate through the membrane, inhibited absorption by 30 %. This inhibition depended weakly on PCMBS concentration in the range of 0.5–5 mM. It seems likely that this 30 % inhibition is a maximal one and only 30 % of transmembrane absorption is determined by symporters; a "passive" translocator of SUF type that may participate in the uptake of other part of sucrose. A protonophor CCCP exerted much stronger inhibition, evidently affecting intracellular metabolization of absorbed sucrose. SA affected sucrose absorption from the solution: 5×10^{-4} and 10^{-4} M SA suppressed sucrose uptake, whereas 10^{-6} M SA weakly but significantly stimulated it (Table 3).

3.4 Sugar Transport Along the Phloem (Long-Distance Transport)

Sucrose synthesized in the mesophyll cell cytoplasm moves to the phloem of vascular bundles. The main pathway for its movement between the mesophyll cells and parenchymal cells of vascular bundle is a symplastic one. During this movement, the regulatory mechanisms described in the Sect. 3.2 operate. Into the phloem (sieve element/companion cell), sucrose can penetrate differently (Gamalei 2004). Different plant species used varied loading ways (Rennie and Turgeon 2009; Slewinski and Braun 2010; Liesche and Schulz 2012): (1) symplastic loading, when plasmodesmal routes are not interrupted and sucrose is transported

passively along the gradient created in the mesophyll; (2) apoplastic loading, when sucrose exits into the apoplast and is absorbed actively across the membranes of specific companion cells (intermediate cells); (3) the mechanism of Suc polymer trapping, when in companion cells oligosaccharides are synthesized from sucrose and this synthesis affects the osmotic potential of these cells. The oligosaccharides and sugar alcohols are transported along the phloem together with sucrose. After loading into the phloem, sugars move by bulk flow along the gradient of the hydrostatic pressure generated on the both ends of sieve tubes (Srivastava et al. 2009; Turgeon and Medville 2011).

Long-distance transport occurs along the vascular system, sieve tubes consisting of sieve elements separated by sieve plates. Transport along sieve elements is metabolically and energetically provided by companion cells connected with sieve elements by specific, branched plasmodesmata with opened pores from the side of the sieve element. Sieve element and companion cells represent a complex for long-distance transport.

It is believed since long that the phloem transports assimilates over long distances in isolation from the surrounding tissues (Kempers et al. 1998). However, exchange with flanking tissues is inevitable because sugars are required for the development of these tissues and to perform the function of nutrient storing. Therefore, along the entire pathway of phloem transport, sucrose exits from the transport channel into the apoplast may be retrieve into the phloem cells with the help of transporter AtSUC2 (Srivastava et al. 2008). According to Minchin et al. (Minchin and Thorpe 1987), the output from the transport phloem is about 6 % of transported sugars, and return loading in the phloem is about 3 %.

After reaching the terminal sink tissues, sucrose abandons sieve elements and is distributed between the cells. The way of exit from the conducting complex differs in different tissues. In growing sinks, both exit and further distribution occurs in symplast (Ruan and Patrick 1995; Stadler et al. 2005a, b). In the organs accumulating compounds and at the sites of contacts between daughter and mother tissues, symplastic pathway is interrupted by apoplastic one (Patrick 1997; Lalonde et al. 2003; Zhang et al. 2006, 2007).

Sieve elements are connected with each other by sieve plates performed with numerous pores; the endoplasmic reticulum and cytoplasmic strands pass through these pores. Pore field of sieve plates manifests enhanced permeability. This is the main pathway providing for mass transport to the consuming sinks. The rate of phloem transport is determined by the length of the channel, density of pores on the sieve plate and their diameter (Thompson 2006).

Sieve pores are functional analogues of plasmodesmata. Therefore, many features of regulation of pore permeability are similar to those of plasmodesma conductivity regulation. This is well known characteristic of callose to clog sieve pores. There is a correlation between callose deposition and assimilate transport in plants. Thus, when sieve elements are damaged, callose deposition on sieve plates is activated (Eschrich 1965; Esau and Thorsch 1985; Cronshaw and Esau 1968; Evert and Derr 1964; McNairn and Currier 1968). Callose deposition is detected as early as within 20 min, after injury (Mullendore et al. 2010). In this way, damaged

sieve tubes, non-functioning, for example, in the winter or those, which do not cope with too rapid assimilate flow, are temporarily excluded from the path of assimilate movement. Callose mechanic rigidity makes plugging the conductive pathways the important way to protect plant against the spread of pathogens (Van Bel 2003a, b).

Callose deposition on the sieve plates is reversible: callose disappears when the damaging factor is no more. Maize mutant deficient in sucrose export (*sxd*1) is characterized by enhanced callose deposition in vascular tissues of source leaves, between bundle sheath and vascular parenchyma cells (Botha and Cross 2000). It might be that just callose causes retardation of assimilate loading into the phloem of such mutants. In another mutant with disturbed glucan synthase-like7, one of the callose synthase isoforms, callose content is reduced only on sieve plates. This mutation did not affect general plant phenotype but disturbed flowering stem growth and reduced the size of all flower parts. Since assimilate movement suppression induced a decrease in sugar content in the flowering axis, it was assumed that growth retardation was a consequence of carbohydrate starvation. Thus, a regulated callose deposition on the sieve plates is a process necessary for normal phloem transport regulation (Barratt et al. 2011).

There is a lot that is common in the regulation of sieve plate pore and plasmodesma conductivity. Thus, the remnants of destroyed plastids and filamentous P proteins, natural components of sieve elements, can accumulate in pores and interfere with the assimilate flow in the phloem. A dense network of endoplasmic reticulum can also inhibit phloem flow (Knoblauch and van Bel 1998). Since the rate of accumulation of such cytoplasmic components depends on the rate of flow, therefore the rate of phloem transport may also affect the conductivity of sieve plate pores. However, the significance of this physical pore blockage has been questioned (Froelich et al. 2011). The causes and effects on phloem transport of other protein structures of sieve elements, forisomes, are also doubtful. Only recently, when phloem flow was simulated artificially via "sieve elements," the confirmation of such forisome function was obtained. It was shown that, in the presence of Ca^{2+}, lens-like forisomes swelled so strong that clog "sieve pores" (Hafke et al. 2009; Knoblauch et al. 2012).

Besides considerations related to the analogy between the transport through plasmodesmata and the sieve pores, we have no information on the participation of SA in the mechanism of substance long-distance transport.

3.5 Effect of Salicylic Acid on the Electric Potential of the Membranes

Since SA affects cell membrane permeability and ion fluxes across them (review by Krasavina 2007), it may not be surprising to correlate it with the observed effects on changes of the membrane potential (MP). As early as in 1974, Glass and

Fig. 4 Salicylic acid action on the local response of cucumber leaf to cooling. *Notes a* control, *b* 0.1 mM SA, *c* 10 mM SA. Extracellular recording
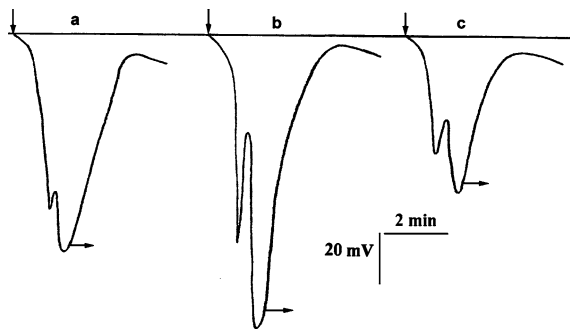
Fig. 5 SA action on action potential in the *Chara corallina* cells. *Notes 1* Control; *2* 0.1mMSA , *3* 1 mM SA. Recording with microelectrodes. Irritation with the electric current pulse
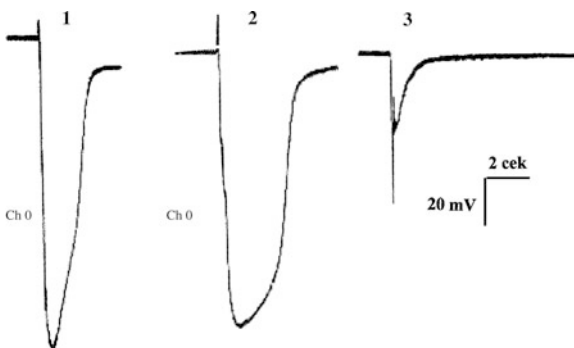
Dunlop (1974) described membrane depolarization in the presence of SA. Later, similar fact was noted by other researchers (Lyalin et al. 1986; Gordon et al. 2002). In plant cells, low negative MP values were recorded (–200 mV and even lower). The value of MP is determined by two groups of processes. Firstly, passive ion diffusion across the membrane along the gradient of concentration and membrane permeability for a corresponding ion. Secondly, an active proton ejection from the cell by H^+-ATPase. SA can affect both processes.

The main ions determining potential in plants are H^+, K^+, Ca^{2+} and anions (Cl^- mainly). Sugars are known to be transported through plant membranes together with proton. During this process, the membrane is temporarily depolarized. Stimulation of proton absorption by the cells simultaneously with sugars is one of the ways of sugar transport interaction with electrogenesis. Another ion, which transport results in membrane depolarization may be K^+. Potassium can entrance into the cell, like proton, and this shifts the potential to the region of positive values. In stress condition the initial step in changes of processes participating in the generation of electrical charge difference on the membrane is Ca^{2+} entrance. Ca^{2+} flux induces small initial depolarization, which is enhanced by fluxes of other ions. Cell membrane depolarization is the basics for essentially all electrical responses and is the initial step of the chain of signal transmitting. Extreme factors induce rapid MP changes and generation of diverse impulse activity. Thus, short-term (105 s) cooling to 8–9 °C of the leaf region of the cucumber plant growing at room temperature

induced generation of local, not spreading over long distances impulses (Prudnikov et al. 2010). The pharmacological analysis showed the involvement of ionic channels in pulse generation. The first depolarizing stage of the pulse activity was sensitive to inhibition of voltage gated and mechanosensitive calcium channels of plasmalemma. Lantan, a non-specific Ca^{2+} competitor for binding sites, suppressed initial depolarization efficiently. Gadolinium, the inhibitor of mechanosensitive Ca^{2+} channels, and ruthenium red, inhibitor of Ca^{2+} exit from intracellular depots, and also verapamil inhibited this process. The action of these inhibitors indicates the involvement of calcium penetration into the cytoplasm during initial steps of depolarization. Calcium can entry from the apoplast (effects of lantan, gadolinium, verapamil), as well as from intracellular depots (action of ruthenium red). These data demonstrate clearly the role of calcium in the formation of local responses, primarily at the stage of initial depolarization. The initial slow depolarization was followed by a fast depolarizing shift, which was sensitive to the anion channel inhibitor 4-acetamido-4′-isothiocyano-stilbene-2,2′-dilsulfonic acid. At the next stage repolarization developed. The blockers of potassium channels, tetraethylammonium and quinine sulfate, suppressed the second stage of the impulse, that means the participation K^+ channels at stage of repolarization. Both stages of electric pulse generation were retarded by the H^+-ATPase inhibitors, sodium orthovanadate and dicyclohexylcarbodiimide, which implies the involvement of the proton pump in the origin of electric pulses examined.

Pretreatment of intact leaf on plant with SA for 1 h changed parameters of electric response to cooling of the leaf region (Fig. 4). At the concentration of 0.1 mM SA increased markedly the amplitude of response and lowered the speed of initial depolarization possibly through the effect on the calcium entry. The higher SA concentrations resulted in the stress response and a decrease in the rate and amplitude of depolarization.

SA also affected the generation of another form of electric response to irritation, e.g., the action potential (AP). As distinct from the local response, AP spreads over the plant and can induce changes in distant plant parts. After *Chara* cell treatment with SA, the shape of generated impulse changed (Fig. 5). The response of Characea algae to excitation is much more rapid than the local response recorded for the whole tissue. In our experiments *Chara* cells were not detected the initial slow phase of depolarization. However, it was well notable that in the presence of 0.1 mM SA the shape of impulse changed: the phase of repolarization determined by potassium outflow started later and its rate was lower. The inhibitor of potassium channels TEA induced similar changes in repolarization phase, which confirms dependence of K^+ exit from the cell on SA. SA action is not explained by its properties as an acid because another weak acid, acetate, did not change the shape of the impulse. The inefficient, in defensive responses, SA analogue, 4-oxybenzoic acid, was also inactive in the action on the shape of impulse. This suggests a specificity of SA action.

In these experiments, tissue pretreatment with SA was used. In the suspension of tobacco cells, during SA treatment the content of free Ca in the cytosol increased rapidly, as early as in 10 s, whereas the highest increase was observed in 90 s.

Lantan and gadolinium inhibited Ca^{2+} accumulation in the cells. These inhibitors suppressed initial phases of electric pulse generation. Ca-chelators, BAPTA and EGTA, eliminated Ca^{2+} content elevation in the cells, but did not interfere with membrane depolarization, i.e., only Ca^{2+} removal from the apoplast is not sufficient for depolarization; Ca-channels should operate (Kawano et al. 1998).

4 Conclusions

Salicylic acid is a phenolic compound manifesting many features of phytohormones. This includes numerous physiological responses that depend on its presence. The spectrum of SA action is wide; therefore, it is very difficult to identify a single key process determining such diverse effects. There are some reports about the early action of SA: this is redox balance in the apoplast. This effect was observed as early as several seconds, when other changes were yet undetectable.

The presence of benzolic ring with several double bonds, hydroxyl and carboxyl groups allow SA to be involved in redox reactions. In fact, SA addition to the tobacco cell suspension culture resulted in the formation of superoxide O_2^- in the redox reaction with the involvement of H_2O_2 as an electron acceptor. The accumulation of O_2^- was suppressed by exogenous SOD converting superoxide into hydrogen peroxide. In this case, SA served an electron donor. Kawano et al. (1998); Kawano (2003); Kawano and Furuichi (2007) suggested the following scheme for this process. SA induces the accumulation of hydrogen peroxide in apoplast by inhibiting catalase. Electron transfer from guaiacol peroxidase secreted into the apoplast to H_2O_2 converts initial form of enzyme (Fe^{3+}) into oxidized form (compound-1, Fe^{5+}). Compound-1 interacts with SA molecule and is reduced to compound-II (Fe^{4+}). As a result the free radical SA' is produced. Transfer of the second electron from another SA molecule to peroxidase (compound II) returns the enzyme to the initial form (Fe^{3+}) and the second SA-radical is generated. Free SA' radical reduces molecular oxygen to generate superoxide O_2^-. The SA devoid of two electrons on oxidation produces (SA^+ is formed). Thus, in this series of reactions, not only superoxide is formed but SA is conversed into the free radical enhancing oxidative burst (Kawano et al. 1998; Kawano 2003). Not only guaiacol peroxidase but also ascorbate peroxidase may be a participant in this process, even though the latter reaction is very slow. Low SA concentrations do not induce ROS accumulation leading to the cell death. In contrast, non-toxic elevation of ROS content serves a signal for the activation of defensive responses in the cell. High SA concentrations can inhibit antioxidant activity and trigger other processes leading to the cell death.

Sugars are also involved in redox processes in the cells. They enter into metabolic pathways that generate both ROS (Oxidative Phosphorylation) and the reducing force NADH and NADPH (oxidative pentose-phosphate pathway), which dissipate (scavenges) these ROS (Couee et al. 2006). Another mechanism of interaction between sugars and redox reactions are non-enzymatic oxidation of hexoses (autooxidation) (Russell et al. 2002). Glucose can be oxidized in the presence of

trace amounts of transition metals or reduce molecular oxygen. As a result, ketoaldehydes, H_2O_2, and various free radicals are generated. Hydroxyl radical oxidizes thiol bonds in proteins and induces their structural changes and even fragmentation. Ketoaldehydes can bind to lysine groups of proteins inducing their oxidative damage. Thus, even without engaging in enzymatic reactions, sugars and SA can cause oxidative stress (Russell et al. 2002; Wolff and Dean 1987; Hunt et al. 1988). This is the first stage of interaction between SA and sugars with ROS.

ROS generated in the apoplast affect ionic channels in the plasma membrane. As early as in several seconds after ROS generation, ROS-sensitive Ca^{2+}-channels are activated (Pei et al. 2000; Keller et al. 2008) and cations start to come into the cell. Further situation is complex and contradictory (Qudeimat and Frank 2009). Primarily, initial elevation of calcium level near the membrane can trigger membrane depolarization, which is dependent on the activation of potential-dependent and redox-dependent anionic and potassium channels (Schroeder and Hagiwara 1989; Zeidne et al. 2001; Siegel et al. 2009). Symport of sugars and proton contributes to the process of depolarization. Thereafter, potential- and Ca-dependent ionic channels are activated and diverse forms of electric activity are generated (local nonspreading pulses, variation potential, and action potential) or their character is changed.

The other way of events development is as follows: Ca^{2+} entrance activates NADPH-oxidase in the plasma membrane, produces superoxide and elevates ROS level. SOD dismutes superoxide into H_2O_2, which via peroxiporin-channels comes into the cytoplasm and induces Ca^{2+} exit from the intracellular depots, enhancing Ca^{2+} accumulation in the cytosol. Then Ca^{2+}-ATPase is activated controlling calcium content. The interaction of processes leading to the calcium entrance into the cell and Ca^{2+}-ATPase functioning releasing the cation from the cell results in the formation of Ca-waves specific for a given acting factor and differing in amplitude, duration, localization, and frequency (McAinsh and Pittman 2009; Allen et al. 2001; Arimura and Maffei 2010; Qudeimat and Frank 2009).

The change in the content of free Ca^{2+} and H_2O_2 in the cytosol may be a key signal for many regulatory processes. The subsequent pathway of calcium signal transmission includes kinases and hormones (jasmonic and salicylic acids) activities. An increase in H_2O_2 content can also induce SA synthesis, for example, in tobacco (Neuenschwander et al. 1995) and *Arabidopsis* (Summermatter et al. 1995). The signal of SA is enhanced. The sequence of responses observed in tissue culture is as follows: superoxide is detected in several seconds after SA addition, and later, approximately in 10 s, Ca^{2+} accumulates. Changes in H_2O_2 and Ca^{2+} equally depend on inhibitors—these processes are interrelated.

Thus, in spite of the complex tangle of interactions between SA, sucrose, calcium, and ROS, it becomes clear that each element can act as a source of the signal path and cause multiple chains of reactions.

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Chapter 7
Endogenous ABA as a Hormonal Intermediate in the Salicylic Acid Induced Protection of Wheat Plants Against Toxic Ions

F. M. Shakirova, M. V. Bezrukova and D. R. Maslennikova

Abstract We have previously suggested that endogenous abscisic acid (ABA) may play a role of hormonal intermediate in the implementation of the salicylic acid (SA) induced protection of wheat plants against abiotic stress factors. With the use of an inhibitor of ABA biosynthesis fluridone there were obtained experimental arguments in favor of the key role of rapid reversible accumulation of ABA during the SA-treatment and maintaining elevated levels of ABA in SA-pretreated seedlings subjected to cadmium stress and salinity in the implementation of pre-adaptive and protective action of SA on wheat plants, respectively. Thus, it was detected that pretreatment of wheat seedlings with fluridone prevented SA-induced accumulation of ABA under normal conditions and maintenance under stress of increased ABA content in plants pre-treated with SA. This was manifested in inhibition of SA-induced effects: generation of ROS, activation of phenylalanine ammonia-lyase and antioxidant enzymes and deposition of lignin in the cell walls of roots, as well as the accumulation of wheat germ agglutinin, proline and enhanced transcription of *TADHN* gene coding for dehydrin that are making an important contribution to the development of plant resistance to oxidative stress and dehydration. In general, this is reflected in the prevention of SA-induced wheat resistance to the effects of toxic ions, as judged by the level of accumulation of MDA, release of electrolytes from the tissues and growth parameters of wheat seedlings. These data provide strong argument in favor of the likelihood of implementation of the endogenous ABA as a hormonal intermediate in triggering the defensive reactions under the influence of SA that form the basis for the development of SA-induced plant resistance to cadmium stress and sodium chloride salinity.

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1 Introduction

Salicylic acid (SA) being an endogenous regulator of growth and development of
phenolic nature has gained great attention due to its practical importance for
increasing plant resistance to stress and productivity. The great attention to SA was
initiated by the discovery of its key role in induction of systemic acquired resistance
(SAR), on the basis of which is the expression of SA-sensitive genes for PR-
(pathogenesis-related) proteins (Metraux 2002; An and Mou 2011). The knowledge
about SA signaling is still limited, which is due to the absence of information about
its receptors although a range of SA binding proteins have been discovered: SABP2,
having the greatest affinity to SA, chloroplast carbo-anhydrase, as well as catalase
and cytoplasmic ascorbate peroxidase, which indicates the important role of H_2O_2 in
the development of SA-induced SAR (Vlot et al. 2009). Information about other
components particularly necessary for development of SAR is available due to the
use of a set of mutants and transgenic plants (Bari and Jones 2009; An and Mou
2011). Transduction of SA-signal demands the presence of a regulatory protein
NPR1 (non-expressor of PR genes1), also known as NIM1 or SAI1, which contains
an ankyrin-repeat motif and a BTB/POZ domain, enabling protein–protein inter-
actions (Vlot et al. 2009; An and Mou 2011). Interaction of nuclear localized NPR1
with the trans-factors of TGA family and structurally related NIMIN proteins that
result in the transcription of genes of PR proteins sensitive to SA. The promoter
region of the *NPR1* gene also contains W-box sequences, which are binding sites of
WRKY family protein, suggesting that WRKY transcription factors play an
important role in mediating signaling between SA and NPR1 (An and Mou 2011).
PR1 is the best studied gene containing in its promoter activation sequence-1 (*as-1*)
motive necessary for binding of TGA-factors of transcription and gene expression
and serving as a marker of SAR (An and Mou 2011).

At the same time SA participates in the regulation of different physiological
processes in the course of plant ontogenesis under normal growth conditions
including germination, flowering, leaf senescence, fruit ripening, thermogenesis,
stomatal conductivity, ion transport, gravitropism (Raskin 1992; Shakirova 2001;
Hayat et al. 2007, 2010; Vicente and Plasencia 2011). Moreover, a lot of infor-
mation was obtained by now about participation of exogenous and endogenous SA
in plant protection not only from biotic, but also from a wide range of abiotic stress
factors (Shakirova and Bezrukova 1997; Yang et al. 2004; Liu et al. 2006; Janda
et al. 2007; Shakirova 2007; Hayat et al. 2010; Gemes et al. 2011).

Previously it was shown by us that SA-treatment causes in wheat plants fast
shifts in the state of hormonal system, associated with parallel reversible

accumulation of abscisic acid (ABA) and indoleacetic acid (IAA) on the background of the absence of changes in cytokinin level. This allowed us to suggest that endogenous ABA may serve as a hormonal intermediate in the realization of SA-induced pre-adaptation of plant to the forthcoming stress (Shakirova et al. 2003; Shakirova 2007).

Increased synthesis and accumulation of ABA having frequently a transitory pattern may be characterized as the universal plant response to stressful impacts leading to disturbance of water relations (Xiong et al. 2002). ABA is known to play a key role in regulation of stomatal closure (Wilkinson and Davies 2010), resulting in a decline in transpiration and reduction of water loss. Stomatal closure is one of early plant responses to salinity caused by ABA-induced increase in Ca^{2+} concentration in cytoplasm, subsequent activation of ion channels in plasmalema and turgor losses by guard cells also linked with ABA-induced enhancement of H_2O_2 production serving as ABA signal intermediate in stomatal closure (Kim et al. 2010). At the same time ABA is involved in up-regulation of antioxidant enzyme genes and enhancement of corresponding enzyme activity (Xiong 2007) providing a protection against oxidative stress caused by conditions unfavourable for plant growth.

ABA is of pivotal importance for the induction of biosynthesis and accumulation of prolin, which functions as osmoprotectant participating in the stabilization of biopolymers and cell membranes and protection against injurious action of reactive oxygen species (ROS) (Yu et al. 2008; Szabados and Savoure 2009), as well as for production of many other ABA-induced components of plant protection (Rock et al. 2010). Among ABA-induced genes, important role belongs to those for dehydrins. Their massive accumulation is observed in plant seed embryos during their dehydration. However, sharp increase in expression of dehydrin genes and accumulation of their protein products is registered in vegetative plant tissues subjected to dehydration, dehydrins being the most abundant among stress proteins induced under these conditions (Close 1996; Hara 2010).

The gene coding for wheat germ agglutinin (WGA) also belongs to ABA-responsive genes (Skriver and Mundy 1990; Shakirova et al. 2001). WGA, being a typical representative of cereal lectins, is a constitutive wheat protein, whose presence in plant tissues increases significantly during ontogenesis. Thus, significant reversible increase in WGA content was observed in wheat plants in response to salinity, drought, osmotic stress and heat shock (Cammue et al. 1989; Shakirova et al. 1993, 1996; Singh et al. 2000; Shakirova and Bezrukova 2007). Data showing a decline in stress-induced oxidative damage in seedlings pretreated with WGA and an accelerated restoration of growth processes during the post-stress period in these plants confirm that WGA is an active participant in ABA-induced wheat resistance (Bezrukova et al. 2008). It is of interest that in the series of components of plant protection controlled by exogenous and endogenous ABA there are also those involved in the range of protective action of SA (Shakirova and Bezrukova 1997; Shakirova 2001, 2007; Shakirova et al. 2003; Fatkhutdinova et al. 2004; Rajjou et al. 2006; Hayat et al. 2010; Nazar et al. 2011). These data indicate in favor of possible implication of endogenous ABA as a hormonal intermediate in the regulation of realization of pre-adaptive and protective action of SA on plants.

2 Endogenous ABA in the Regulation of Pre-adaptive Effect of Salicylic Acid on Wheat Plants

In order to assess the regulatory role of ABA in SA-induced defense responses, we carried out experiments with the pre-treatment of wheat plants with ABA biosynthesis inhibitor fluridone effective in preventing stress-induced accumulation of ABA, when applied at concentration of 5 mg/l (Shakirova et al. 2009).

Figure 1 shows that pretreatment with fluridone in selected concentration completely prevented SA-induced accumulation of ABA in wheat seedlings. Consequently, ABA accumulated in SA-treated plants was new born indicating participation of SA in the control of *de novo* ABA synthesis in wheat plants.

In connection with this, it was of interest to estimate the importance of SA-induced accumulation of ABA in the regulation of protective reactions of wheat seedlings developing in plants in response to SA-treatment.

We have previously shown that SA-treatment itself leads to a significant accumulation of WGA and increased transcription of *TADHN* gene coding for dehydrin in wheat seedlings (Shakirova and Bezrukova 1997; Shakirova 2007), and in connection with this, it was of interest to conduct a comparative analysis of the impact of SA on the level of WGA and *TADHN* gene transcripts in wheat seedlings, untreated and pretreated with fluridone.

Figure 2 shows that treatment with SA causes an accumulation of WGA and TADHN dehydrin transcripts in seedlings, which is preceded by a rapid transient accumulation of ABA, whereas pretreatment of seedlings with fluridone completely prevents the stimulatory effect of SA on these processes. The results evidence in favor of an important role of SA-induced synthesis of ABA in triggering defense reactions, which may contribute to the development of the pre-adaptive effect of SA to further stresses and reducing their damaging effects on wheat plant.

Deposition of lignin in the root cell walls resulting in strengthening of their barrier functions is known to contribute significantly to the development of plant resistance to the stress factors leading to dehydration (Moura et al. 2010).

Fig. 1 The effect of 50 μM SA on ABA content in 4-day-old wheat seedlings pretreated or not treated with 5 mg/l fluridone for 24 h. Mean data of three independent replicates and their SEs are presented
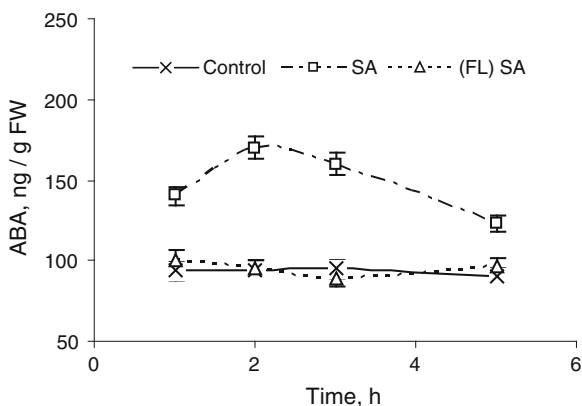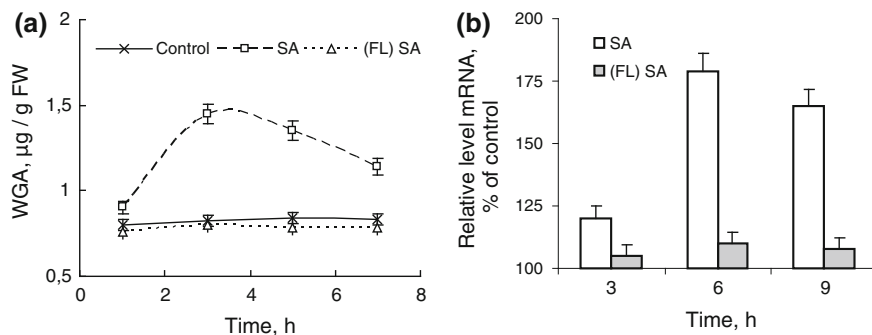
Fig. 2 The effect of 50 μM SA on (**a**) WGA content and (**b**) relative level of *TADHN* gene transcripts in 4-day-old wheat seedlings pre-treated or not treated with 5 mg/l fluridone for 24 h. Mean data of three independent replicates and their SEs are presented

Biosynthesis of this biopolymer is implemented with the help of H_2O_2, as well as enzymes phenylalanine ammonia-lyase (PAL) and peroxidase, ABA and SA being involved in the control of expression of their genes and enzyme activity (Thulke and Conrath 1998; Hiraga et al. 2001; Fatkhutdinova et al. 2004; Fernandes et al. 2006; Chen et al. 2006; Moura et al. 2010). In connection with this it was of interest to compare the effect of SA on dynamics of concentration of H_2O_2 and anionic peroxidase isoform ($pI \sim 3.5$), total activity of peroxidase and PAL as well as that of lignin deposition in the cell walls of the central cylinder of the basal part of roots of wheat seedlings and to estimate the role of ABA in the control of these processes.

The treatment with SA itself have been discovered by us earlier to result not only in the transitory accumulation of ABA, but in enhancement of O_2^- and H_2O_2 in wheat seedlings, accompanied by activation of SOD and peroxidase, which was beneficial for plants as judged from the growth promoting effect of 50 μM SA (Fatkhutdinova et al. 2004; Shakirova 2007). In connection with this, it was important to carry out further a detailed analysis of the effect of SA on H_2O_2 and anionic isoperoxidase concentration and dynamics of total peroxidase and PAL enzyme activity in the seedlings untreated and pretreated with fluridone for 24 h under normal conditions for plant growth.

Figure 3 shows that pre-treatment of wheat seedlings with fluridone completely inhibited the enhancement of H_2O_2 production, anionic isoperoxidase accumulation, peroxidase and PAL activity exerted by SA alone. These results demonstrate that under normal conditions balanced increase of H_2O_2 and activation of peroxidase and PAL in wheat plants in response to SA-treatment is due to SA-induced *de novo* ABA production.

The obtained data indicate the key role of ABA in regulation of H_2O_2 production and confirm that under different external influences leading to ABA accumulation pretreatment of plants with inhibitors of ABA synthesis inhibits both H_2O_2 production and activation of the antioxidant defense system (Jiang and Zhang 2002; Ye et al. 2011).
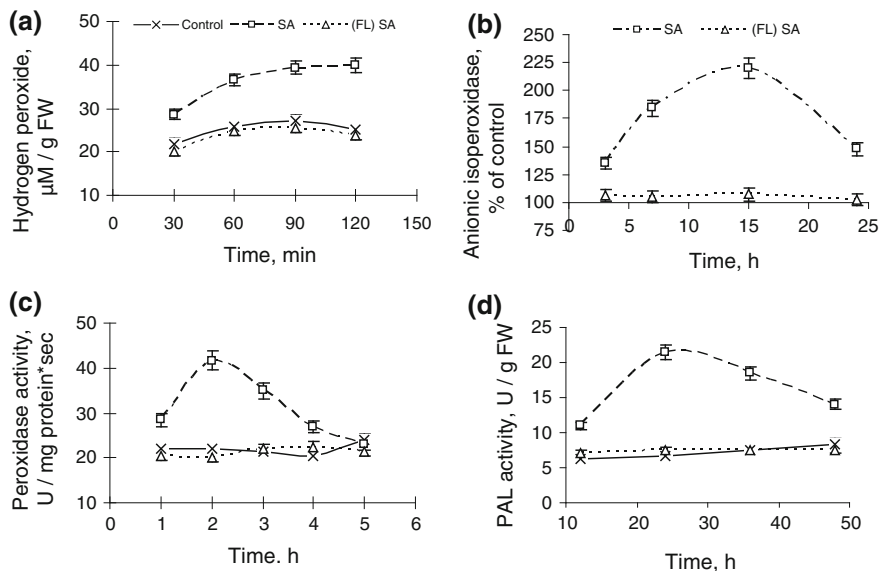
Fig. 3 The effect of 50 μM SA on (**a**) H_2O_2 production and (**b**) anionic isoperoxidase content, (**c**) total peroxidase and (**d**) PAL activity in 4-day-old wheat seedlings pretreated or not treated with 5 mg/l fluridone for 24 h. Mean data of three independent replicates and their SEs are presented

We further carried out the analysis of dynamics of lignin deposition in the central cylinder of the basal part of the control and treated wheat plants (Table 1). It shows that treatment of seedlings with SA for 24 h contributes to acceleration of lignification in the cell walls of root xylem vessels as compared to the control: staining of cell walls with phloroglucinol was clearly revealed in the roots of 5-d-old seedlings, while in 6-d-old seedlings it was additionally enhanced. At the same time controlled lignin deposition in the cell walls was discovered later on, starting from the 6th day (Table 1).

Pretreatment of 3-day-old seedlings with fluridone for 24 h inhibited deposition of lignin in cell wall of roots not only in 5-day-old but also in 6-day-old seedlings (Table 1). Consequently, preventing SA-induced accumulation of endogenous

Table 1 Qualitative assay of specific lignin staining with phloroglucinol in the basal part of roots of wheat seedlings pre-treated with 50 μM SA during 24 h in the presence or absence of fluridone (5-day-old) and then 24 h after exposure of seedlings on 4 μM ABA (6-day-old)

Variant	5 days	6 days
Control	−	+
SA	+	++
(FL) SA	−	−
(FL + SA) + ABA		++

"−" indicates absence of staining, number of "+" reflects the extent of staining intensity

ABA by the pretreatment with fluridone inhibited the increase in H_2O_2 and anionic peroxidase level and activation of PAL and peroxidase being the key enzymes in lignin biosynthesis. As a consequence, lignin deposition in the cell walls of the root central cylinder was inhibited. Those results convincingly indicate the regulatory role of endogenous ABA in acceleration of lignin deposition in the cell walls of roots of SA-treated seedlings as also evident from the data showing a complete recovery of this process during the subsequent action of exogenous ABA for 24 h on seedlings pretreated with fluridone and SA (Table 1).

The sum of obtained data indicate the important role of SA-induced synthesis of ABA in the regulation of SA-induced activation of the key components of lignin biosynthesis significantly contributing to the strengthening of barrier properties of root cell walls and pre-adaptation of plants to possible forthcoming action of environmental stress factors.

3 Endogenous ABA in SA-Induced Activation of Defensive Reactions in Wheat Seedlings Subjected to the Influence of Toxic Ions

Frequently changing environment demands plant adaptation to the conditions of their growth, which implies the development of a complex network of protective reactions aimed on the struggle for survival under the stressful environment. Based on this there is the integration of the effective systems of regulation of cell metabolic activity for switching genetic programs from norm to stress aimed on development of an adequate protection at the level of whole organism. Drought, disturbance of temperature regime and salinity are most important environmental factors, which are critical for survival of plants and lead to significant losses of crop yield. Responses to these stresses are known to be interconnected, employing common signaling pathways, which allow cell adaptation and lead to similar changes in plants on morphological, physiological, biochemical and molecular/or genetic levels (Verslues et al. 2006; Shinozaki and Yamaguchi-Shinozaki 2007; Potters et al. 2009; Arbona et al. 2010; Des Marais and Juenger 2010). Due to the increasing anthropogenic pollution of soils increasing attention is paid to the study of the effects of excess concentrations of heavy metals (HM) on the plants, which are also manifested in the disturbance of the processes of their growth and development (Pál et al. 2006; DalCorso et al. 2008; Yadav 2010).

Survival of plants themselves is known to be due to their ability to struggle against extreme environment, protecting their vital potential. However, realization of natural protective mechanisms taking place in plants, when conditions become worse, is well known to be accompanied by a decline in their productivity and quality. This raises important question about regulation of stress resistance. The problem of stress-resistance is most important for plant breeding and is under a steadfast attention of researchers all over the world. This is indeed the case, since information concerning the chain of reactions taking place in plants in response to

extreme external conditions may really contribute to an increase in plant resistance and productivity. These goals are achieved not only by means of selection of stress tolerant cultivars, but also through the purposeful manipulation of adaptation with the help of natural plant growth regulators. To those, in particular, can salicylic acid be attributed.

3.1 Cadmium Stress

Due to the progressive contamination of soils with salts of toxic heavy metal (HM), causing not only a decrease in yield, but also the deterioration of soil quality (Pál et al. 2006; DalCorso et al. 2008). The investigation of the molecular mechanisms of plant resistance to HM becomes a serious goal. Cadmium can be attributed to the most toxic of HM, because it is not a necessary element for the normal functioning of plants and does not perform any physiological function in plants (DalCorso et al. 2008).

The reactions of plants in response to cadmium, mainly, are nonspecific and are similar to those of the effects of other HM. The most characteristic of them could be the accumulation of ABA, stomatal closure, the inhibition of uptake and transport of water, the inhibition of chlorophyll synthesis and photosynthesis, the imbalance of pro- and antioxidant components and disturbance of integrity of the cell membrane structure, which in general is reflected in a delay in plant growth and development and reduction of its productivity (Hsu and Kao 2005; Pál et al. 2006; DalCorso et al. 2008; Yadav 2010).

In recent years, special attention is paid to the study of molecular mechanisms of plant resistance to cadmium and other HM as well as of the ways of their regulation. Thus, transcriptomic and proteomic analysis allowed to identify a wide range of proteins involved in the responses of plants to cadmium. Among them an important place is occupied by the proteins associated with antioxidant protection and detoxification of ROS (Fusco et al. 2005; Ahsan et al. 2009; Wang et al. 2011). In addition, there has also been identified proteins involved in phytohormone signaling including salicylic acid (Ahsan et al. 2009), which indicates the involvement of this hormone in the regulation of plant resistance to HM. Since exposure to cadmium causes a disturbance of water relations, it is not surprising that under these conditions there has been detected the activation of the transcription of ABA sensitive genes (Fusco et al. 2005), playing, as is known, a key role in the regulation of plant defense responses during dehydration. Those, in particular, include genes coding for proteins dehydrins and lectin wheat germ agglutinin, whose synthesis and accumulation increases sharply under disturbance of the water regime, including the effects of HM (Hara 2010; Bezrukova et al. 2011).

To date, a lot of information accumulated, indicating the effectiveness of application of salicylic acid for reduction of the toxic effects of cadmium on different crops (Metwally et al. 2003; Janda et al. 2007; Meng et al. 2009; Hayat et al. 2010). Thus, it is shown that pretreatment with SA helps to prevent

cadmium-induced shifts in the composition of fatty acids and in the integrity of membrane structures (Ivanova et al. 2008; Krantev et al. 2008), as well as to reduce the extent of the inhibitory effect of cadmium on the activity of key photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenol pyruvate carboxylase and on photosynthesis, in general (Krantev et al. 2008; Zhang and Chen 2011), and to reduce the level of reactive oxygen species and lipid peroxidation (Choudhury and Panda 2004; Zhang and Chen 2011). This, in general, is reflected in the maintenance of plant growth processes under these conditions at a level close the control (Hayat et al. 2010). In addition, there is evidence of the ability of SA to influence the lignin deposition in the cell walls of roots, making an important contribution to improve their barrier properties and to protection of plants from toxic effects of cadmium (Kováčik and Klejdus 2008).

Visual manifestation of the toxic effect of cadmium on plants is the inhibition of growth processes. Indeed, exposure to 1 mM cadmium acetate has a very pronounced inhibitory effect on wheat seedlings, as evidenced by the inhibition of mitotic activity of cells of root tips and root relative growth rate (RGR) (Fig. 4a, b). Shoots also experience negative effects of cadmium, but this is much less than in roots as suggested by the data on RGR (Fig. 5).

These results are in accordance with the observations that roots suffer from the toxic effects of cadmium to a greater extent, since it is in the roots it accumulates in larger quantities (Seregin and Ivanov 1997; Bezrukova et al. 2011). Comparison
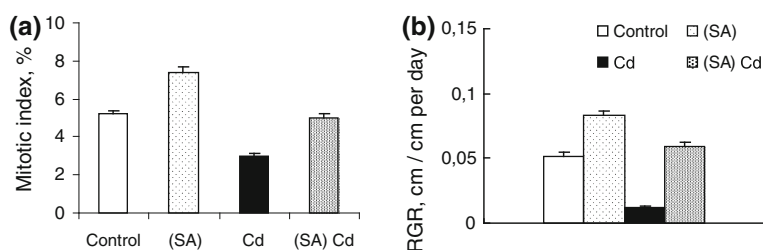
Fig. 4 The effect of pretreatment 3-day-seedlings with 50 μM SA during 24 h on (**a**) mitotic index of the roots and (**b**) relative rate of root growth of wheat seedlings exposed to 1 mM cadmium acetate for 24 h ours. Mean data of three independent replicates and their SEs are presented

Fig. 5 The effect of pretreatment 3-day-seedlings with 50 μM SA during 24 h on relative rate of shoot growth of wheat seedlings exposed to 1 mM cadmium acetate for 24 h. Mean data of three independent replicates and their SEs are presented
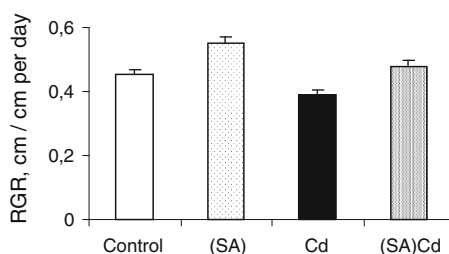
of the growth of SA-pretreated plants with/without cadmium showed that the pretreatment does not prevent, but significantly reduces the extent of the negative effects of stress on the seedlings, and helps to maintain the activity of the growth processes of these plants, at least at the level of control (Figs. 4, 5). Thus, pretreatment with SA has a clear protective effect on the growth of wheat plants under cadmium stress.

It is known that plant growth is controlled by hormonal system, responsive to environmental changes (Wang et al. 2008; Shakirova et al. 2010), and a simultaneous analysis of different concentrations of phytohormones in the same plants provides a comprehensive picture of the stress-induced rearrangements in a state of hormonal system.

The results of analysis of changes in the balance of ABA, IAA and cytokinins in the wheat seedlings, pretreated and untreated with SA and exposed to cadmium acetate, are shown in Fig. 6. As can be seen, cadmium causes dramatic changes in the hormonal balance of seedlings, as can be evidenced by the decrease in the ratio of IAA and cytokinins to ABA associated with a sharp reversible accumulation of ABA and persistent fall in the concentration of cytokinins and IAA in particular, that in general is reflected in the inhibition of growth of these plants (Figs. 4, 5).

Pretreatment with SA did not prevent, but significantly reduced the amplitude of the cadmium induced changes in the concentration of ABA, auxin and cytokinins. Moreover, 5 h after the start of the experiment the ratios of IAA/ABA and cytokinins/ABA in SA-pretreated seedlings completely recovered (Fig. 6), which

Fig. 6 Coefficients of ratio cytokinins/ABA (**a**) and IAA/ABA (**b**) in roots of 4-day-old wheat seedlings pretreated and untreated with 50 μM SA during exposure of 1 mM cadmium acetate
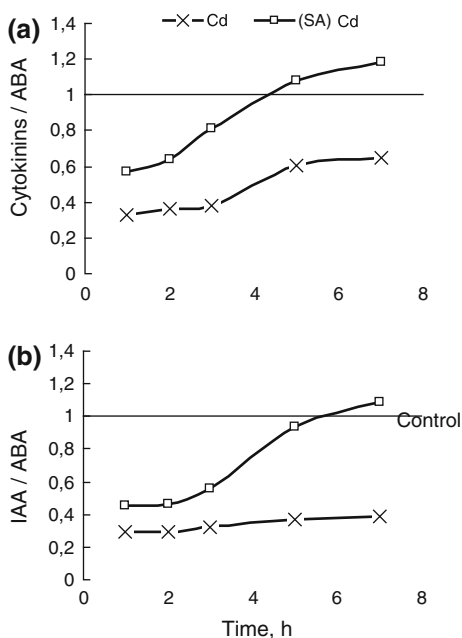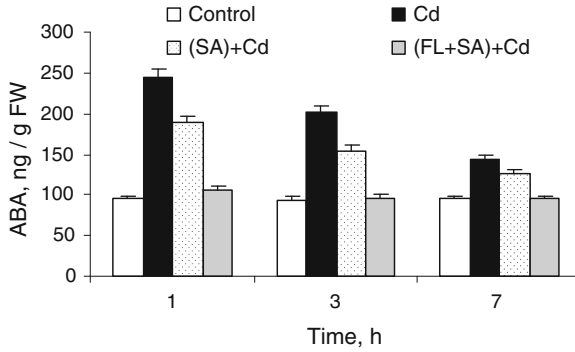
Fig. 7 Effect of 1 mM cadmium acetate on the ABA content in 4-day-old wheat seedlings, untreated and pretreated for 24 h with 50 μM SA in the presence or absence of 5 mg/l fluridone

is reflected in the maintenance of the activity of growth processes in these plants at the control level (Figs. 4, 5).

Furthermore it was important to compare the effects of pre-treating the plants with SA and a mixture of SA and fluridone on ABA content in the seedlings exposed to cadmium stress.

Figure 7 shows that incubation of seedlings in cadmium led to a rapid reversible accumulation of ABA in wheat seedling untreated with SA, which is not surprising, since this response is a typical stress reaction (Shakirova et al. 2010). SA-pretreated seedlings were characterized by visibly lower level of cadmium-induced accumulation of ABA, which may be an indicator of lower degree of the damaging stress effect in these plants due to pre-adaptive action of SA in the course of pretreatment. At the same time pretreatment of seedlings initially with fluridone and then with a mixture of fluridone and SA during 24 h completely prevents cadmium-induced accumulation ABA.

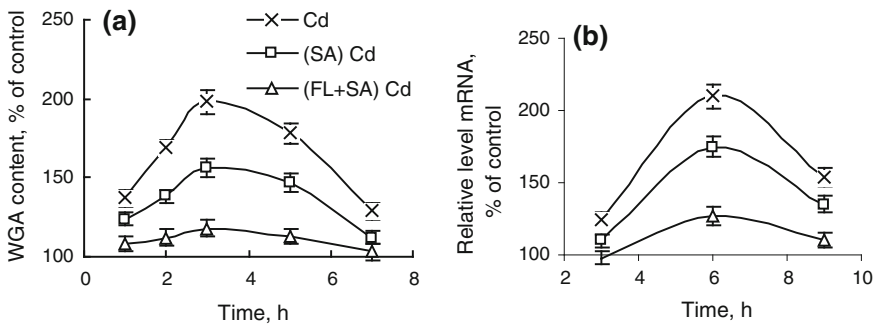**Fig. 8** Effect of 1 mM cadmium acetate on the (**a**) WGA content and (**b**) the relative level of TADHN transcripts dehydrin in 4-day-old wheat seedlings, untreated and pretreated for 24 h with 50 μM SA in the presence or absence of 5 mg/l fluridone

Figure 8a and b shows that the effect of cadmium acetate leads to a transient increase in WGA content and level of transcription of *TADHN* gene in the seedlings, which is also not surprising, since the literature contains information on the involvement of wheat lectin and dehydrins in the responses of plants not only to drought, salinity, hypothermia, and also to HM (Shakirova et al. 1993, 2009; Hara 2010; Tamás et al. 2010; Bezrukova et al. 2011).

At the same time plants pretreated with SA within 24 h are characterized by a lower level of accumulation of the lectin and transcripts of *TADHN* gene coding for wheat dehydrin, indicating a less damaging effect of cadmium on them, in contrast to SA-untreated plants (Fig. 8). Co-pretreatment with fluridone and SA although did not prevent, but sharply reduced the stress-induced accumulation of WGA and the up-regulation of dehydrin gene transcription (Fig. 8a, b). These results are the indication in favor of the key role of endogenous ABA in the regulation of these protective components in the plants pretreated with SA. However, alongside with the ABA-dependent signaling pathway there was revealed the presence of ABA-independent pathways of regulation of protective reactions, which is in agreement with literature data (Verslues and Bray 2006; Shinozaki and Yamaguchi-Shinozaki 2007). Nevertheless, growth results showed (Figs. 4, 5) that these ABA-independent responses were not sufficient for the manifestation of defense related effects of SA in the presence of fluridone on cadmium-treated wheat plants.

The obtained results indicate in favor of an important role of maintaining increased concentration of endogenous ABA in SA-pretreated seedlings in the regulation of the content of WGA and the level of transcription of *TADHN* gene, contributing to the development of plant resistance to cadmium stress.

It is known that exposure to toxic cadmium ions leads to the overproduction of reactive oxygen species and increased lipid peroxidation, the intensity of which can be judged by the level of accumulation of malondialdehyde (MDA) (Hsu and Kao 2007; Wang et al. 2011). The data presented in Fig. 9 shows that cadmium causes a sharp increase in the concentration of MDA in comparison with the control, whereas in the plants pretreated with SA this characteristic is much lower. This is probably due to the ability of SA to induce, during its pretreatment, the

Fig. 9 The effect of pretreatment 3-day-seedlings with 50 μM SA or initially with 5 mg/l fluridone alone during 3 h and then with the mixture of fluridone with SA for 24 h on MDA content in wheat seedlings after 24-h-cadmium acetate. Mean data of three independent replicates and their SEs are presented
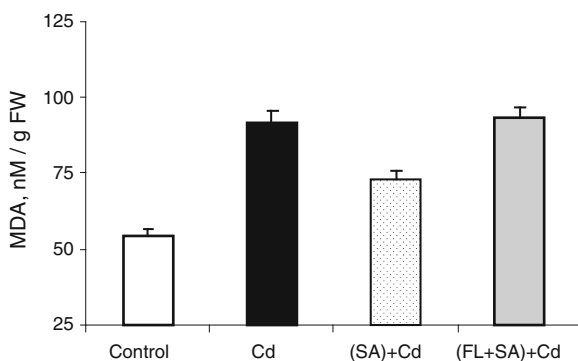
Table 2 Qualitative assay of specific lignin staining with phloroglucinol in the basal part of roots of 5-day-old wheat seedlings pre-treated with 50 μM SA during 24 h in the presence or absence of 5 mg/l fluridone and then 24 h after exposure of seedlings on 1 mM cadmium acetate

Variant	5-day-old
Control	−
1 mM cadmium acetate	+
(SA) + cadmium acetate	++
(FL + SA) + cadmium acetate	−/+

"−"indicates absence of staining, "−/+" very weak staining, number of "+" reflects the extent of staining intensity, "++" being very strong staining

transient activation of antioxidant enzymes in wheat seedlings (Fig. 3) involved in neutralization of oxidative stress-induced burst.

An additional argument in support of involvement of ABA in the regulation of SA-induced protective reactions reducing the damaging effect of cadmium stress on wheat plants is in the data showing significant decline in lignin accumulation in the cell walls of the basal part of seedling roots pretreated with the mixture of fluridone with SA (Table 2). This is likely to be due to prevention by fluridone of the SA-induced production of H_2O_2 and activation of the key enzymes of lignin biosynthesis (PAL and peroxidase) being under the control of ABA (Moura et al. 2010).

Since, as noted above, pretreatment with SA reduced the extent of the damaging effect of cadmium on the growth processes of wheat seedlings, one would expect that the manifestation of the protective effect of SA on the plants under cadmium stress is due to the increased barrier properties of the cell walls of roots bringing about the inhibition of the entry of toxic ions into the root tissues and their subsequent delivery to the shoot.

Thus, the histochemical analysis with the help of dithizone reagent (Seregin and Ivanov 1997) revealed the presence of cadmium in all tissues of the transverse sections of roots of 5 days-old wheat seedlings untreated with SA (Table 3). At the same time in the experiments with SA-pre-treatment cadmium was detected only in rhizoderm and outer layers of the primary cortex (Table 3). These data demonstrate the important contribution of SA-induced acceleration of lignin deposition in the basal part of seedlings roots and additional intensification of the process

Table 3 The effect of pretreatment of 3-day-seedlings with 50 μM SA or initially with 5 mg/l fluridone alone during 3 h and then with the mixture of fluridone with SA for 24 h on cadmium ion distribution over root tissues of 5-day-old wheat seedlings

Treatment	Tissue					
	Rhizodermis	Primary cortex	Endodermis	Pery cycle	Stellar parenchyma	Conducting tissues
Control	−	−	−	−	−	−
Cd acetate, 1 mM, 24 h	+	+	+	+	+	+
(SA) + Cd	++	−	−	−	−	−
(FL + SA) + Cd	+	+	+	+	+	+

under stress to enhancement of the barrier properties of cell walls and consequent inhibition of the entry of toxic ions into the internal tissues of roots.

In a joint pretreatment of wheat seedlings with SA and fluridone there was observed an inhibition of cell wall lignification (Tables 1, 2), resulting in the prevention of SA-induced inhibition of cadmium entrance into root tissues (Table 3), which, in turn, indicates the involvement of endogenous ABA in the regulation of this protective mechanism by salicylic acid.

Therefore, for the first time inhibition analysis allowed to obtain experimental evidence indicating in favor of the key role of endogenous ABA in the regulation of SA-induced protective mechanisms making an important contribution to the development of resistance of wheat seedlings exposed to the toxic ions of cadmium.

3.2 Sodium Chloride Salinity

Salinity caused by increased content of soluble salts in soil is one of the most widely spread abiotic stress factors resulting in significant inhibition of plant growth and decline in crop productivity, with sodium chloride being the most detrimental (Munns and Tester 2008; Cambrolle et al. 2011).

The decline in cell growth processes is due to dehydration resulting from osmotic effect of salts accumulating in the root zone and due to toxic effect of sodium and chloride accumulation in the plant tissues causing great damaging effect on the most important physiological processes and cell membrane integrity (Munns and Tester 2008; Nazar et al. 2011). It is necessary to emphasize that plants are able to develop a broad spectrum of protective reactions aimed on diminishing the detrimental effects of salinity (Flowers 2004; Munns and Tester 2008). Stress hormone ABA, whose fast and significant accumulation is a characteristic response to salinity, makes an important contribution to protective reactions (Rock et al. 2010; Shakirova et al. 2010). At the same time attention is drawn to the similarity in the several of the plant responses to sodium chloride salinity and cadmium stress.

We have previously reported that pretreatment with SA did not prevent, but significantly reduced the salinity-induced transient accumulation of ABA in wheat plants, suggesting that the maintenance of increased content of ABA in SA-pretreated plants plays an important regulatory role in the manifestation of the protective effect of SA (Shakirova 2007). To estimate the importance of maintaining increased level of endogenous ABA in the manifestation of the protective effect of SA on wheat seedlings under salt stress there was also used fluridone in the experiments. Like cadmium, salinity stress also caused a rapid reversible accumulation of ABA in untreated plants, whereas in pre-treated with SA seedlings increase of ABA level was much lower. This is reflected in the fact that SA-pretreated wheat seedlings are characterized by lower levels of stress-induced accumulation of WGA (Fig. 10a), as well as of osmoprotectant proline (Fig. 10b),

Fig. 10 The effect of pretreatment 3-day-seedlings with 50 μM SA or initially with 5 mg/l fluridone alone during 3 h and then mix fluridone with SA for 24 h on (a) WGA and (b) proline content in 4-day wheat plants under salinity. Mean data of three independent replicates and their SEs are presented
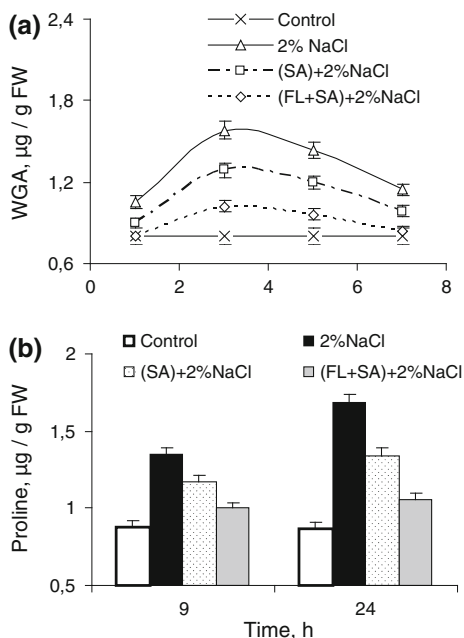
which make an important contribution to the protection of plants against oxidative burst caused by salinity (Bezrukova et al. 2008; Hara 2010). The pretreatment of seedlings initially with fluridone and then with a mixture of fluridone and SA completely prevented the SA-induced increase in the level of ABA under salt-stress conditions.

At the same time, attention is drawn to the fact that the joint fluridone pre-treatment with SA although greatly reduced, but not completely prevented the increase in WGA and proline content in seedlings subjected to salinity (Fig. 10). Similar results were obtained during the study of the activity of antioxidant enzymes. Salinity induces a fast increase initially in activity of SOD followed by peroxidase in wheat seedlings (Fig. 11), which is a typical plant response to stress-induced enhancement of ROS production (Jaspers and Kangasjarvi 2010). Stressed plants pretreated with SA are characterized with a significantly lower activity of antioxidant enzymes as compared to untreated plants (Fig. 11), which is likely to be due to the lower production of ROS in the plants (Shakirova 2007). This is associated with the fact that SA-treatment itself causes a balanced increase in production of ROS and activity of antioxidant enzymes (Fig. 3), sufficient for effective neutralization of salt-induced oxidative burst (Shakirova 2007).

These results support the belief of a key role of endogenous ABA in the regulation of investigated protective components in the plants pretreated with SA. This notion is supported by 50 % decline in the level of MDA in comparison with the plants untreated with SA under salt stress (Fig. 12a).

Fig. 11 The effect of
pretreatment of 3-day-
seedlings with 50 µM SA or
mix 5 mg/l fluridone with SA
for 24 h on activity of
(**a**) SOD and (**b**) peroxidase
in 4-day wheat plants under
salinity. Mean data of three
independent replicates and
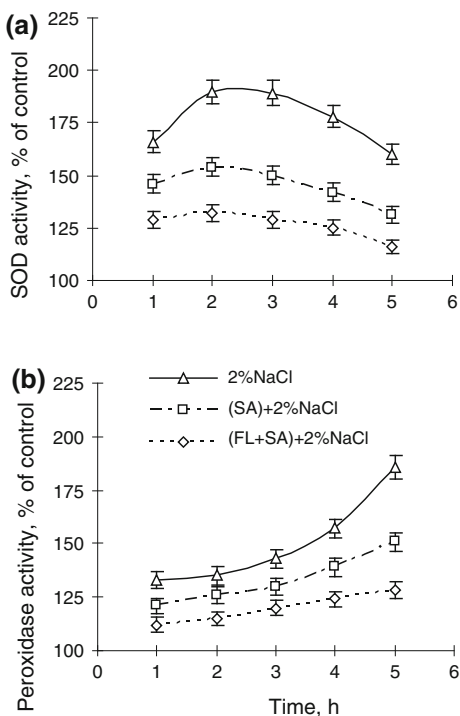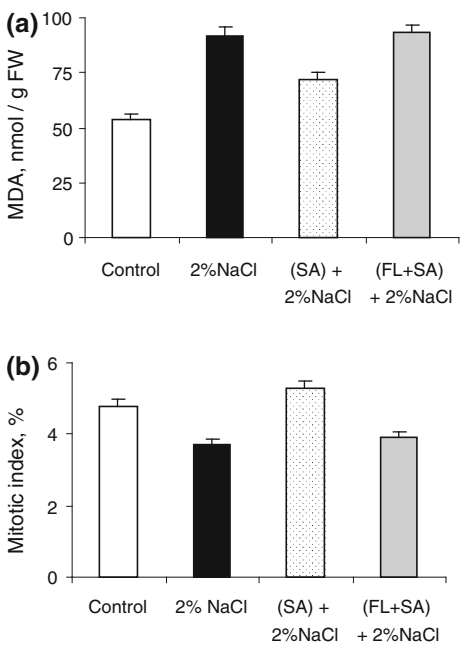their SEs are presented

Fig. 12 The effect of
pretreatment 3-day-seedlings
with 50 µM SA or mix 5 mg/
l fluridone with SA for 24 h
on (**a**) MDA content in wheat
plants and (**b**) mitotic index
(%) of root meristem cells
after 7 h exposure on 2 %
NaCl. Mean data of three
independent replicates and
their SEs are presented

Similar to cadmium stress the presence of fluridone in the medium for incubation of the seedlings did not prevent, but significantly reduced the SA-induced activation of antioxidant enzymes under conditions of sodium chloride salinity (Fig. 11). This was reflected in that in the variant of treatment with the mixture of fluridone and SA, the plants were characterized by the same level of MDA accumulation as in the salt-stressed plants untreated with SA (Fig. 12a).

Pretreatment with SA significantly decreased the degree of the negative stress effect on the seedlings, while the treatment with a mixture of SA with fluridone prevented the protective action of SA on growth (Fig. 12b) indicating the important role of endogenous ABA in realization of SA protective action in stressed plants. This suggests inhibition of ABA-mediated protective reactions and consequent prevention of protective action of SA on plants under salinity.

The obtained data illustrate the importance of ABA in realization of pre-adaptive effect of SA on wheat plants manifested in maintaining enzyme activity of SOD and peroxidase in SA-pretreated salt-stressed plants at the level sufficient for their protective effect and in significant decline in the level of the damaging effect of salinity on the integrity of cell membranes and their permeability in SA-pretreated plants under salinity.

It can be assumed that the SA-induced enhancement of the barrier properties of cell walls of root and the inhibition of the penetration of toxic ions into plant tissues play an important role in the manifestation of the protective effect of SA on wheat seedlings under sodium chloride salinity, as well as under cadmium stress.

Thus pretreatment with SA reduced the extent of the damaging effect of salinity on the growth of wheat seedlings, which is largely due to the ability of SA to cause reversible accumulation of ABA during the treatment with SA and to maintain the increased content of ABA in plants under salt-stress.

4 Conclusion

Based on our earlier data, we assigned an important role of SA-induced reversible accumulation of ABA in the development of SA pre-adaptive effect on plants and the importance of maintaining increased content of ABA in SA-pretreated plants in development of resistance to abiotic stresses, it was suggested that endogenous ABA may serve as an intermediate in the implementation of the protective action of SA on wheat plants.

Using an effective inhibitor of ABA biosynthesis fluridone we have obtained for the first time experimental evidence for the key role of SA-induced rapid transient accumulation of endogenous ABA in the regulation of SA pre-adaptive effect on wheat plants exposed to subsequent stressors of abiotic nature. It was found that, preventing ABA accumulation, by pretreatment with fluridone completely inhibits SA-induced accumulation of WGA, which is a typical representative of cereal lectins and a component of ABA-controlled responses of wheat to drought, salinity, hypo- and hyperthermia, cadmium ions, making an important contribution

to the protection of plants from the damaging effect of osmotic and oxidative stress induced by these adverse environmental factors. Important role in protecting cellular structures from osmotic and oxidative stress is performed by dehydrins, belonging to the group of 2 LEA proteins, which are just as WGA responsive to abscisic acid (RAB) proteins, having also chelating properties. We found that the presence of fluridone in the medium for incubation of the seedling prevents SA-induced reversible enhancement of transcription *TADHN* dehydrin gene. It was shown that pretreatment with fluridone prevents the SA-induced rapid production of H_2O_2, activation of peroxidase and PAL, involved in the formation of lignin, which is reflected in the inhibition of SA-induced deposition of lignin in the cell walls of the central cylinder of the basal part of roots. Moreover, treatment of these plants with ABA restores completely the intensity of the deposition of lignin in the cell walls of roots.

Pretreatment with SA substantially reduces the damaging effects of cadmium stress and sodium chloride salinity on growth processes of wheat seedlings. This seems to be primarily due to the fact that the seedlings pretreated with SA and subjected to stress factors, are characterized by lower amplitude of imbalance of ABA, IAA and cytokinins, which could be due to the triggering in the course of pretreatment with SA (prior to stress) of the various protective mechanisms forming the basis of its pre-adaptive effect on plants, in particular, the activation of the systems osmoprotection and antioxidant defense, including the accumulation of WGA, and TADHN transcripts. In this regard, it is not surprising that under saline conditions or when exposed to toxic cadmium ions stress-induced activation of these important components of protection in the SA-pretreated seedlings occurs at notably lower level, which in general is reflected in a decrease in the levels of MDA and electrolyte leakage from plant tissues, as well as in maintaining the growth of these plants, at least at the level of control. It should be noted that the seedlings pre-treated with SA, in contrast to untreated with SA, are characterized by an additional acceleration of deposition of lignin in the cell walls of roots, contributing to the strengthening of their barrier properties, and inhibition of the entry of toxic ions into the internal tissues of roots, which is clearly demonstrated in particular for cadmium by histochemical methods using dithizone reagent.

The important role of maintaining high concentration of ABA in the development of the resistance of SA-pretreated wheat seedlings to cadmium and salinity was manifested in a significant inhibition of all studied defense responses in seedlings pretreated with SA in combination with fluridone. Despite the fact that fluridon completely prevents maintenance of elevated ABA content in plants pretreated with SA, attention is drawn to the fact of existence of alternative ABA-independent pathways of regulation of stress-induced activation of protective mechanisms, although, as can be seen in terms of growth, MDA level and release of electrolytes from the tissues, this was not sufficient for effective development of SA-induced wheat plant resistance.

Thus, the summary of the data presented in this chapter, obtained by using an inhibitor of ABA synthesis, fluridone, clearly demonstrates the key role of endogenous ABA in the implementation of pre-adaptation and protective action of

SA on wheat plants and the likelihood of implementation of ABA as a hormonal intermediate in triggering the complex defensive reactions forming the basis for the development of SA-induced resistance of plants to abiotic stress factors.

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Chapter 8
Salicylic Acid Biosynthesis and Role in Modulating Terpenoid and Flavonoid Metabolism in Plant Responses to Abiotic Stress

T. Tounekti, I. Hernández and S. Munné-Bosch

Abstract Salicylic acid (SA) is a simple phenolic acid with hormonal function synthesized from the amino acid phenylalanine or chorismate depending on the plant species, developmental stage and growth conditions. This compound plays a key role in plant growth and development, and in plant responses to abiotic stresses such as salinity and drought stress. Under these environmental constraints, plants synthesize a number of secondary metabolites, including flavonoids and terpenoids, with a defence-related function. Here, we will discuss the role of SA in modulating plant responses to abiotic stress, particularly as an inducer of defence responses against salinity and drought stress. Emphasis will be put on discussing the SA signalling pathways that affect flavonoid and terpenoid metabolism as defense compounds against stress.

Keywords Drought stress · Salicylic acid · Salt stress · Terpenoids · Flavonoids

1 Introduction

Salycilic acid (SA) or *ortho*-hydroxy benzoic acid has a broad distribution in the plant kingdom (Raskin et al. 1990). Basal SA levels differ widely among species, with up to 100-fold differences between plants, even among members of the same family. For instance, in the *Solanaceae*, while tobacco contains low basal levels of

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SA ($<$100 ng g^{-1} FW) (Yalpani et al. 1991; Malamy et al. 1992), potato might contain up to 10 μg of total SA g^{-1} FW (Navarre and Mayo 2004). In rice, crabgrass, barley, and soybean plants the level of SA is about 1 μg g^{-1} FW. In the model plant *A. thaliana*, basal levels of total SA range from 0.250 μg to 1 μg g^{-1} FW (Nawrath and Metraux 1999; Wildermuth et al. 2001; Brodersen et al. 2005). The highest levels of SA have been found in the inflorescence of thermogenic plants and in spice herbs (Raskin et al. 1990). SA is considered to be a potent plant hormone because of its varied regulatory roles in plant metabolism, growth, development, interaction with other organisms and the responses to environmental stresses (Raskin 1992; Yalpani et al. 1994; Popova et al. 1997; Senaratna et al. 2000). Exogenously applied SA can be used to hasten the negative effects of ozone and UV light (Yalpani et al. 1994; Sharma et al. 1996; Rao and Davis 1999), heat stress (Senaratna et al. 2000; Larkindale and Knight 2002; Chakraborty and Tongden 2005), chilling and drought (Senaratna et al. 2000), and salt and osmotic stresses (Borsani et al. 2001). However these responses are highly dependent of the SA concentration applied, so that moderate doses of SA improve the antioxidant status and induce stress resistance, while higher concentrations trigger a hypersensitive cell death pathway and enhance stress sensitivity. SA is best known as an important signalling molecule involved in disease resistance and accumulated in response to various pathogenic attacks (Enyedi et al. 1992; Alvarez 2000; Nishimura and Dangl 2010). Increases in the levels of SA and pathogenesis-related (PR) proteins have been reported in plants exposed to UV-C light and ozone, which imply a common signal transduction pathway in plant responses to biotic and abiotic stresses (Yalpani et al. 1994).

Environmental stresses are among the factors most limiting to plant productivity. Unravelling the mechanisms by which plants perceive and transduce these stresses is crucial if we are to understand the plant stress response and provide tools to improve stress tolerance. Exposure to prolonged drought or salinity may lead to an excess of excitation energy in chloroplasts and then to photooxidative stress (Chaves et al. 2003). Plants have evolved several mechanisms to dissipate this excess energy in photosynthetic membranes, some of which engage isoprenoid and flavonoid compounds (Munné-Bosch and Alegre 2003; Tattini et al. 2004; Delfine et al. 2005; Owen and Peñuelas 2005; Agati et al. 2007). Some of the isoprenoid compounds (e.g. α-tocopherol and carotenoids) represent a conserved mechanism of photoprotection, while others (e.g. for instance monoterpenes) represent an additional or alternative photoprotection mechanism (Peñuelas and Munné-Bosch 2005). Tocopherol and carotenoids display antioxidant activity, and contribute to keep thylakoid membrane structure and function under stress (Havaux et al. 2005; Munné-Bosch 2005). The production of monoterpenes has been linked to an increased thermo- and water stress tolerance only in some species (Copolovici et al. 2005; Delfine et al. 2005). Anthocyanins and other flavonoids have also been associated with stress tolerance either providing indirect photoprotection by light scattering or acting directly as antioxidants (Agati and Tattini 2010). Several other reviews have focused on the role of SA, isoprenoids and flavonoids in plant responses to several abiotic stress including drought and

salinity (Rivas-San Vicente and Plasencia 2011; Peñuelas and Munné-Bosch 2005; Hernández et al. 2009; Agati and Tattini 2010). Here, we will focus on the role of SA in modulating the response of plants to abiotic stress, including salinity and drought stress. Emphasis will be put on discussing the SA signalling pathways that affect flavonoid and terpenoid metabolism.

2 SA Biosynthesis

SA is synthesized through two distinct and differentially compartmentalized pathways that employ different precursors: the phenylpropanoid route starting in the cytoplasm from phenylalanine (phenylalanine ammonia-lyase (PAL) pathway), and the isochorismate (IC) pathway operative in the chloroplast of the cell. Both pathways derive ultimately from chorismate, the end product of the shikimate pathway. Although up to date neither of these biosynthetic routes have been entirely defined, it is becoming clearer that both IC and PAL pathways contribute to SA synthesis.

2.1 The PAL Pathway

It was often accepted that SA synthesis occurred via PAL pathway (Raskin 1992; Lee et al. 1995; Coquoz et al. 1998). Early radiolabelling studies with phenylalanine (Phe), *trans*-cinnamic acid (*t*-CA), or benzoic acid (BA) suggested that SA is synthesized from Phe via *t*-CA, which is then transformed to SA via two possible intermediates: *ortho*-coumaric acid or BA, depending on the plant species and the plant growth conditions (El-Basyouni et al. 1964; Chadha and Brown 1974). Through the PAL pathway, plants can potentially develop three biosynthetic routes to BA, including a β-oxidation route from cinnamoyl Co-A (Fig. 1, route 2c-1), a non-oxidative route from cinnamoyl Co-A (Fig. 1, route 2c-2), and a non-oxidative route from *t*-CA to BA (Route 2b; Wildermuth 2006). Radiolabelling studies using Phe or putative pathway intermediates performed in TMV-infected tobacco or cucumber detected incorporation of radiolabelled C onto BA and SA but not benzaldehyde, suggesting that SA is synthesized by the cinnamoyl-CoA β-oxidation route (Fig. 1, route 2c-1; Ribnicky et al. 1998; Jarvis et al. 2000). However, a study of BA production in developing seeds identified an *Arabidopsis thaliana* aldehyde oxidase 4 (AAO4) that catalyzes the conversion of benzaldehyde to BA, which is then incorporated into benzoyl glucosinolates (Ibdah et al. 2009). The conversion of BA to SA has been suggested to take place via an inducible benzoic acid 2-hydroxylase (BA2H). However, there has not been any report describing a BA2H-encoding gene in plants (Ford et al. 2010). The first two biosynthetic routes of SA (Route 2c-1 and Route 2c-2), have been reported in tobacco (Yalpani et al. 1993) and in rice (Silverman et al. 1995). For instance, healthy and virus-
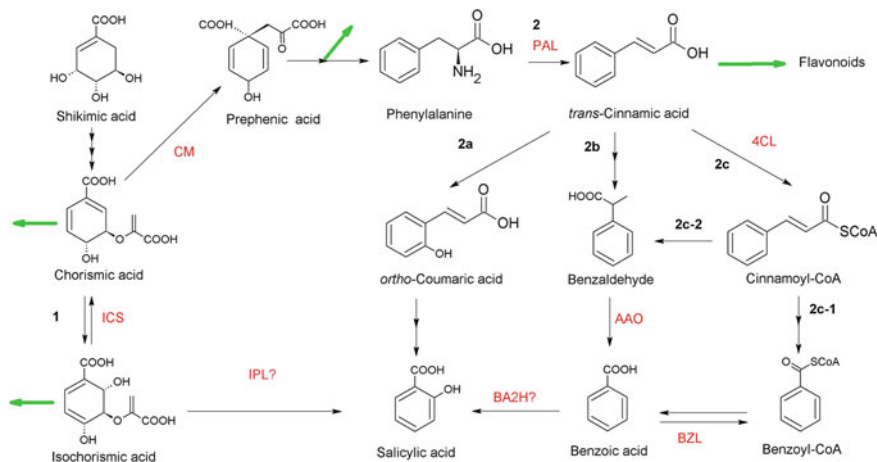
Fig. 1 Biosynthesis of salicylic acid in Arabidopsis thaliana. Key enzymes are shown in red (*AAO* Arabidopsis aldehyde oxidase; *BZL* benzoyl-CoA ligase; *BA2H* benzoic acid 2-hydroxylase; *4CL* 4-coumaroyl:CoA ligase; *CM* chorismate mutase; *ICS* isochorismate synthase; *IPL* isochorismate pyruvate lyase; *PAL* phenylalanine ammonia lyase). Enzymes the identity of which has not been identified so far are marked with a question tag. *Single arrows* indicate enzymatic reactions; *green arrows* indicate reactions (or sets of reactions) leading to other pathways competing for the same substrate. Tags in *bold letters* indicate subsets of reactions. Adapted from Dempsey et al. (2011)

inoculated tobacco synthesized [^{14}C]SA after being fed with [^{14}C]BA or [^{14}C]CA (Yalpani et al. 1993) via the activation of a 2-benzoate hydroxylase (León et al. 1993). The formation of [^{14}C]BA from [^{14}C]Phe through [^{14}C]CA was also shown in *Tsuga canadiensis* (Zenk and Muller 1964), in young *Glautheria procumbens* tissue (Ellis and Amrhein 1971), and in uninfected tomato seedlings (Chadha and Brown 1974). In addition, the direct conversion of [^{14}C]BA to [^{14}C]SA was also reported in etiolated *Helianthus annuus* hypocotyls, *Solanum tuberosum* tubers, and *Pisum sativum* internodes (Klämbt 1962). Labeled [^{14}C]SA in infected cucumber plants was found not only after feeding [^{14}C]BA but also after using [^{14}C]Phe as precursor (Meuwly et al. 1995). The enzyme that catalyzes the transformation of CA to BA has been identified in *Quercus pedunculata* (Alibert et al. 1972). The third route for the biosynthesis of SA (Route 2b) involves a 2-hydroxylation of CA to *O*-coumaric acid which is then decarboxylated to SA and the reaction is catalyzed by the enzyme *trans*-cinnamate-4-hydroxylase (Alibert and Ranjeva 1972), which was first detected in pea seedlings (Russell and Conn 1967). This enzyme was also identified in *Quercus pedunculata* (Alibert and Ranjeva 1972) and in *Melilotus alba* (Gestetner and Conn 1974). In support of the *O*-coumaric acid pathway, leaves of *G. procumbens* or *Primula acaulis* accumulated both labeled *O*-coumaric acid and SA after feeding with [^{14}C]CA or Phe (Grisebach and Vollmer 1963; El-Basyouni et al. 1964). The growth conditions could favour one of the aforesaid SA biosynthesis routes in the plant. It was

suggested, for example, that tomato seedlings infected with *A. tumefaciens* synthesized SA via *O*-coumaric acid whereas the BA- involving routes (Route 2c-1 and Route 2c-2) operated in noninfected plants (Chadha and Brown 1974). Using pathogen-inoculated tobacco and cucumber, elicitor-treated potato, or healthy rice seedlings, radiolabelling studies suggested that SA was synthesized from Phe via BA (Yalpani et al. 1993; Silverman et al. 1995; Coquoz et al. 1998). Further support to the PAL role in SA biosynthesis came from the combined findings that tobacco and *A. thaliana* resisting pathogen infection showed increases in *PAL* expression (Mauch-Mani and Slusarenko 1996) and endogenous SA levels (Dempsey et al. 1999). Moreover, loss of PAL activity, due to sense-suppression or treatment with the PAL inhibitor 2-aminoindan-2-phosphonic acid (AIP), reduced pathogen-induced SA accumulation in tobacco, cucumber, and *A. thaliana* (Meuwly et al. 1995; Pallas et al. 1996).

2.2 The IC Pathway

Genetic studies in *A. thaliana* showed that SA was also formed when the SA biosynthetic routes mentioned before were either inhibited or the specific activity of radiolabeled SA in feeding tests was lower than expected. In this additional pathway (the IC pathway), SA is synthesized from chorismate by means of isochorismate synthase (ICS) in chloroplasts. The SA synthesized by this pathway is responsible for providing local and SAR in plants (Wildermuth et al. 2001) and was also shown to be implicated in several physiological processes including modulation of flowering time (Martínez et al. 2004) and leaf senescence (Abreu and Munné-Bosch 2008). Furthermore, the importance of this pathway for SA synthesis has been explored in response to biotic and abiotic stresses. The IC pathway was found to be the major route for SA synthesis in both basal and induced thermotolerance in *A. thaliana*, although it should be kept in mind that the PAL pathway was also operational in this study (Garcion et al. 2008).

The IC pathway for SA biosynthesis was firstly studied in bacteria. In fact several bacterial genera have been shown to synthesize SA, which is employed in the production of iron-chelating siderophores (Garcion and Métraux 2006). In the bacterial pathway, chorismate is converted to SA through IC (Verberne et al. 1999). In some bacterial species a unifunctional enzyme, ICS, isomerizes chorismate to IC which is then transformed to SA and pyruvate by another unifunctional enzyme, isochorismate pyruvate lyase (IPL; Serino et al. 1995; Mercado-Blanco et al. 2001). However, the SA synthesis in *Yersinia enterocolitica* and *Micobacterium tuberculosis* is mediated by a sole, bifunctional enzyme termed SA synthase (SAS). This enzyme directly converts chorismate to SA via an IC intermediate (Kerbarh et al. 2005; Harrison et al. 2006). The ICS and SAS enzymes are structurally very similar and contain preserved active sites (Parsons et al. 2008).

In plants, Wildermuth et al. (2001) characterized two putative *ICS* genes (*ICS1* and *ICS2*) implicated in the SA biosynthesis from IC in *A. thaliana*. *ICS1* expression correlated with SA accumulation and expression of the SA-induced *PR-1* gene. More recent analyses revealed that *ICS1* transcripts are also accumulated in response to several biotic and abiotic stresses, including UV light, ozone, biotrophic pathogens, and exogenous SA treatment (Ogawa et al. 2005; Nobuta et al. 2007; Harrower and Wildermuth 2011). Overexpression of bacterial ICS and IPL in tobacco or *A. thaliana* led to higher levels of SA and SA glucoside as compared to wild-type plants, provided the enzymes were targeted to the chloroplasts (Verberne et al. 2000; Mauch et al. 2001). Biochemical and molecular analyses of ICS1 further supported a role for this enzyme in SA biosynthesis. The high affinity of ICS1 for chorismate allows ICS1 to compete successfully with other pathogen-induced enzymes that use chorismate as their substrate, such as anthranilate synthase (Ziebart and Toney 2010). ICS1 functions as a unifunctional ICS (Strawn et al. 2007). Moreover, SA accumulation is not totally blocked by mutations in ICS1, so ICS2 seems to have a redundant function (Garcion et al. 2008). Some studies in tobacco cells indicated that ICS2 is a functional, chloroplast-localized ICS (Garcion et al. 2008), and further analysis of the recombinant ICS2 confirmed it to be a unifunctional ICS that is imported into the chloroplast stroma (Strawn et al. 2007). Still, genetic analyses revealed that ICS2 accounted for small level of SA biosynthesis (Garcion et al. 2008). In addition to Arabidopsis, *ICS* homolog has been characterized in several other plant species (Van Tegelen et al. 1999; Ogawa et al. 2005; Uppalapati et al. 2007; Catinot et al. 2008; Yuan et al. 2009). Knowing their role in phylloquinone synthesis, it is very likely that ICS homologues will be identified in all plant species. Hence, identification of *ICS* gene in a given plant species is not enough to show that SA synthesis occurs via IC. However, analysis of [1-^{13}C]-D-glucose incorporation in *Catharanthus roseus* cells, revealed that most of the SA synthesized after elicitation with a *Pythium aphanidermatum* extract was generated via the IC pathway (Mustafa et al. 2009). Still, it is remarkable that no plant gene encoding an IPL activity has been identified so far (Chen et al. 2009).

3 Roles of SA

3.1 Regulation of SA Accumulation

The SA metabolism may differ slightly between plant species. In some species, IC metabolism is more complex than in *A. thaliana*, with a major flux of IC channelled to the formation of anthraquinones (Stalman et al. 2003). In other species, including rice and poplar, SA synthesis is not markedly induced by biotic and abiotic stress, while SA and their derivatives play a key role in disease and herbivore resistance (Smith and Mètraux 1991). Based on the current knowledge in *A.*

thaliana, Dempsey et al. (2011) suggested an integrated model of SA metabolism regulation. According to this model, SA metabolism is mostly regulated at the transcriptional level. In the absence of an inducing stress or hormone, the genes involved in SA synthesis and modification are expressed at low levels. Following a biotic and abiotic stress, activation of *ICS1* expression likely requires both de-repression of negative transcriptional regulators, such as *ETHYLENE INSENSI-TIVE3* (*EIN3*) and *EIN3-LIKE1* (*EIL1*), and activation of positive regulators, including *CALMODULIN-BINDING PROTEIN60-LIKE G* (*CBP60g*), *SAR-DEFICIENT1* (*SARD1*), and/or *WRKY28* (see Dempsey et al. 2011 for review). However, the mechanisms leading to these modifications still remain unknown. Once sufficient levels of SA have been generated, SA-mediated activation of pathogenesis-related genes (PR) such as *PR1* leads to a feedback inhibition of *ICS1* expression, therefore preventing excessive SA accumulation. Transcriptional regulation of SA modifying genes such as *BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE 1* (*BSMT1*) and *GH3 ACYL ADENYLASE FAMILY MEMBER 3.5* (*GH3.5*) by auxin, jasmonic acid and ethylene may also play a role in controlling cellular SA levels (Goda et al. 2008). In this manner, these hormones can limit SA accumulation, which in turn suppresses the activation of SA-induced defence responses. Furthermore, ethylene and jasmonic acid pro-mote the expression/stabilization of EIN3, which negatively regulates *ICS1* expression and thus suppresses SA levels. On the other hand, SA has the ability to form conjugates with a range of molecules either by glycosylation, methylation, and amino acid conjugation or by esterification. Most of these modifications make SA inactive, so in addition to the transcriptional regulation, SA levels can be rapidly tuned at the biochemical level, by modulating the activities of SA modi-fying enzymes, including benzoic acid/salicylic acid carboxyl methyltransferase 1 (BSMT1), methyl esterase 1 and 9 (MES1/9), salicylic acid glucosyltransferase 1 (SGT1) or UDP-glucosyltransferase 74F1 (UGT74F1), among others (Koo et al. 2007; Forouhar et al. 2005; Song et al. 2009; Dean and Delaney 2008). Hence, local hormone concentrations and the comparative catalytic efficiencies of these enzymes, as well as their level of expression, play a role in determining the level of free SA. Interestingly, it was shown that the SA modifying enzymes have very large differences in their Km for SA, so their activity on SA change in concert with the temporal and spatial variations in free SA levels. For instance, a local threshold of approx. 200 mM free SA appears to be necessary for glucosylated SA formation by SAGT1 and UGT74F1 and robust activation of defence gene expression. By contrast, enzymes that convert free SA to inactive forms for transport or catalysis appear to have a higher affinity for SA. Moreover, the proper balance between SA synthesis and catabolism may involve temporal and/or spatial separation of these competing processes (Dempsey et al. 2011).

   Most of the SA accumulated in plants is glucosylated and/or methylated. Glucose conjugation at the hydroxyl group of SA results in formation of the SA glucoside (SAG, SA 2-*O*-β-D-glucoside) as a major conjugate, while glucose conjugation at the SA carboxyl group produces the SA glucose ester in small amounts. These conjugation reactions are initiated by cytosolic SA

glucosyltransferases that are induced by SA application or pathogen attack in tobacco and *A. thaliana* plants (Lee and Raskin 1998; Song 2006). The lack of SAG in phloem exudates makes it an implausible candidate for the translocatable form of SA. In addition to lowering cellular SA, SAG may function as a slow-release storage form of SA that keeps SAR over extended periods of time (Dean and Mills 2004; Dean et al. 2005). Another alternative is that the SAG formation is the first step in SA catabolism. SA can also be converted to methylsalicylate (MeSA) by SA carboxyl methyltransferase (Chen et al. 2003; Park et al. 2007a, b; Vlot et al. 2008). Methylation inactivates SA, while increasing its membrane permeability, as well as its volatility, thus allowing more effective long distance transport. It is hypothesized that volatile MeSA may function as an airborne signal for both intra- and interplant communication (Shulaev et al. 1997). MeSA can be further glucosylated to produce MeSA 2-*O*-β-D-glucose, but this SA-conjugated form is not stored in the vacuole (Dean et al. 2005). Amino acid conjugation of SA is less well characterized, but may be involved in SA catabolism. Additionally, SA has been shown to be sulfonated in vitro by members of the SOT family of sulphotransferases in *A. thaliana* (Baek et al. 2010). The characterization of SOT12 revealed that it catalyzes the transfer of a sulphuryl group (SO_3^{-1}) to the 2-OH position of SA in vitro (Baek et al. 2010). Still, sulphonated SA has not been detected *in* planta so far.

3.2 Role of SA in the Response of Plants to Water and Salt Stress

SA was initially regarded as an important signal molecule during plant-pathogen interactions, a chemical defence against microbes, and an allelopathic compound (Malamy and Klessig 1992; Raskin 1992). Since then, numerous new roles have been proposed for SA, including mediation of the response of plants to a number of abiotic stresses, such as heat stress (Dat et al. 2000; He et al. 2005; Larkindale et al. 2005), chilling (Janda et al. 1999; Senaratna et al. 2000), salt and osmotic stress (Borsani et al. 2001; Molina et al. 2002), and ozone and UV- light exposure (Yalpani et al. 1994; Sharma et al. 1996; Rao and Davies 1999). It is now clear that the role of SA during plant biotic and abiotic stress responses is coupled to the production of ROS (Dat et al. 2000; Mittler 2002). These compounds can alter the cellular redox homeostasis and lead to oxidative damage to lipids, proteins and nucleic acids (Reviewed by Møller et al. 2007).

When applied exogenously at adequate (usually low) concentrations, SA was found to improve the efficiency of the antioxidant system in plants (Knorzer et al. 1999). SA treatment was found to alleviate the oxidative stress generated by paraquat in tobacco and cucumber (Strobel and Kuc 1995). SA was also found to boost the activities of antioxidant enzymes, CAT, peroxidase (POX) and super-oxide dismutase (SOD), when sprayed to drought-stressed tomato plants (Hayat

et al. 2008) as well as to salt-stressed mustard plants (Yusuf et al. 2008). In contrast, the treatment with SA resulted in transitory reduction of catalase (CAT) activity and increased H_2O_2 level (Janda et al. 2000) which played a key role in providing the SAR (Chen et al. 1993) and tolerance against the oxidative stress (Gechev et al. 2002). In rice, soaking seeds with SA prior to sowing results on a decline in the activities of the antioxidant enzymes CAT, POX, SOD and gluta-thione reductase (Choudhury and Panda 2004). These discrepancies are probably due to the differences in the experimental set up used. Exogenous SA has been shown to bind and inactivate CAT and to inactivate cytosolic APX which under a stress situation such as drought may lead to enhanced formation of ROS such as H_2O_2 (Mittler 2002). This initial accumulation of ROS leads to acclimation, which includes enhanced antioxidant defences. Thus, the timing of the experiments determines whether the initial inhibition of the antioxidant machinery to cause an oxidative burst or the subsequent reinforcement of the antioxidant protection, is recorded (see Fig. 2). However, further research is needed in order to verify that the discrepancies in the behavior of antioxidants, especially CAT, can be indeed ascribed to the temporal resolution of the mentioned reports.

Several studies indicate that the exogenous supply of SA can notably alleviate the salt-induced toxic effects in plants including barley (El Tayeb 2005) and *A. thaliana* seedlings (Borsani et al. 2001). In carrot, the supply of exogenous SA markedly improved plant growth under combined salt stress and boron toxicity, which was asociated to an increase in the carotenoids and anthocyanin contents

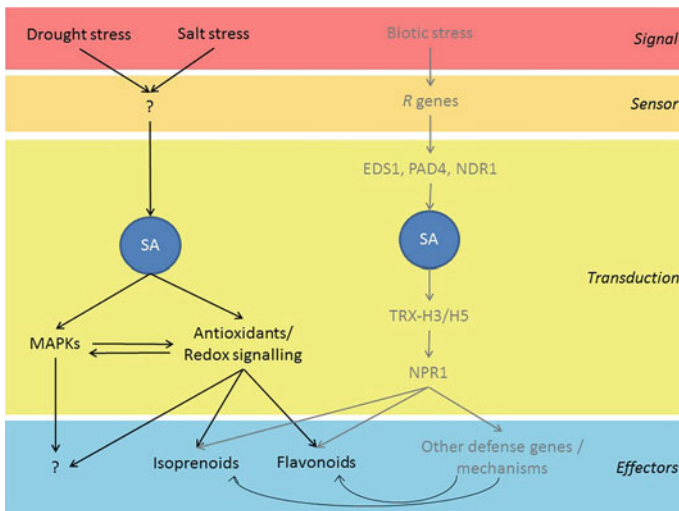**Fig. 2** Comparision between the SA-dependent signal transduction pathways leading to the accumulation of flavonoids and terpenoids under biotic stress and abiotic stress (drought or salt stress). *Grey lines and text* correspond to events related to biotic stress signalling, and *lines and text in black* correspond to those involved in drought or salt stress signalling. TRX-H3/H5 stands for thioredoxin H3 or H5

and total antioxidant activity of shoots and roots (Eraslan et al. 2007). An enhanced tolerance to salt stress was also observed in wheat seedlings raised from grains soaked in SA (Hamada and Al-Hakimi 2001). Under such a stress, these seedlings accumulated high amounts of proline, which increased further when SA was applied exogenously, thereby alleviating the damaging effects of salt stress (Shakirova et al. 2003). The exogenous SA barred the decrease of both auxin and cytokinin levels in salt stressed wheat plants resulting in a better cell division in root apical meristem, and thus increasing growth and productivity of plants (Shakirova et al. 2003). These authors also reported that the pre-treatment with SA resulted in the accumulation of ABA which might contribute to the pre-adaptation of seedlings to salt stress as ABA induces the synthesis of several anti-stress proteins that protected the plants. Besides, the SA treatment was shown to decrease the activities of SOD and POX in the roots of young wheat seedlings (Shakirova et al. 2003); which clearly indicated that their activities are directly or indirectly regulated by SA (Sakhabutdinova et al. 2004). Furthermore, the pre-sowing soaking of wheat seeds with SA positively affected the osmotic potential, shoot and root dry mass, K^+/Na^+ ratio, and contents of chlorophylls and carotenoids in wheat seedlings, under both saline and non-saline conditions (Kaydan et al. 2007). The loss of growth, photosynthetic parameters and the nitrate reductase and carbonic anhydrase activities, as a result of salt stress in mustard, were recovered when SA was sprayed to the foliage. Furthermore, the activities of various antioxidant enzymes (CAT, POX and SOD) and proline content were increased under salinity exposure and/or SA treatment, providing enhanced tolerance against salinity stress (Yusuf et al. 2008).

Compelling evidence indicate that SA plays a key role in providing tolerance to plants exposed to water deficit. Hayat et al. (2008) showed that the treatment of the water-stressed tomato plants with low SA concentrations improved their tolerance to drought stress. Furthermore, similar conditions provided tolerance to the harmful effects of drought in bean plants, whereas, higher concentrations did not show successful results (Senaratna et al. 2000). Higher tolerance to drought stress was also observed in plants raised from the grains soaked in aqueous solution of acetyl SA, which also improved dry matter accumulation (Hamada and Al-Hakimi 2001). Wheat seedlings subjected to drought stress and treated with SA exhibited higher water content and also higher dry matter accumulation, carboxylase activity of Rubisco, SOD and total chlorophyll content compared to the untreated control (Singh and Usha 2003). The SA application also alleviated the damaging effects of water deficit on cell membranes of barley plants and concomitantly increased the ABA content in leaves, which might have contributed to the enhanced tolerance of plants to water scarcity (Bandurska and Stroinski 2005).

Salt and drought stresses induce severe metabolic disfunctions by boosting ROS formation and accumulation, which results on an oxidative stress that may lead to damage in DNA, inactivation of enzymes and lipid peroxidation (Smirnoff 1993; Dat et al. 2000; Mittler 2002; Chaves et al. 2003). As a result, there exist several

examples of SA and ROS interplay during abiotic stress situations. The rapid and transient production of ROS during these stress conditions is analogous to the "oxidative burst" commonly observed during plant-pathogen interactions but also to that reported in mammalian macrophage and neutrophils during pathogen invasion (Langebartels et al. 2002). During many biotic and abiotic stress situations SA-induced ROS formation is crucial for the development of an appropriate response. *Arabidopsis eto1* and *eto3* mutants, ethylene overproducers, constitutively accumulate high SA levels and exhibit a rapid increase in free SA prior to lesion formation in response to ozone fumigation (Rao et al. 2002). In contrast, in rice, which contains much higher basal levels of free SA (at least 10 times more than infected tobacco or *Arabidopsis* plants), SA does not appear to be an effective signal molecule during disease responses (Yang et al. 2004). SA-deficient transgenic rice has higher ROS levels and reduced antioxidant capacity, as well as spontaneous lesion formation in an age- and light-dependent manner. Symptom development was in fact suppressed by exogenous application of the SA analog, benzothiadiazol (Yang et al. 2004). Although unexpected, these results may be explained by the fact that in plants SA may directly function as an antioxidant as reported for animals (Castagne et al. 1999). SA may scavenge hydroxyl radicals and thus protect plants against catalase inactivation by H_2O_2 (Durner and Klessig 1996). Hence, in rice for instance, SA may play a critical role in modulating the cell redox balance, hence protecting the plant against oxidative damage (Yang et al. 2004).

Despite of the evident involvement of SA in plant responses to drought and salt stress, the drought or salt stress signal transduction mechanisms downstream SA remain obscure. The extensive search for a SA receptor has yielded several SA-binding proteins. One of these proteins is CAT, which is specifically inactivated upon SA binding. Moreover, SA also inhibits cAPX activity directly, thus indicating that SA may inhibit ROS scavenging systems to facilitate an oxidative burst (reviewed by Vlot et al. 2009; Fig. 2). In biotic interactions the task of SA sensing lies largely upon NONEXPRESSION OF PR GENES1 (NPR1) (Pieterse and Van Loon 2004) (Fig. 2). Whether NPR is involved in SA-dependent abiotic stress responses is still unclear, but *npr1* mutants show delayed senescence phenotype (Morris et al. 2000). The involvement of several Mitogen-Activated Protein Kinases (MAPKs or MPKs) such as the tobacco SA-INDUCED PROTEIN KINASE (SIPK) and WOUND-INDUCED PROTEIN KINASE (WIPK), and their respective Arabidopsis orthologues AtMPK3 and 6, has been shown in plant responses to both drought and osmotic stress (a component of salt stress) (reviewed by Vlot et al. 2009) and, in agreement, MAPK cascades operating under drought and salt stress have been partially described (MEKK1-MKK2-MPK4 and MKK1-MPK4, respectively) (see Zhang and Klessig 2001 for review). Still, both the stress sensor upstream the MAPK cascades and the effectors downstream them remain unknown (Fig. 2).

3.3 Role of SA in Modulating Terpenoid and Flavonoid Metabolism

Unfavourable conditions such as drought or salt stress lead to an imbalance between the energy harvested by plants and their ability to process it (Chaves et al. 2003). Plants display a number of mechanisms to avoid and dissipate this excess excitation energy, some of which engage isoprenoid and flavonoid compounds (Munné-Bosch and Alegre 2003; Delfine et al. 2005; Owen and Peñuelas 2005; Agati and Tattini 2010). Many of these mechanisms represent conserved tools for photoprotection. For instance, α-tocopherol and carotenoids display antioxidant activity and contribute to keep cell functionality under stress (Havaux and Kloppstech 2001; Havaux et al. 2005; Munné-Bosch 2005). Others, such as monoterpenes and flavonoids (including anthocyanins and flavonols) represent additional or alternative protective mechanisms (Feild et al. 2001; Peñuelas and Munné-Bosch 2005; Hernández et al. 2009). Stress protection by terpenoids and flavonoids has been ascribed to their antioxidant capacity, which in many cases, especially terpenoids but also some flavonoids such as (−)-epigallocatechin gallate, is exerted in membranes (Munné-Bosch 2005; Hernández et al. 2006).

The biosynthesis of terpenoids has been extensively reviewed in the literature (see Cheng et al. 2007 for a recent review). In summary, it consists in the sequential condensation of isopentenyl diphosphate (IPP) molecules. In plants, the biosynthesis of this 5-carbon intermediary is achieved by two different pathways, the 2-methyl-D-erytritol-4-phosphate (MEP) and the mevalonate (MEV) pathways, which operate in plastids and cytosol, respectively. All terpenoids are synthetized by the sequential action of a set of prenyltransfereases that elongate the prenyl chain, initially IPP, by the repetitive addition of this C_5 unit, generating mono-, sesqui-, di-, tri-, tetraterpenes or even longer compounds. The genes encoding for the 1-deoxy-D-xylulose-5-phosphate reductoisomerase, from the chloroplastic MEP pathway, and 3-hydroxy-3-methylglutaryl-CoA reductase, from the cytosolic MEV pathway, have been shown to be transcriptionally upregulated by SA in *Salvia miltiorhiza* and *Michelia chapensis*, respectively (Yan et al. 2009; Cao et al. 2011). The expression of farnesyl diphosphate synthase (FDPS), geranylgeranyl diphosphate synthase (GGDS) and squalene synthase (SQS), three prenyltransferases from the core terpenoid biosynthetic pathway, has been shown to be upregulated by SA in different species as well (Cao et al. 2012; Shabani et al. 2010; Kai et al. 2010). In agreement, the levels of many isoprenoids such as α-tocopherol or β-carotene have been shown to be upregulated during drought or salt stress in parallel to increases in SA levels (see for instance Munné-Bosch and Peñuelas 2003; Eraslan et al. 2007). In addition, holm oaks fumigated with SA showed higher monoterpene levels in leaves and enhanced volatile monoterpene emision (Peñuelas et al. 2007). Thus, as a general rule, SA upregulates the terpenoid biosynthetic pathway at the transcriptional level.

However, not all terpenoids behave equally upon drought or salt stress exposure and SA levels, and some studies report on decreases of the levels of particular

terpenoids associated with increases of SA levels. For instance, water-stressed *Phillyrea angustifolia* plants showed increased α-tocopherol levels but decreased amounts of β-carotene and chlorophylls (Munné-Bosch and Peñuelas 2003). Extreme drough and salt stress provokes senescence, with the concomitant loss of chlorophylls and carotenoids (Abreu and Munné-Bosch 2008), and SA has been shown to positively regulate, at transcriptional level, some senescence-associated genes such as *SAG102*, which encodes a cysteine protease (Morris et al. 2000). Moreover, *A. thaliana NahG* transgenic lines, which constitutively degrade SA, show delayed senescence symptoms such as chlorophyll and carotenoid loss (Abreu and Munne-Bosch 2009). In agreement, *A. thaliana flu* mutants (which overaccumulate $^1O_2$) expressing transgenic NahG are somehow protected from the cell death provoked by the release of $^1O_2$ (Danon et al. 2005). The role of SA as a both senescence and terpenoid biosynthesis promoter seems contradictory. Still, despite of the clear effect of SA in the expression of core isoprenoid biosynthetic genes, its effect on the genes encoding for downstream terpenoid modifying enzymes is yet to be explored. For instance, it has been shown that the phytol derived from chlorophyll degradation during senescence can bind the aromatic ring of homogentisic acid to form 2-methyl-6-phytyl benzoquinone, and subsequently tocopherols (Valentin et al. 2006). Further research is needed in order to determine the effects of SA on the biosynthesis of particular terpenoids (especially in the committed steps leading to these particular terpenoids), and to establish the possible relationship between the biosynthetic pathways leading to different individual terpenoids.

Flavonoids are a group of compounds derived from the phenylpropanoid pathway. Although multiple variations and side branches exist to synthetize more than 9,000 different flavonoids identified so far in nature (Williams and Grayer 2004), their biosynthesis is essentially as follows. The shikimate pathway produces chorismate, which is sequentially transformed into phe; phe is de-aminated and sequentially transformed into 4-coumaroyl-CoA by the simple phenylpropanoid pathway; and 4-Coumaroyl-CoA is condensed with 3 molecules of malonyl-CoA by the action of a chalcone synthase (CHS) to yield a chalcone in the first committed step of the flavonoid biosynthetic pathway. Thereafter, chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS) form the core flavonoid biosynthetic pathway (reviewed by Winkel-Shirley 2001; and Grotewold 2006). Exogenous SA is known to boost the accumulation of flavonoids in several plant species (e.g. Yang et al. 2008; Xu et al. 2009). To enhance flavonoid accumulation SA has been reported to trigger the expression and/or increases the activity of the gene products of most of the core flavonoid biosynthetic genes (e.g. *CHS*, *CHI*, *F3H* and *ANS*) (Campos et al. 2003; Yu et al. 2006; Xu et al. 2008). Although it falls out of the scope of this chapter, SA in plant pathogen interactions triggers the expression of genes leading to the accumulation of flavonoid phytoalexins such as resveratrol (reviewed by Ahuja et al. 2012). However, constitutively SA-producing (CSA) tobacco plants obtained by inserting two bacterial genes encoding for ICS and IPL in tobacco plants (thus CSA plants constitutively produce SA from chorismate) show a strong

inhibition of the accumulation of the flavonoids quercetin, kaempferol and rutin (Verberne et al. 2000; Nugroho et al. 2002). This seems an extreme case since the constitutive production of SA in these plants appears to divert all the isochorismate available towards SA production, this way preventing its utilization for phe biosynthesis and therefore for flavonoid biosynthesis. In support of this idea TMV-inoculation or SA-treatment to CSA plants did not affect the expression of *PAL*, *CHS* or *CM* (Guo et al. 2000). Under high light stress, the flavonoid biosynthetic genes have been shown to be coordinately upregulated mainly -not exclusively, though- by two types of transcription factors (MYB and bHLH) (reviewed by Grotewold 2006).

4 Conclusion

SA has been recognized as a regulatory signal mediating plant response to abiotic stress such as drought and salinity. SA may affect both isoprenoid and flavonoid accumulation in leaves both under control and stress conditions. Further research is however required to better understand how SA mediates the changes in isoprenoid and flavonoid metabolism, whether biosynthetic or degradation pathways are affected and how isoprenoid and flavonoid building blocks are diverted to specific biosynthetic routes.

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Chapter 9
Salicylic Acid-Mediated Stress-Induced Flowering

K. C. Wada and K. Takeno

Abstract Plants have a tendency to flower under unsuitable growth conditions. Stress factors, such as poor nutrition, high or low temperature, high- or low-intensity light, and ultraviolet light, have been implicated in this stress-induced flowering. The stressed plants do not wait for the arrival of a season when photoperiodic conditions are suitable for flowering, and such precocious flowering might assist in species preservation. Stress-induced flowering has been well studied in *Pharbitis nil* (synonym *Ipomoea nil*), *Perilla frutescens* var. *crispa*, *Lemna paucicostata* (synonym *Lemna aequinoctialis*) and *Arabidopsis thaliana*. The phenylalanine ammonia-lyase (PAL) inhibitor suppresses stress-induced flowering in *P. nil*, and this effect was reversed with salicylic acid (SA). The *PAL* gene expression, PAL enzyme activity and SA content in the cotyledons increased during stress-induced flowering. These results suggest that SA mediates stress-induced flowering.

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1 Introduction

After a period of vegetative growth, plants switch to reproductive growth to produce the next generation. The switch from vegetative to reproductive growth is called flowering. Flowering is regulated through both endogenous and environmental factors. One endogenous factor is the autonomous pathway for the regulation of flowering, which has been well characterized in *Arabidopsis thaliana* (Simpson 2004). Day-neutral plants switch from vegetative to reproductive growth in response to endogenous signals after a designated period of time (McDaniel 1996). Environmental factors that regulate flowering include the duration of the day and night periods in photoperiodic flowering and low temperature in vernalization (Thomas and Vince-Prue 1997). The flowering stimulus, which is produced in leaves under adequate photoperiodic conditions, is transferred to the shoot apical meristem, and induces the transition from the vegetative apical meristem to floral meristem, is called florigen. The isolation and identification of florigen has been long considered as an important research subject in plant physiology. Recently, the proteins derived from the floral pathway integrator gene of *A. thaliana*, *FLOWERING LOCUS T* (*FT*), and its homologs have been characterized as florigens in several species (Corbesier et al. 2007; Lin et al. 2007; Tamaki et al. 2007). Epigenetic mechanisms, such as DNA demethylation, are involved in the regulation of vernalization, i.e., flowering induced through exposure to low temperatures ranging from 0 to 5 °C (Michaels and Amasino 2000, 2001). Occasionally, flowering that cannot be classified as photoperiodic flowering or vernalization has also been reported. For some plants, flowering is induced under unsuitable growth conditions. Stress has been implicated in many cases of non-photoperiodic flowering. This flowering is called stress-induced flowering. It is becoming apparent that SA is involved in the regulation of the stress-induced flowering.

2 Stress-Induced Flowering

The short-day (SD) plant, *Pharbitis nil* (synonym *Ipomoea nil*), can be induced to flower under long-day (LD) conditions when grown in tap water (poor-nutrition conditions), at 12–15 °C (low-temperature conditions) or under 15,000–20,000 lux light (high-intensity light conditions) (Hirai et al. 1993, 1994; Shinozaki 1985; Shinozaki and Takimoto 1982; Shinozaki et al. 1982, 1988a, b, 1994; Swe et al. 1985). Phenylalanine ammonia-lyase (PAL) activity increases when flowering is induced under these growth conditions (Hirai et al. 1995). Although the factors that can induce such LD flowering are not related, PAL is involved in flowering induced under any of these conditions. This observation suggests that these factors might stimulate flowering through a common signal transduction pathway. Poor nutrition, low temperature and high-intensity light can be regarded as stress factors, and PAL activity increases under stress conditions (Dixon and Paiva 1995).

Accordingly, we assumed that LD flowering in *P. nil* might be induced by stress (Wada et al. 2010b). Non-photoperiodic flowering is not restricted to *P. nil*. We observed that the SD plant *Lemna paucicostata* (synonym *Lemna aequinoctialis*) flowered under LD conditions when grown under poor-nutrition conditions (Shimakawa et al. 2012). Further, we observed that the SD plant *Perilla frutescens* var. *crispa* exhibited LD flowering when grown under low-intensity light (Wada et al. 2010a). Low-intensity light can also be regarded as a stress factor. Non-photoperiodic flowering has been sporadically reported in several plant species. Experienced flowering physiologists have noticed that plants have a tendency to flower under unsuitable growth conditions. However, this unusual flowering has not been studied systematically. We surveyed the flowering behavior reported in the literature, and found that most of the factors responsible for such flowering could be regarded as stress, although many of those reports did not show that stress was responsible for flowering. These factors include high or low light intensity, high or low temperature, ultraviolet (UV) light, drought, crowdedness, nitrogen deficiency, salt, poor nutrition, oxygen debt, root removal, girdling, and mechanical stimulation (Takeno 2012; Wada and Takeno 2010). Approximately 20 plant species belonging to a wide range of taxonomic groups that undergo stress-induced flowering have been reported. Therefore, flowering induced through stress might be a universal phenomenon in the angiosperms. Accordingly, this flowering has been named 'stress-induced flowering' (Hatayama and Takeno 2003; Takeno 2012; Wada and Takeno 2010). The concept and term of stress-induced flowering have recently become acceptable concepts in the area of flowering physiology (Blanvillain et al. 2011; Dezar et al. 2011; Marín et al. 2011; Rivas-San Vicente and Plasencia 2011; Segarra et al. 2010; Yaish et al. 2011).

Stress can be defined as a condition in which the vegetative growth of plants is suppressed. Because flowering is the change from vegetative growth to reproductive growth, it is quite natural that the suppression of vegetative growth through stress accelerates flowering. Stress-induced flowering results in the production of fertile seeds in plants, and the progeny develop normally in both *P. nil* and *P. frutescens* (Wada et al. 2010a, b). Plants modify their developmental processes to adapt to stress conditions. Stress-induced flowering is an adaptation to stress. Plants flower as an emergency response if stressed to ensure the production of the next generation. Through this mechanism, plants preserve the species, even under unfavorable conditions. The stressed plants do not need to wait for photoperiodic conditions that are suitable for flowering, and such precocious flowering might assist in species preservation. Thus, stress-induced flowering can be regarded as an ultimate adaptation to stress and should be considered a central component, along with tolerance, resistance and avoidance, of stress physiology. Stress-induced flowering might have a biological benefit and should be considered as important as photoperiodic flowering and vernalization to plant physiology.

3 Involvement of Salicylic Acid in Flowering

The plant hormone, salicylic acid (SA), induces flowering in many species belonging to the Lemnaceae (Kandeler 1985). SA was first identified in a unique and elegant experiment in the quest of photoperiodic flowering stimulus (Cleland 1970, 1978). The flowering stimulus moves through the phloem from photo-induced leaves to shoot apices. Aphids feed on phloem sap by inserting their stylet into the sieve element and produce large quantities of honeydew that is qualitatively quite similar to phloem sap. Abscicic acid (ABA), gibberellins and indole-acetic acid (IAA) pass through aphids and appear in the honeydew without any apparent loss in biological activity. By analogy, it was proposed that the chemical substances responsible for the control of flowering would behave in a similar manner and thus make it possible to isolate them from aphid honeydew. Therefore, honeydew was collected from aphids feeding on the SD plant *Xanthium strumarium*. The honeydew from the aphids was collected on glass plates placed around the base of the plants. The collected honeydew was dissolved in phosphate buffer, extracted with ethyl acetate at pH 2.5, and purified using thin-layer chromatography. The purified sample was added to the culture medium of the LD plant *Lemna gibba* G3 to test the effect on flowering. The flower-inducing activity was obtained, and the active flower-inducing substance was identified as SA (Cleland 1974; Cleland and Ajami 1974). Authentic SA induces the flowering of *L. gibba* under non-inductive photoperiodic conditions. However, SA had no apparent effect on flowering when administered to *X. strumarium*. Furthermore, SA was present in honeydew collected from aphids feeding on both vegetative and flowering *X. strumarium*. These negate the determining role of SA in the control of photoperiodic flowering in *X. strumarium*.

The SA-induced flowering was observed in several species of *Lemna* and the related genus *Spirodela*. SA analogs, such as benzoic acid, induce flowering in *L. gibba* G3 (Cleland 1974). Therefore, the endogenous level of benzoic acid, a direct precursor of SA, was quantified in *L. gibba* G3 and three strains of *Lemna paucicostata* (synonym *Lemna aequinoctialis*) (Fujioka et al. 1983); no difference was detected between vegetative and flowering plants. Thus, the endogenous level of benzoic acid, and most likely also SA, does not regulate the photoperiodic flowering of *Lemna*.

SA is not considered as the endogenous flowering stimulus for regulating photoperiodic flowering. However, it is apparent that SA can induce flowering when exogenously applied to the Lemnaceous plants. Thus, SA most likely plays some important role in the regulation of flowering.

4 Flowering Substances of Stress-Induced Flowering

Stress-induced flowering is accompanied by an increase in PAL activity (Hirai et al. 1995). PAL activity is known to increase under stress conditions (Dixon and Paiva 1995). These suggest that some compound(s) in PAL-regulated metabolic

pathways that might act as flowering stimuli. PAL regulates the biosynthesis of phenylpropanoids, such as chlorogenic acid (CGA), which were prominent candidates for flowering stimulation in early studies. Phenylpropanoids accumulate in the cotyledons during conditions of poor nutrition, low temperature or high-intensity light (Hirai et al. 1993, 1994; Shinozaki et al. 1988a, b, 1994). Many reports have indicated a close correlation between CGA content and the flowering response (Ishimaru et al. 1996). Aminooxyacetic acid (AOA), which functions as a PAL inhibitor (Kessmann et al. 1990), inhibits flowering (Hatayama and Takeno 2003; Shinozaki et al. 1988a, 1994). These findings suggested that endogenous CGA might be involved in LD flowering. However, the exogenous application of CGA and the related phenylpropanoids did not induce flowering (Ishimaru et al. 1996; Shinozaki et al. 1988a, 1994), suggesting that other PAL-regulated compounds might be involved in this flowering.

When plants are stressed, they generate stress substances, such as reactive oxygen species, nitric acid, jasmonic acid, SA, ethylene and ABA, which regulate gene expression to adapt to the stress conditions (Hey et al. 2010; Jaspers and Kangasjärvi 2010; Liu and Zhang 2004; Moreau et al. 2010; Xiong et al. 2002). Among these stress substances, SA and ethylene have been reported to induce flowering. Ethylene induces flowering in the members of the Bromeliaceae, which include pineapple. However, this is an exceptional case, and ethylene generally inhibits flowering in many plant species, including *P. nil*. SA induces flowering in the Lemnaceae plants, and PAL regulates the biosynthesis of SA. Although SA is synthesized from *t*-cinnamic acid, which is converted from phenylalanine by PAL in the majority of plant species (Yalpani et al. 1993), SA is also derived from isochorismate in bacteria and some plant species, including *A. thaliana* (Chen et al. 2009). Generally, however, stress induces PAL activity, resulting in the accumulation of SA (Dixon and Paiva 1995; Scott et al. 2004). Therefore, the most likely stress substance involved in stress-induced flowering is SA.

5 Salicylic Acid-Mediated Stress-Induced Flowering in *Pharbitis nil*

5.1 Stress-Induced Flowering in P. nil

P. nil is an obligatory SD plant with no vernalization requirement (Imamura 1967). However, *P. nil* cv. Violet was induced to flower under LD conditions when grown in 1/10- or 1/100-strength nutrient solution or tap water without the addition of any nutrients for 2 or more weeks (Wada et al. 2010b), or at 13 °C for 10 days (Hatayama and Takeno 2003). The other cultivar, Tendan, was not induced to flower under the poor nutrition conditions but flowered with greater sensitivity than cv. Violet at low temperatures. Thus, the response to stress differs depending on the cultivar.

Stressed plants might flower to produce the next generation as an emergency response to adapt to stress conditions. For this to be a biologically advantageous response, stress-induced flowering plants must produce fertile seeds and the progeny must develop normally. Therefore, we tested the ability of *P. nil* plants grown under continuous stress to produce normal progeny. Violet was grown in 1/10-strength nutrient solution throughout the plant life. The plants that were induced to flower through poor-nutrition stress reached anthesis, fruited and produced seeds (Wada et al. 2010b). These seeds germinated and the progeny developed normally. Furthermore, they responded to SD treatment and formed floral buds. The third generation of the stressed plants also developed normally. Therefore, it is apparent that stressed plants flower to produce the next generation as an emergency response when they can not adapt to unfavorable environmental conditions. It is apparent that stress-induced flowering has a biological benefit.

A transmissible flowering stimulus, such as florigen, which is involved in photoperiodic flowering, has not been reported in stress-induced flowering. To investigate this possibility, we performed grafting experiments to detect the transmission of stress-induced flowering stimuli. The presence of cotyledons is necessary for the flowering of *P. nil* seedlings in response to poor nutrition or low temperature (Shinozaki 1985; Shinozaki and Takimoto 1982). This suggests that a flowering stimulus is produced in cotyledons. If the stress-induced flowering stimulus is transmissible, defoliated scions might flower when grafted onto rootstocks with cotyledons and under stress conditions. Violet and Tendan were grafted in several combinations and the grafted plants were grown in tap water without nutrition under LD conditions for 20 days. The defoliated Violet scions grafted onto Violet rootstocks with cotyledons were induced to flower. The flowering might reflect the influence of the rootstocks because all the leaves were removed from the scions. Therefore, we conclude that a transmissible flowering stimulus is involved in the stress-induced flowering of *P. nil*. We predicted that Tendan would not produce such a flowering stimulus because Tendan did not flower in response to the poor-nutrition stress conditions. If this were the case, then Violet would not be expected to flower when grafted onto Tendan rootstocks. However, defoliated Violet scions grafted onto Tendan rootstocks with cotyledons were induced to flower. The flowering response of the scions grafted onto Tendan was slightly weaker than those grafted onto Violet, but the difference was not statistically significant. Therefore, Tendan might produce almost the same amount of the flowering stimulus as Violet. Conversely, the Tendan scions grafted onto Violet rootstocks were not induced to flower. These results indicate that Tendan produces a transmissible flowering stimulus but does not respond to it.

The flowering of *A. thaliana* is induced through the activation of *FT* expression. Two orthologs of *FT*, *PnFT1* and *PnFT2*, have been identified in *P. nil* (Hayama et al. 2007). Therefore, the expression of *PnFT* genes in response to poor-nutrition stress conditions was examined. *P. nil* Violet was induced to flower in tap water. The cotyledons and true leaves of these plants were collected, and the expression of *PnFT1* and *PnFT2* was examined using reverse transcription-polymerase chain reaction (RT-PCR). The expression of *PnFT1* and *PnFT2* was induced in

cotyledons given a single SD treatment, but neither gene was expressed under LD conditions (Wada et al. 2010a; Yamada 2011). The expression of *PnFT2* was induced in the cotyledons and true leaves of plants grown under the poor-nutrition conditions for 2 weeks or longer. The level of mRNA expression was closely correlated with the flowering response. However, *PnFT1* was not expressed in the cotyledons or true leaves regardless of nutritional conditions. These results suggest that *PnFT2*, but not *PnFT1*, is involved in the poor-nutrition stress-induced flowering of *P. nil*. *PnFT2* is involved in both photoperiodic flowering and stress-induced flowering, whereas *PnFT1* is only involved in photoperiodic flowering. The two *PnFT* genes might play different roles in the regulation of flowering depending on the inductive cue. It is also possible that the essential gene for flowering is *PnFT2* and that *PnFT1* expression is induced only under SD treatment and redundantly enhances the activity of *PnFT2*.

5.2 Involvement of the Metabolic Pathway Regulated by PAL in Stress-Induced Flowering

The PAL activity increases when *P. nil* is induced to flower under poor-nutrition or low-temperature conditions. Additionally, we observed that the roots of the stressed plants produced a red color when *P. nil* was induced to flower in response to poor-nutrition stress. Accordingly, we determined the anthocyanin content of roots, stem, cotyledons and true leaves that were harvested from Violet grown in nutrient solution or tap water for 20 days. The anthocyanin content was increased in the roots and stem of Violet grown in tap water, although there was no significant difference in the anthocyanin content in the cotyledons, and the anthocyanin concentration was reduced under stress conditions in the true leaves. PAL regulates the biosynthesis of anthocyanin. The activity of PAL increases when plants are stressed (Borsani et al. 2001; Dixon and Paiva 1995; Larkindale et al. 2005; Mateo et al. 2006; Scott et al. 2004). The PAL inhibitor AOA inhibited low-temperature or poor-nutrition-induced flowering in Violet; therefore, it is hypothesized that some compound(s) in the metabolic pathway regulated by PAL act as flowering stimuli (Hatayama and Takeno 2003; Hirai et al. 1995). PAL catalyzes the conversion of phenylalanine to *t*-cinnamic acid, and several metabolic intermediates are derived from *t*-cinnamic acid. Dihydrokaempferol-7-*O*-*β*-D-glucoside, which is derived from the pathway from *t*-cinnamic acid to anthocyanin via *p*-coumaric acid, has been reported to promote the flowering of *P. nil* (Nakanishi et al. 1995). Therefore, metabolic intermediates were applied simultaneously with AOA to the plants grown under low temperature stress conditions. Among them, *t*-cinnamic acid and benzoic acid negated the inhibitory effect of AOA in low-temperature-induced flowering, but *p*-coumaric acid and caffeic acid did not (Hatayama and Takeno 2003). The AOA-mediated inhibition of flowering under poor-nutrition conditions was rescued when SA was applied simultaneously

in a dose-dependent manner, and this effect was completely eliminated with SA at 10^{-4} M (Wada et al. 2010b). These results suggest that SA is involved in stress-induced flowering, but phenylpropanoids and dihydrokaempferol-7-O-β-D-glucoside are not. AOA also inhibits 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. ACC synthase catalyzes the conversion of S-adenosylmethionine (SAM) to ACC, which is subsequently converted to ethylene. However, the involvement of ethylene in the stress-induced flowering is excluded because flowering was completely inhibited in the presence of ACC (Hatayama and Takeno 2003). This is consistent with the observation that ethylene inhibits the photoperiodic flowering of *P. nil* (Suge 1972). SAM is also a precursor of polyamines; the excess SAM may have been metabolized to polyamines. Polyamines, especially putrescine, have been reported to induce flowering of *P. nil* under poor nutritional conditions (Wada et al. 1994). Recently, AOA and L-2-aminooxy-3-phenylpropionic acid (AOPP), which are known inhibitors of PAL (Appert et al. 2003), were reported to inhibit the biosynthesis of IAA (Soeno et al. 2010). However, IAA is known to inhibit flowering of *P. nil* (Takeno 1996).

Stress increases PAL activity and induces SA biosynthesis (Rasmussen et al. 1991; Wen et al. 2005). Stress also induces reactive oxygen species (Leon et al. 1995; Okuda et al. 1991), and reactive oxygen species promote the conversion of benzoic acid to SA (Gidrol et al. 1996; León et al. 1995; Mauch-Mani and Slusarenko 1996; Neuenschwander et al. 1995; Summermatter et al. 1995). The treatment of *P. nil* with SA, its precursor benzoic acid and some benzoic acid derivatives prior to low-temperature treatment enhances the flower-inducing effect of low temperature (Shinozaki 1985; Shinozaki et al. 1982, 1985). In addition to these effects, the flower-inhibiting effects of PAL inhibitors, which might have decreased the endogenous SA level in *P. nil*, provided new evidence to suggest that SA acts as an endogenous regulator of stress-induced flowering (Wada et al. 2010b). The flowering response of cultured plumules excised from SD treated *P. nil* seedlings was enhanced with benzoic acid (Ishioka et al. 1990). Amagasa et al. (1992) reported that AOA inhibited the photoperiodic flowering of *P. nil*. These observations suggest that SA is also involved in photoperiodic flowering.

5.3 PAL Gene Expression, PAL Activity and SA Content

Based on these results, we hypothesize that stress-induced flowering is regulated through SA, which is synthesized in the pathway mediated by PAL. However, there is no evidence to indicate that endogenous SA levels increase when *P. nil* plants are induced to flower through the application of stress factors. Accordingly, the gene expression and enzyme activity of PAL were determined, and the increase in the endogenous SA content in *P. nil* during flowering under poor-nutrition stress was calculated (Wada 2012). *P. nil* plants were induced to flower in 1/100-strength nutrient solution under LD conditions for 10–20 days. The cotyledons were harvested at the end of the stress treatment to analyze *PAL* expression. The *PAL*

expression was stronger in the plants that were induced to flower under poor-nutrition stress than in the control plants grown in the standard nutrient solution. The *PAL* expression in the plants grown under poor-nutrient stress conditions increased from the 10th to the 15th day. The expression level of *PAL* in plants grown in the standard nutrient solution remained constant, regardless of the growth period. *P. nil* plants were induced to flower in the same manner as mentioned above, except that the plants were grown for 1–3 weeks. Subsequently, PAL activity in the cotyledons was measured. The PAL activity was higher in the stressed plants than in the control plants. The PAL activity increased with an increase in the growth period in the stressed plants, and PAL activity in the control plants remained low throughout the growth period.

P. nil plants were induced to flower under the same conditions as mentioned above, except these plants were grown for 1–2 weeks, and the SA content in the cotyledons was measured using high-performance liquid chromatography-mass spectrometry (LC-MS) with deuterium-labeled SA as an internal standard. The SA content was higher in the stressed plants than in the control plants. These results suggest that poor-nutrition stress-induced flowering in *P. nil* is induced through SA, and PAL promotes the synthesis of SA.

5.4 Flower-Inducing Effect of Exogenously Applied SA

The effect of SA on the flowering of *P. nil* under non-stress conditions was examined under LD conditions. The exogenously applied SA did not induce flowering in plants grown in mineral nutrient solution. SA may be necessary but not sufficient to induce flowering. Stress might induce the production of other factors necessary to induce flowering.

DNA demethylation is a possible candidate for flowering induction. Low-temperature stress-induced DNA demethylation in *Zea mays* has been observed (Steward et al. 2002). Low temperature conditions can be replaced with DNA demethylation to induce flowering in vernalization-requiring plants (Burn et al. 1993). Among the photoperiodism-regulated plants, *P. frutescens* and *P. nil* were induced to flower through DNA demethylation without SD treatment (Iwase et al. 2010; Kondo et al. 2006, 2007, 2010). DNA demethylation might be induced under stress conditions, which act together to induce flowering. However, treatment with DNA demethylating reagents, 5-azacytidine or zebularine, together with SA did not enhance the flowering response.

Stress substances are also possible candidates for the induction of flowering. ABA promotes floral evocation of *P. nil* (Takeno and Maeda 1996) and flowering of *Lemna minor* (Krajnčič 1985). However, ABA did not promote the SA effect. Jasmonic acid (JA), which is also a stress hormone (Reymond and Farmer 1998),

induces the flowering of *Spirodela polyrrhiza* (Krajnčič and Nemec 1995) and *L. minor* (Krajnčič et al. 2006); therefore, the influence of JA should be examined in *P. nil*.

It is also conceivable that stress enhances the sensitivity to SA. In the induction of systemic-acquired resistance in potato, injury stress did not change the endogenous SA level but increased the sensitivity to SA (Yu et al. 1997). SA is not essential for seed germination in *A. thaliana*, but increased activity under salt stress conditions was observed (Lee et al. 2010). If SA acts only under stress conditions or SA sensitivity is enhanced by stress in *P. nil*, it explains the reason why exogenously applied SA did not induce flowering under non-stress conditions.

The interaction between SA and gene expression should also be considered. The expression of orthologous gene of the floral pathway integrator gene *FT*, *PnFT2*, was induced in plants that flower under poor-nutrition conditions (Wada et al. 2010b). SA application induced the expression of *A. thaliana FT* and sunflower *HAFT*, which is an ortholog of *FT* (Dezar et al. 2011; Martínez et al. 2004), indicating that *FT* and SA might regulate flowering. In *P. nil*, stress could induce both SA biosynthesis and *PnFT2* expression, and SA and the PnFT2 protein might act together to induce flowering. Moreover, SA might induce the expression of *PnFT2*, or the product of *PnFT2* could induce the expression of genes involved in the biosynthesis, response or signaling of SA.

6 Salicylic Acid-Mediated Stress-Induced Flowering in *Perilla frutescens*

6.1 Stress-Induced Flowering in *P. frutescens*

Two forms of *P. frutescens* var. *crispa,* an obligatory SD plant, were grown under LD conditions under different light intensities. All of the red-leaved plants flowered under an intensity of 30 μmol m^{-2} s^{-1}, whereas the plants grown under 60 or 120 μmol m^{-2} s^{-1} did not (Wada et al. 2010a). The green-leaved form was also induced to flower under 30 μmol m^{-2} s^{-1}, although the flowering response was lower than that of the red-leaved form. The stem length was shorter under lower light intensity than under normal light intensity in both forms. The reduction of vegetative growth results from stress (Hatayama and Takeno 2003), and therefore the flowering of *P. frutescens* plants under low-intensity light might be a stress-induced response. The response to low-intensity light appeared to be a shade avoidance response. An important component of the shade avoidance syndrome is an acceleration of flowering, but the most typical phenotype of the shade avoidance response is rapid stem elongation (Adams et al. 2009). Because the stem length of *P. frutescens* plants was shortened under low-intensity light, the response of *P. frutescens* plants to low-intensity light might not be a shade avoidance response. Photosynthetic activity could be reduced under low-intensity light

conditions. However, it is unlikely that the photosynthetic deficiency induced flowering because the photoassimilate is a flower-inducing factor (Bernier and Périlleux 2005; Seo et al. 2011). In fact, the application of sucrose induced flowering in *P. frutescens* plants cultured in vitro under LD conditions (Purse 1984). We treated the red-leaved *P. frutescens* plants with several other stress factors. None of the tested factors induced flowering, although a reduction in vegetative growth was observed. This indicates that not all stresses induce flowering.

Young red-leaved *P. frutescens* plants respond to low-intensity light treatment, even immediately after their cotyledons were expanded. They were induced to flower with the 3 week treatment, and 100 % flowering was obtained under the 4 week treatment. Flowers were formed even at the cotyledonal nodes. The plants induced to flower under the low-intensity light conditions reached anthesis and formed seeds. The seeds germinated, grew normally and were induced to flower in response to SD treatments.

6.2 Effect of PAL Inhibitors on the Flowering Induced by Low-Intensity Light

It was observed that the leaves of red-leaved plants were deep green under low-intensity light (Wada et al. 2010a). The greening might be associated with stress-induced flowering. Accordingly, we extracted the leaves with methanol and obtained absorption spectra for the extract. The color of the red-leaved *P. frutescens* plants resulted from the presence of anthocyanin, which has an absorption peak at 529.5 nm. With decreased light intensity, the absorbance at 529.5 nm ($A_{529.5}$) decreased and that at 652.5 nm increased, indicating that the greening of the leaves increased with decreasing anthocyanin content and increasing chlorophyll content. Although the greening of the leaves partially resulted from the increase in chlorophyll content that might not be directly related to flowering because an increase in chlorophyll content under low-intensity light was observed even in green-leaved plants, which exhibited only a slight flowering response. The relationship between anthocyanin content ($A_{529.5}$) and flowering was examined in experiments concerning the effect of different light intensities on flowering. There was an apparent negative correlation between these variables ($r = -0.71$). The absence of flowering was also observed when the threshold reaction reached an $A_{529.5}$ of greater than 0.8. Therefore, the metabolic pathway related to anthocyanin synthesis might be involved in the regulation of flowering. Namely, low-intensity light stress could reduce anthocyanin synthesis and induce flowering. However, this conflicts with previous reports. Stress generally promotes anthocyanin biosynthesis through increasing PAL activity (Chalker-Scott 1999; Christie et al. 1994; Dixon and Paiva 1995), and stress-induced flowering in *P. nil* was accompanied by an increase in PAL activity. Thus, two hypotheses have been proposed: low-intensity light stress could either decrease or increase PAL activity

in *P. frutescens*. To verify which hypothesis is correct, we examined whether the PAL inhibitor could induce flowering under normal-intensity light conditions in *P. frutescens* plants and whether the PAL inhibitor could suppress low-intensity light-induced flowering. AOPP, a PAL inhibitor, did not induce flowering when applied under non-inductive normal-intensity light conditions and inhibited flowering when applied under inductive low-intensity light conditions (Wada et al. 2010a). Treatment with another PAL inhibitor, AOA, showed the same results. Flowering induced through low-intensity light was inhibited with AOPP, and the inhibition was partially rescued with SA. These results suggest that stress increased PAL activity in *P. frutescens* and *P. nil*, suggesting that the same mechanism is involved in the flowering induced through low-intensity light in *P. frutescens* and poor-nutrition stress in *P. nil*.

The preliminary analysis using RT-PCR suggested that the expression of the *PAL* gene was suppressed under low-intensity light conditions in red-leaved *P. frutescens* plants (Wada 2007). Thus, we must consider that AOPP and AOA did not suppress flowering through an inhibition of PAL activity, but rather through some unknown mechanism. Although AOPP is a specific inhibitor of PAL (Kessmann et al. 1990; Mavandad et al. 1990; Ni et al. 1996; Orr et al. 1993), it was recently reported that AOPP and AOA also inhibited the synthesis of IAA (Ishii et al. 2010). We therefore consider the possibility that AOPP and AOA suppresses flowering through the inhibition of IAA synthesis. Further investigation is required to determine the mechanism that regulates the low light-induced flowering.

7 Salicylic Acid-Mediated Stress-Induced Flowering in *Lemna paucicostata*

7.1 Stress-Induced Flowering in *L. paucicostata*

Three strains of the SD plant *L. paucicostata*, 151, 441 and 6746 were induced to flower in tap water without addition of nutrients under non-inductive LD conditions (Shimakawa et al. 2012). The control plants cultured in a nutrient medium showed almost no flowering response under LD conditions. The flowering response under the poor-nutrition conditions was the strongest in strain 6746. In comparison with the flowering response under SD conditions, the poor-nutrition conditions produced a weaker flowering response in each strain examined. However, virtually no flowering occurred under nutrient conditions. The number of fronds per flask cultured in tap water was much smaller than that cultured in the nutrient medium for all of the tested strains. The vegetative multiplication of the fronds was restricted when cultured in tap water, which indicates that the vegetative growth of the plants was suppressed in tap water, implying that the plants were stressed (Hatayama and Takeno 2003). Therefore, poor-nutrition stress

induces the flowering of *L. paucicostata* plants. A weak flowering response was detected after 2 weeks of stress treatment in strains 151 and 6746, and an apparent flowering response was detected after 3 weeks of stress treatment in all of the strains.

7.2 PAL-Inhibitor Inhibition of the Flowering Induced by Poor Nutrition

SA has long been implicated in the regulation of flowering in *Lemna* spp. However, the current supporting evidence merely shows that exogenously applied SA can induce flowering. Therefore, we attempted to reduce the endogenous SA level by inhibiting its biosynthesis to ascertain whether this effect inhibits flowering. Strain 6746, which is highly sensitive to poor-nutrition stress, was cultured in tap water under LD conditions to induce flowering, and PAL inhibitors were added to the tap water to examine their effects on poor-nutrition stress-induced flowering. The flowering was completely inhibited with AOA at 10^{-5} M and AOPP at 10^{-4} M (Shimakawa et al. 2012). The number of fronds per flask was decreased with AOA at 10^{-5} M, whereas the multiplication of fronds was not suppressed with AOPP at 10^{-4} M.

7.3 Endogenous SA Contents

Strain 6746 was cultured in tap water for 3 weeks to induce flowering, and SA was extracted from the fronds for quantification using LC-MS. Significantly higher amounts of SA were detected in fronds that flowered under the poor-nutrition conditions when compared with the vegetative fronds cultured under nutrient conditions (Shimakawa et al. 2012). This is the first evidence showing that the SA content increases when flowering is induced in Lemnaceous species.

7.4 Promotion of Flowering by Exogenously Applied SA

Strain 6746 was cultured in tap water containing SA and grown under non-inductive LD conditions for 3 weeks. The addition of SA at 3×10^{-5} M promoted flowering (Shimakawa et al. 2012). Taken together, the results suggest that endogenous SA plays a role in the promotion of stress-induced flowering. Cleland and his colleagues previously observed that SA induces flowering in many Lemnaceous species in the study regarding photoperiodic flowering. It is now apparent that SA regulates stress-induced flowering but not photoperiodic flowering.

Yamaguchi et al. (2001) isolated norepinephrine and the α-ketol of octadeca-dienoic acid (KODA), from which flower-inducing factors are derived, from *L. paucicostata*. KODA was found to be induced by drought, heat or osmotic stress conditions (Yokoyama et al. 2000). These results are consistent with the previous finding that *L. paucicostata* has a mechanism that responds to stress and promotes flowering. However, clarification of the relationship between these flower-promoting factors and SA requires further investigation.

8 Salicylic Acid-Mediated Stress-Induced Flowering in *Arabidopsis thaliana*

8.1 Stress-Induced Flowering in *A. thaliana*

Poor-nutrition conditions accelerated flowering of LD plant *A. thaliana* (Kolár and Senková 2008). The authors suggested that this precocious flowering was due to stress. When plants were grown in a full-strength nutrient solution for 3–5 weeks and then transferred to a 1/10- to 1/1,000-strength media, the flowering time was notably shortened. This effect was stronger when the stress was applied earlier and increased with increasing dilutions. This acceleration was more pronounced in SD than in LD conditions. The response was stronger in the wild ecotype Landsberg *erecta* (L*er*) than in Columbia (Col). The nutrient-deficient L*er* plants formed normal flowers and fruits with seeds.

UV-C light stress promoted flowering in *A. thaliana* (Martínez et al. 2004). UV-C irradiation accelerated flowering in Col in a dose-dependent manner between 0 and 200 mJ cm^{-2}. UV-C induced the expression of the flowering gene *FT* and moderately induced *CONSTANS* (*CO*) expression.

General stress leads to early flowering in *A. thaliana*. High-intensity light (800 μmol m^{-2} s^{-1}), high temperatures of 26 °C, photochilling with 800 μmol m^{-2} s^{-1} of light at 16 °C or continuous light treatments lead to earlier flowering in wild type L*er*, but not in the *fca1 co-2 ga1-3* triple mutant (Marín et al. 2011).

8.2 Involvement of SA in Stress-Induced Flowering in *A. thaliana*

Similar doses of UV-C irradiation did not promote flowering in *NahG* transgenic plants expressing bacterial salicylate hydroxylase, and these plants did not accumulate SA because of the rapid and efficient conversion of SA to catechol. UV-C light irradiation increased the expression of the SA-responsive *PR1* gene in wild type plants but not *NahG* plants. The expression of the *SA induction*

deficient 2/isochorismate synthase 1 (*SID2/ICS1*) gene, which encodes the SA biosynthetic enzyme, increased under UV-C light irradiation in wild type but not *NahG* plants. Although UV-C induced expression of *FT* and *CO* in wild type, these genes were not induced in *NahG* plants.

The exogenous application of SA at 100 μM accelerated flowering in Col, but the *NahG* plants were not responsive to SA treatment. SA also regulates flowering time in non-stressed plants. SA-deficient *NahG* plants exhibit late flowering (Martínez et al. 2004). The *siz1* mutant with elevated SA level shows an early flowering phenotype under SD, and this phenotype is suppressed by the expression of *NahG* (Jin et al. 2008). Exogenous SA treatment reduced the expression of the flower-inhibiting gene, *FLC*. Thus, UV-C-induced flowering requires the enhanced expression of *FT* and the reduced expression of *FLC*.

The genome-wide analyses of transcriptomes revealed the down-regulation of *Pathogen and Circadian Controlled 1* (*PCC1*) in SA-deficient *A. thaliana* plants (Segarra et al. 2010). *PCC1* was initially characterized as a circadian clock-regulated gene that is rapidly up-regulated after pathogen inoculation. The expression of *PCC1* was strongly activated under UV-C light irradiation in Col but not in *NahG* plants. SA application also activated *PCC1* expression. The activation of *PCC1* expression required *CO*. RNAi transgenic plants contained lower levels of *FT* transcript. The over-expression of *PCC1* did not accelerate flowering, but the RNAi-mediated suppression of its expression delayed flowering. UV-C light irradiation of plants accelerates flowering through a SA-dependent process in wild type but not RNAi transgenic plants with reduced expression of *PCC1*, suggesting that neither SA nor PCC1 alone is sufficient to accelerate flowering in *A. thaliana*.

These results suggest the involvement of SA in UV-C stress-induced flowering in *A. thaliana*.

9 Conclusion

It is apparent that plants flower in response to several stress conditions. Constantly exposed to stresses that negatively affect growth and development, plants establish protection and adaptation strategies to minimize stress influences. However, protection or adaptation might not be sufficient under severe stress conditions. Thus, precocious flowering might assist in species preservation under such conditions. Thus, stress-induced flowering can be considered as the ultimate adaptation to stress and should be considered a central component, along with tolerance, resistance and avoidance, of stress physiology. The production of SA, through the activation of PAL, and the expression of *FT* have been suggested to be involved in the regulation of stress-induced flowering in several plant species. Therefore, an important subject to be studied in the future is the interaction between SA and *FT*.

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Chapter 10
Salicylic Acid-Mediated Abiotic Stress Tolerance

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Abstract Plants are exposed to many environmental stresses, which are further aggravated by the effects of global climate change. So investigations on compounds capable of reducing the stress sensitivity of plants are of great importance. Salicylic acid is a phenolic compound produced to varying extents by a wide range of plant species. Its usefulness in human medicine was recognized much earlier than its role in plants. This endogenous plant growth regulator participates in many physiological and metabolic reactions. It was first demonstrated to play a role in responses to biotic stress. Soon afterwards; however, it became increasingly clear that salicylic acid also plays a role during the plant response to abiotic stresses such as heavy metal toxicity, heat, chilling, drought, UV-light and osmotic stress. Two kinds of evidence have accumulated to support this. First, endogenous salicylic acid levels rise in several species when they are exposed to abiotic stress conditions. Secondly, the application of salicylic acid at suitable concentrations induces stress tolerance in various plant species. The use of mutants and transgenic plants in which the synthesis, accumulation or translocation of salicylic acid is modified could help to clarify its molecular modes of action in physiological processes. Crosstalk with other hormones such as jasmonic acid, ethylene, abscisic acid, gibberellic acid and cytokinin is important part of a finely tuned immune response network. It can be seen that SA exerts an effect at several levels and its effect also depends on several factors, such as the mode of application, the concentration, environmental conditions, plant species and organs, etc. In the present chapter a summary will be given of the relationship between SA and various abiotic stress factors in relation to biotic stress and other plant hormones, followed by a summary of the known physiological and biochemical effects of SA that may explain the change in stress tolerance.

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1 Introduction

Plants containing large quantities of salicylates were used medicinally long before these compounds were identified. Salicylates have been known to have healing properties since the 5th century B.C., when Hippocrates prescribed the chewing of willow leaves and bark to relieve labour pains (Rainsford 1984). The use of plants containing salicylate continued and was widespread not only in the old World but also in the New World, where Native Americans used extracts of willow bark for therapeutic purposes (Vane and Botting 1992). Salicylic acid (SA), or *ortho*-hydroxy benzoic acid, is ubiquitously distributed in the whole plant kingdom (Raskin et al. 1990).Its name is derived from the word *Salix,* the scientific name for the willow tree. Salicin, the glucoside of salicylic alcohol, was first isolated from willow bark in 1828, while the synthetic derivate of SA, acetyl SA, which is one of the best known "antistress compounds" used by human beings was registered as "aspirin" in 1899 (Rainsford 1984).

Despite its broad distribution in plants, the basal levels of SA differ widely among species, and differences of up to 100-fold have been recorded. SA is generally present in plants in quantities of a few μg/g fresh mass or less (Raskin et al. 1990), either in the free state or in the form of glycosylated, methylated, glucose-ester or amino acid conjugates (Lee et al. 1995). Among the all plants surveyed, rice leaves have the highest endogenous SA content (Raskin et al. 1990; Silverman et al. 1995), although rice roots have very low contents of SA (Chen et al. 1997). SA can be detected in the largest quantities in thermogenic flowers at flowering, or after pathogenic infection (Raskin 1992a). In plants SA can be synthesized via two distinct and compartmentalized enzymatic pathways both requiring the primary metabolite chorismate. L-phenylalanine, derived from chorismate, can be converted into SA via the precursors free benzoic acid (BA), benzoyl glucose or *ortho*-coumaric acid (*ortho*-hydroxy-cinnamic acid: *o*HCA), depending on the plant species, but chorismate can also be converted into SA via isochorismate (Catinot et al. 2008; Chen et al. 2009; Dempsey et al. 2011) (for more details see Chap. 12).

Although the therapeutic effect of SA in humans has been well studied for about 200 years, its role in plants has only been recognized in the last three decades and the full picture is still not clear. It was thought for a long time that phenolic compounds were non-essential for all organisms, and were therefore called "secondary metabolites". A large amount of evidence has since accumulated demonstrating that phenolics are involved in various plant processes. The first plant physiological processes in which SA was reported to play a role were growth regulation (DeKock et al. 1974) and flower induction, which was demonstrated in a long-day *Lemna gibba* L. strain (Cleland 1974; Cleland and Ajami 1974).

Another well-known effect of SA is that it increases the temperature of certain thermogenic plants. Heat production, known as thermogenesis, occurs in male reproductive structures of cycads and in the flowers of some angiosperms. In *Sauromatum guttatum* S. (voodoo lily), a 100-fold increase in SA, which precedes the onset of thermogenesis in the spadix, is responsible for the induction of heat production (Raskin et al. 1987). Later, evidence was found to show that the increase in alternative oxidase expression as the result of SA played a role in the induction of thermogenesis (Rhoads and McIntosh 1992). Interestingly, SA was also shown not only to induce cyanide-resistant respiration but also to block electron flow from the substrate dehydrogenases to the ubiquinone pool in isolated mitochondria, and to act as an uncoupler of the mitochondrial electron transport chain in non-thermogenic plant species (Norman et al. 2004).

Since then several studies have focused on the role of both endogenous and exogenous SA in plant growth and development and have proved that this hormone regulates processes such as seed germination, vegetative growth, uptake and loss of water, nutrient uptake, nitrogen metabolism, photosynthesis, respiration and the activity of the enzymes involved in these processes. Seed production and especially seed quality parameters are also affected by SA, which may also induce senescence, and a type of cell death that is not associated with the hypersensitive response (reviewed by Amunullah et al. 2010; Asghari and Aghdam 2010; Hayat et al. 2010; Rivas-San Vicente and Plasencia 2011) (for more details see Chap. 12). SA could contribute to maintain cellular redox homeostasis, through the regulation of antioxidant enzyme activity (Durner and Klessig 1995, 1996; Slaymaker et al. 2002) and the induction of the alternative respiratory pathway (Moore et al. 2002), and to regulate gene expression by inducing an RNA-dependent RNA polymerase,which is important for post transcriptional gene silencing (Xie et al. 2001). It has been demonstrated that SA and related compounds are capable of inhibiting the stomatal closure induced by abscisic acid (ABA) (Rai et al. 1986). Interactions between SA and other phytohormones, especially ABA, jasmonic acid and ethylene have been much studied, but have not yet been clarified (Singh et al. 2011; Vlot et al. 2009; Pieterse et al. 2009). SA treatment can influence pigment and protein contents, lignin biosynthesis and photosynthesis (Çag et al. 2009; Gallego-Giraldo et al. 2011; Janda et al. 2012). Both endogenous and exogenous SA have been shown to have various effects (Raskin 1992b), but these cannot always be generalised, as the studies were carried out on various plant species in various systems (from the whole plant to cell suspensions).

The analysis of plants responding to microbial pathogen infection revealed another function of SA. As a key signal, it has an important role in the activation of disease resistance (see Chap. 11). The role of SA in the signal transduction processes of biotic stress tolerance has already been widely studied. SA is involved in the development of the hypersensitive reaction (HR): in tobacco leaves infected with tobacco mosaic virus there is an increase in the level of endogenous SA (known as the salicylate burst) both in the necrotic lesion and in surrounding tissues (Enyedi et al. 1992). The external application of SA induces the expression of pathogenesis-related (PR) proteins in tobacco (Malamy et al. 1990; Yalpani et al. 1991) and rice

(Rakwal et al. 2001). A large body of evidence indicates that SA is also required for the development of systemic acquired resistance (SAR). The level of endogenous SA increased in cucumber plants when acquired resistance developed (Métraux et al. 1990). Transgenic tobacco plants incapable of accumulating SA due to the presence of a salicylate-hydroxylase enzyme gene (*nahG*) of bacterial origin were unable to develop systemic acquired resistance (Gaffney et al. 1993). Nevertheless, SA does not appear to be the signal molecule transported from the site of infection to more distant tissues, though the accumulation of SA in the given tissues is essential if SAR is to develop (Vernooij et al. 1994). Most of the SA synthesized in plants is glucosylated and/or methylated. Glucose conjugation generally takes place at the hydroxyl group of SA, resulting in the formation of SA glucoside [SA 2-O-b-D-glucoside] (SAG), whereas the less frequent glucose conjugation at the SA carboxyl group produces SA glucose ester. Both reactions are catalysed by cytosolic SA glucosyl transferases (Lee and Raskin 1999). SAG is actively transported from the cytosol into the vacuole, where it may function as an inactive storage form (Dean and Mills 2004). SA is also converted to methyl salicylate (MeSA) by SA carboxyl methyl transferase, and this volatile derivate is an important long-distance signal in systemic acquired resistance (Shulaev et al. 1997).

There is an increasing body of evidence suggesting that SA is involved not only in biotic stress, but also in abiotic stress, as the endogenous SA content changes during abiotic stresses (see Chap. 3) and the protective effect of exogenously applied SA has also been demonstrated (Horváth et al. 2007; Hayat et al. 2010). Yang et al. (2004) divided plants into SA-sensitive and SA-insensitive species and suggested that in SA-insensitive plants such as rice, although SA may play an important role in modulating the redox balance and protecting plants from the oxidative damage caused by various biotic and abiotic factors, it is unable to act as an effective secondary signal for the activation of defence genes and induced resistance. In the present chapter a summary will be given of the relationship between SA and various abiotic stress factors in relation to biotic stress and other plant hormones, followed by a summary of the known physiological and biochemical effects of SA that may explain the change in stress tolerance.

2 Studies on the Role of Salicylic Acid During Abiotic Stress

2.1 Toxic Metals

2.1.1 Effect of Toxic Metals

The term "heavy metals" is often used as a synonym of toxic metals, but other metals may also be toxic and not all heavy metals are particularly toxic. The definition may also include trace elements when they are present in abnormally

high, toxic doses. Some heavy metal ions play an important role in many metabolic processes, making them essential for metabolism, growth and development in trace element quantities. Heavy metals are only able to exert a stimulatory or inhibitory effect on plants if they are present in a form available to the plants. Availability is influenced by a range of abiotic and biotic factors. The abiotic factors include the ionogenicity of the toxic metal, its solubility in water and its ability to form complexes, and the pH and redox potential of the soil. The biotic parameters include the protons and organic acids (e.g. citric acid, amino acids) exuded into the rhizosphere by plant roots, the symbiosis of higher plants with mycorrhizal fungi, and the quantity of humic acids and humin present in the soil as the result of organic matter decomposition. Problems only arise when the cells encounter a high concentration of heavy metal ions, which cause cell damage. The heavy metals contaminating the soil, largely due to various anthropogenic activities, constitute one of the major environmental contaminants that restrict plant productivity. Their non- biodegradability results in their prolonged persistence in the environment, which is coupled with the tendency for bio-enrichment through food chains (Sharma and Dietz 2009).

The effects of heavy metals on plant species have been well studied (Sanita di Toppi and Gabbrielli 1999; Yadav 2010). Plants may be sensitive or tolerant, based on their ability or inability to adapt to heavy metal ions. In plants sensitive to heavy metals, cell damage, or in severe cases cell death, may be caused by a number of mechanisms. Certain heavy metal ions can readily be exchanged for the essential metal ions in the active centres of enzymes (e.g. Mg^{2+}, Zn^{2+}, $Cu^{2+,}$ Co^{2+}), thus inhibiting the regulatory role of the enzymes. Furthermore, heavy metal ions may form free radicals, leading to the lipid peroxidation of cell membranes and causing them to lose their ion permeability. The results have been found to vary as a function of the type of metal ion, the concentration, the duration of treatment and the plant organ investigated. The evaluation of the results is further complicated by the fact that many symptoms are not specific to the responses induced by heavy metal stress. In ananalogous manner to general stress theory, mechanisms which result in heavy metal stress tolerance can be divided into two groups: avoidance strategies and tolerance strategies (Hall 2002). Avoidance strategies involve the limitation of heavy metal uptake, thus excluding them from the tissues, while plants capable of tolerating heavy metals synthesise amino acids, proteins and peptides that bind heavy metals which are accumulated, stored and immobilised in these forms. Some of the most important compounds involved in heavy metal tolerance are phytochelatins (PCs). These enzymatically synthesized Cys-rich peptides are able to create chelates with heavy metals, thus decreasing their damaging effects (Grill et al. 1989). PCs form a family of structures in which the γ-Glu-Cys dipeptide is repeated a number of times, followed by a terminal Gly: [(γ-Glu-Cys)$_n$-Gly], where n is generally in the range of 2–5. As their glutamic acid content is present in a γ-bond, these peptides are capable of forming thiolate bonds with heavy metal ions. They then transport the bound metal ions from the cytoplasm into the vacuoles, where they can be stored in non-toxic form, bound to organic acid ligands.

2.1.2 Effect of Exogenous Salicylic Acid During Heavy Metal Stress

One of the earliest works to report on the protective effect of SA against abiotic stress factors dealt with heavy metals. SA at a concentration of 0.1 or 0.2 mM was found to reduce the inhibitory effect of Pb^{2+} and Hg^{2+} on seed germination and seedling growth, and its membrane-damaging effect in rice (*Oryza sativa* L.) seeds placed in Petri plates containing filter paper discs moistened with SA and heavy metal solutions (Mishra and Choudhuri 1997, 1999). The higher concentration of SA was more effective, as evident from the better recovery from metal-induced growth inhibition. SA also moderated the inhibitory effect of lead on the activity of the nitrate reductase enzyme in maize (*Zea mays* L.) plants (Sinha et al. 1994). SA-induced aluminium tolerance was also reported in *Cassia tora* L. plants, where the increased citrate efflux induced by 5 μM SA treatment was associated with a decrease in the inhibition of root growth and in the Al content of the root tips (Yang et al. 2003). SA (at concentrations of 0.1–200 μM) also had a protective effect in soybean against cadmium stress (Dražić and Mihailović 2005), against lead stress in *Brassica napus* var. Okapi (Jazi et al. 2011) and against nickel stress in *Brassica napus* L. (Kazemi et al. 2010) when added to the nutrient solution.

By contrast, 10 μM SA treatment stimulated the accumulation of cadmium at the beginning of germination in *Medicago sativa* L. seedlings and was unable to inhibit the damaging effects of Cd treatment on the shoot and root growth (Dražić et al. 2006). The same concentration of SA, applied for 3 h at the beginning of imbibition, stimulated Cd accumulation in a tolerant soybean genotype, while accumulation was inhibited by SA in a susceptible genotype. This could indicate that the Cd-tolerant genotype was better able to regulate the oxidative stress induced by Cd, so mechanisms designed to prevent the further uptake of the heavy metal were not triggered (Dražić and Mihailović 2009). These results suggest that the effect of SA cannot be fully generalised, and that it depends greatly on the genotype. In seedling of the same plant species 200 μM SA for 12 h was found to alleviate mercury toxicity by inducing the antioxidant defence system (Zhou et al. 2009). In sunflower the exogenous application of SA appeared to induce an adaptive response to Cu stress, including the accumulation of organic solutes leading to the protection of photosynthetic pigments and membrane integrity (El-Tayeb et al. 2006). The results obtained when soaking the seeds of *Linum usita-tissimum* L. in SA suggested that it could be used as a growth regulator and a stabilizer of membrane integrity to improve plant resistance to Cd stress (Belkhadi et al. 2010).

Other results indicated that, although 500 μM SA reduced the Cd uptake of maize roots and the inhibitory effect of Cd treatment on photosynthesis, the compound itself stressed the seedlings, so preliminary treatment with SA could aggravate the damaging effect of Cd (Pál et al. 2002). However, soaking maize seeds in 500 μM SA for 6 h was able to reduce the inhibitory effect of 10, 15 and 25 μM Cd on growth, chlorophyll content and the RUBISCO and PEPC enzymes, while also decreasing the proline production, lipid peroxidation and membrane leakage induced by Cd (Krantev et al. 2008). Although the soaking of seeds in SA

solution before exposure to Cd may reduce the level of heavy metal injury, this protection is not directly connected with the altered regulation of phytochelatins (unpublished data).

The analysis of plants expressing targeted modifications of various components in the antioxidant system and the comparison of closely related plant species with different degrees of toxic metal sensitivity led to the conclusion that salicylic acid may be both related to the degree of plant tolerance to metals and to the level of antioxidants (reviewed in Sharma and Dietz 2009). Transcriptome analysis on the antioxidative enzymes in the leaves of pea plants grown in the presence of Cd and treated with compounds that modulate the signal transduction cascade suggested that SA and H_2O_2 were involved in some of the steps (Romero-Puertas et al. 2007; Maksymiec 2007). Pre-treatment with 10 µM SA enhanced Cd tolerance in rice, which can be attributed to the ability of SA to stimulate the production and the activity of enzymatic and non-enzymatic antioxidants and to induce H_2O_2 signalling in rice (Guo et al. 2007a, b; 2009). The protective effect of SA pre-treatment was also accompanied by the modified activity of antioxidant enzymes during other heavy metal stresses, including lead stress in rice (nutrient solution containing 100 µM SA) (Chen et al. 2007) and manganese stress in cucumber, foliar spray with 1 mM SA, (Shi and Zhu 2008).

By contrast, although the pre-treatment of barley plants with 0.5 mM SA for 1d prevented cadmium-induced lipid peroxidation, thus increasing the fresh shoot and root mass, this protective effect was not due to an increase in the antioxidant capacity, because the increased activity of antioxidant enzymes observed in untreated plants during Cd stress could not be detected in plants preliminarily treated with SA (Metwally et al. 2003).

2.1.3 Changes in Endogenous Salicylic Acid Content During Heavy Metal Stress

The role of salicylic acid in various stress signal transduction processes is also supported by the fact that various abiotic stress factors induce the accumulation of SA. The SA content increased in several plant species during treatment with various heavy metals (Metwally et al. 2003; Rodriguez-Serrano et al. 2006; Yang et al. 2003; Zawoznik et al. 2007). The endogenous SA content exhibited a concentration-dependent increase in maize plants treated with Cd (Pál et al. 2005). 25 and 50 µM Cd triggered an approximately 3-fold accumulation of free and conjugated BA and SA, with a higher amount in the bound form. However, the accumulation was not of the same magnitude for SA and BA. The accumulation of conjugated forms of BA indicates the rapid conversion of free forms to conjugated forms. The large-scale accumulation of bound BA can also be explained by the lower rate of SA biosynthesis. The accumulation of free and bound oHCA was also observed in Cd-treated leaves. Among the phenolic compounds the highest accumulation was found in the case of bound oHCA in the leaves. Since oHCA has been demonstrated to have antioxidant properties (Foley et al. 1999), these results

suggest that the increase in the *o*HCA content was induced independently of the SA biosynthesis, but may play a role in the antioxidative response to cadmium. The increased endogenous SA levels in the leaves of maize seedlings may be associated with the oxidative stress observed in the leaves of Cd-stressed plants, suggesting a role for SA in the response of maize to Cd. At the same time other authors found no increase in the endogenous SA content as a result of Cd (7 μM), Cu (3 μM) and Zn (70 μM) treatment, or any difference in the SA content of sensitive and resistant plants of *Salix viminalis* L. in the control (Landberg and Greger 2002).

Heavy metal tolerance is often correlated with intracellular compartmentalization (Brune et al. 1995). Nickel hyperaccumulation is usually due to a highly efficient pumping system that transfers the metal to the central vacuole of the shoot cells, leading to a high level of tolerance to this element (Krämer et al. 2000), but it is clear that a substantial amount of cellular Ni also accumulates outside the vacuole, suggesting the existence of a cytoplasmic-based tolerance mechanism. Due to the constitutively enhanced activity of serine acetyl transferase, the glutathione concentration in hyperaccumulating *Thlaspi* plants is also constitutively elevated, leading to enhanced tolerance to Ni-induced oxidative stress (Freeman et al. 2004). In a later experiment it was also proved that the glutathione-mediated Ni tolerance mechanism observed in Ni-hyperaccumulating *Thlaspi* species is signalled by the constitutively elevated levels of SA. It was also observed that both biochemical and genetic manipulations that increase SA in *Arabidopsis thaliana* (L.) Heynh plants mimic the glutathione-related phenotypes of the hyperaccumulating *Thlaspi*, and that these biochemical changes in the non-accumulator are associated with increased glutathione-mediated Ni resistance. Such observations suggest that SA may be one of the regulators involved in coordinating certain key biochemical differences between Ni/Zn hyperaccumulators and non-accumulator plant species.

2.2 Drought

2.2.1 Drought-Induced Changes in Plants

Plants are exposed to drought stress when there is not sufficient water available, or when, for some reason, the water present cannot be taken up by the plants, e.g. if the ground is dry, if there is intense evaporation or severe frost, or if the soil has a high salt content, leading to strong osmotic water binding (Mishra and Singh 2010). Water deficit is a multidimensional stress affecting plants at various levels of their organization. The first, most sensitive sign of water deficiency is a reduction in turgor, leading to the retardation of growth processes, especially linier growth. Nevertheless the effects of stress are not only manifested at the morphological level but also at the physiological level (growth inhibition, stomatal closure, reduced transpiration rate, decrease in water potential and photosynthetic

rate) and at the biochemical and molecular level (formation of radical scavenging compounds, accumulation of compatible organic solutes and ABA, changes in endogenous phytohormone contents and lipid composition, modifications in the expression of stress responsive-genes, etc.) (Yordanov et al. 2000; Aimar et al. 2011). Some of these responses are directly induced by the changing water status of the tissues, while others are brought about by plant hormones (Chaves et al. 2003).

One of the most characteristic symptoms of drought stress is the formation and accumulation of ABA. The biosynthesis of this stress hormone is induced by the turgor reduction arising due to water loss. ABA-dependent and ABA-independent signal transduction chains have been shown to function between the primary signal induced by drought or cold stress and the expression of specific genes. One pathway of ABA-dependent signal transduction requires protein synthesis, while the other does not. The regulation of one ABA-independent pathway, on the other hand, involves a dehydration-responsive element in the case of both drought and salt- or cold-induced stress, while the other pathway is only initiated by drought or salt stress. The genes induced by dehydration also control the genes responsible for the signal transduction pathway of the response to drought stress. One group contains proteins involved in stress tolerance (e.g. water channel proteins, proteins protecting macromolecules and membranes, such as LEA proteins or chaperones), while the other contains proteins participating in signal transduction and gene expression (e.g. protein kinases, transcription factors, phospholipase C). On the other hand, the exogenous application of ABA enhances the tolerance of plants to drought (Lu et al. 2009).It was also shown that drought-tolerant cultivars have higher ABA levels than susceptible ones (Veselov et al. 2008; Thameur et al. 2011).

2.2.2 Protective Effect of Exogenous Salicylic Acid During Drought Stress

When wheat seeds were soaked in acetyl SA (which may be degraded into SA in aqueous solution) the plants had better resistance to drought stress (Hamada 1998; Hamada and Al-Hakimi 2001). Soaking in 100 ppm acetyl SA for 6 h before sowing not only alleviated the inhibitory effects of drought but also had a stimulatory effect, as the dry matters of both the shoot and the root improved and the transpiration rate showed a marked gain. Treatment with ascorbic acid or thiamine had a similar protective effect, which was attributed to the protection of the photosynthetic apparatus from oxidation and the retardation of dark respiration (Hamada 1998). In another experiment, irrespective of SA concentration (1–3 mM) and the level of water stress, plants treated with SA generally exhibited higher moisture content, dry mass, carboxylase activity of Rubisco, superoxide dismutase (SOD) activity and total chlorophyll content compared to untreated seedlings (Singh and Usha 2003). In the case of water stress, SA treatment protected the nitrate reductase activity and maintained the protein and nitrogen

contents of the leaves compared to water-sufficient seedlings. The protective effect of SA treatment was also demonstrated in tomato (*Lycopersicon esculentum* L.), mainly through enhancing the photosynthetic parameters, membrane stability, water potential and activities of nitrate reductase and carbonic anhydrase (Hayat et al. 2008), and in sunflower, where it was manifested as a less severe water stress-induced decrease in the yield and oil content (Hussain et al. 2008). Along with several other plant growth substances, SA also improved the protoplasmic drought tolerance of free-cell suspensions prepared from fully turgid leaves of *Sporobolus stapfianus* Gandoger (Ghasempour et al. 2001). Spraying with methyl-SA (at 10^{-4} M) was also shown to promote drought-induced leaf senescence in *Salvia officinalis* L. plants (Abreu and Munné-Bosch 2008).

In plants exposed to abiotic stress (e.g. salinity and drought), the accumulation of ROS such as superoxide radical (O_2^-), hydroxyl radical (OH^-), and H_2O_2 is induced. Increasing ROS levels in plants expose lipids, proteins and nucleic acids to oxidative stress, which, in turn, alters the redox homeostasis (Smirnoff 1993). It was reported by Kadioglu et al. (2011) that the exogenous application of SA induced the activity of antioxidant enzymes, at the same time alleviating water stress damage in plants of *Ctenanthe setosa*. Treating water-stressed wheat seedlings with SA or ABA induced the activity of SOD, catalase (CAT), ascorbate peroxidase (APX) and NADPH oxidase was (Agarwal 2005a, b), signifying the role of SA in regulating the drought response of plants and suggesting that SA could be used as a potential growth regulator to improve plant growth under water stress. Plant treatment with SA before stress reduced the damaging effect of water deficit on the cell membranes in the leaves. SA treatment increased ABA content in the leaves of barley genotypes, suggesting that ABA may contribute to the development of the antistress reactions induced by SA (Bandurska and Stroinski 2005).

Soaking tomato and bean seeds in either SA or acetyl SA solution at concentrations of 0.1 and 0.5 mM increased seedling survival subsequent to drought stress. Above and below this concentration range, however, no positive results were recorded (Senaratna et al. 2003). In maize, although a 1 day preliminary treatment with 0.5 mM SA increased the polyamine content of plants, drought tolerance was not improved; in fact, plants treated in this way became more susceptible to drought (Németh et al. 2002). A negative effect was also recorded when Chinese Spring wheat plants were treated in this way, while it had no effect on winter wheat Cheyenne. However, the SA analogue 4-hydroxybenzoic acid (4-HBA) increased drought tolerance of Cheyenne (Horváth et al. 2007). Despite evidence that SA participates in abiotic stress responses, the stress tolerance imparted by SA appears to be dose-dependent, since SA deficiency or very high SA contents increase susceptibility. Furthermore, the results are influenced by the method of treatment and by the developmental stage of the plants. Hence, the role of SA may also differ, depending on the severity of the stress (Yuan and Lin 2008).

2.2.3 Drought-Induced Salicylic Acid Accumulation

Besides reports on the protective effect of exogenous SA application, endogenous SA accumulation during drought stress has also been demonstrated on several occasions (Munné-Bosch and Penuelas 2003; Bandurska and Stroinski 2005; Abreu and Munné-Bosch 2008). The SA level in the leaves of *Phillyrea angustifolia* L. plants exposed to drought showed a strong negative correlation with the relative water content and increased progressively to as much as 5-fold during drought (Munné-Bosch and Penuelas 2003). During recovery, the SA levels decreased, but remained slightly higher than those observed before drought. The SA levels were positively correlated with those of α-tocopherol during drought, but not during recovery, indicating the possible role of endogenous SA in the induction of a protective mechanism during water stress.

In another experiment the effect of moderate or severe water deficit on the SA content in the leaves and roots, and the effect of pre-treatment with SA on the response to water stress were evaluated in barley plants (Bandurska and Stroinski 2005). Water deficit increased the SA content in the roots, whereas the SA content in the leaves did not change. In the leaves of *Panicum virgatum* L., the endogenous SA contents decreased considerably during moderate water stress and then increased significantly after 24 h of rehydration (Aimar et al. 2011).

2.3 Salinity

2.3.1 Salt Stress

Salinity is one of the most important abiotic stresses. Salt stress affects the physiology of the whole plant and cellular levels and from seed germination to maturity. During salt stress plants may suffer four types of stress: osmotic conductance, specific ion toxicity, ion imbalance, and oxidative stress, involving the production of reactive oxygen species (Tester and Devenport 2003). Salinity was found to decrease the contents of dry matter, chlorophyll and soluble proteins, but to enhance those of free amino acids, such as proline. This free amino acid is one of the potential biochemical indicators of salinity tolerance in plants and is involved in plant protection (Gadallah 1999; Ashraf and Harris 2004). Salinity also alters the phytohormone content (Iqbal and Ashraf 2010; Shafi et al. 2011; Javid et al. 2011). The exposure of plants to salinity is known to induce a proportional increase in ABA concentration, which is in most cases correlated with leaf water potential, suggesting that salt-induced endogenous ABA is due to water deficit rather than specific salt effects (Zhang et al. 2006). Salt stress induces oxidative damage and alters the amounts and activities of the enzymes involved in scavenging oxygen radicals (Hernandez et al. 1993; Borsani et al. 2001).

2.3.2 Role of Exogenous and Endogenous Salicylic Acid During Salt Stress

It has been shown that SA could provide multiple stress tolerance, namely drought, chilling and heat to bean and tomato plants when applied in aqueous solution or as soil drenches (Senaratna et al. 2000). Similarly, soaking wheat seeds in SA solution provided protection against not only drought, but also salinity stress (Hamada and Al-Hakimi 2001). Long-term incubation of tomato plants in low concentration of salicylic acid enabled plants to tolerate salt stress caused by 100 mM NaCl (Tari et al. 2002).The application of exogenous SA appeared to induce a pre-adaptive response to salt stress in barley plants, leading to the protection of photosynthetic pigments and the maintenance of membrane integrity, which was reflected in an improvement in plant growth (El-Tayeb 2005). Furthermore, foliar spray with SA significantly decreased the lipid peroxidation induced by NaCl and improved plant growth. This alleviation of NaCl toxicity was related to a decrease in the Na content and increases in K and Mg contents, and also to increase the activity of SOD, CAT, glutathione peroxidase (GPX) and dehydroascorbate reductase and in the ascorbate and glutathione contents (He and Zhu 2009). The stress-induced accumulation of active oxygen species and, therefore, the level of SOD and peroxidase (POD) activity in the roots of young wheat seedlings pre-treated with SA were significantly lowered than in untreated plants, indicating that these enzymes contribute to the protective effect of SA on plants under conditions of salination (Sakhabutdinova et al. 2004). SA pre-treatment also provided protection against salinity due to the increased activity of aldose reductase, GST and APX enzymes, to improve photosynthetic performance and to the accumulation of osmolytes, such as sugars, sugar alcohol or proline in tomato plants (Tari et al. 2002, 2004, 2010; Szepesi et al. 2005, 2008a, b; Gémes et al. 2008) and in *Salvia officinalis* L. (Sahar et al. 2011). A high ABA level was also maintained in wheat seedlings treated with SA. The SA-induced increase in ABA might contribute to the pre-adaptation of plants to stress, since ABA is known to have a key role in inducing the synthesis of a range of stress proteins ensuring the development of antistress reactions, for example, the maintenance of proline accumulation (Sakhabutdinova et al. 2004).

By contrast, SA may promote the formation of ROS in the photosynthetic tissues of *Arabidopsis* plants during salt stress and osmotic stress. Recent results also suggest that the decrease of intracellular K^+ concentration and K^+/Na^+ ratio is a common phenomenonin triggering programmed cell death by lethal concentrations of salicylic acid and NaCl (Poór et al. 2012a). The widespread necrotic lesions observed on the shoots of wild-type plants after NaCl or mannitol treatments were not exhibited by *NahG* plants incapable of SA accumulation (Borsani et al. 2001). Investigations on *Arabidopsis* transgenic lines and mutants, including *snc1* (with a high level of SA), *NahG* (with a low level of SA), *npr1-1* (with SA signalling blockage) and*snc1/NahG* (expression of *nahG* in the *snc1* background), and on wild type plants showed that SA deficit or signalling blockage in *Arabidopsis* plants was favourable to salt adaptation, while a high accumulation of SA

increased salt-induced damage (Hao et al. 2012). SA promotes seed germination under high salinity in *Arabidopsis*. The seed germination of *sid2* mutant (SA induction deficient), which has a defect in SA biosynthesis, is hypersensitive to high salinity, but the inhibitory effects were reduced in the presence of physiological concentrations of SA (Lee and Park 2010).

Salt stress caused a decrease in SA content in *Iris hexagona* (Wang et al. 2001). A lower concentration of SA was also reported in tomato cell suspension cultures after NaCl supplement (Molina et al. 2002). In maize there were no changes in the levels of endogenous free and bound salicylic acid during NaCl stress, but the free *o*HCA content, a putative precursor of SA, in the leaves increased after 7 days, and rose dramatically after recovery. Free SA only increased during recovery in the leaves and roots (Szalai and Janda 2009). In contrast, the endogenous free SA content decreased in soybean under salt stress (Hamayun et al. 2010).

2.4 Heat Tolerance

2.4.1 Heat Stress and Thermotolerance

Heat stress is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to plant growth and development. At moderately high temperatures, injuries or death only occur after long-term exposure. At very high temperatures, severe cellular injury and even cell death may occur within minutes. Heat stress causes morpho-anatomical, physiological and biochemical changes in plants. Direct injuries due to high temperatures include protein denaturation and aggregation, and the increased fluidity of membrane lipids. Indirect or slower heat injuries include the inactivation of enzymes in chloroplasts and mitochondria, the inhibition of protein synthesis, protein degradation and loss of membrane integrity. These injuries eventually lead to starvation, inhibition of growth, reduced ion flux, and the production of toxic compounds and ROS (Wahid et al. 2007). The homeostasis, content, biosynthesis and compartmentalization of hormones are also altered under heat stress (Maestri et al. 2002). The upper developmental threshold, above which growth and development cease differ for different plant species and for genotypes within species, and also depend on plant behaviour, which may differ in various environmental conditions.

Plants manifest different survival mechanisms at elevated temperatures, including long-term evolutionary phenological and morphological adaptation and short-term avoidance or acclimation mechanisms such as changing leaf orientation, transpirational cooling, or alterations in membrane lipid composition. The capacity to survive heat shock varies with the plant species and genotype, and also with the developmental stage. Plants may have basal thermotolerance in the absence of pre-adaptation. In addition, plants subjected to mild heat stress may transiently acquire tolerance to previously lethal high temperatures: this phenomenon is known as acquired thermotolerance or heat acclimatisation, and is probably an adaptation to

the gradual increases in temperature in the natural environment (Clarke et al. 2004). Immediately after exposure to high temperatures the transcription and translocation of heat shock proteins (HSPs) is stimulated. Heat shock proteins belong to multi-gene families, most of which are regulated by high temperature, while a small number are induced by other abiotic stress factors. Proteins known as chaperones are responsible for the secondary and tertiary structure of the polypeptides synthesised in the cell, for linking up the subunits and for transporting them to the required cell component. If this complex process is disturbed, for example by high temperature, newly synthesised proteins form insoluble aggregates. It is currently thought that the majority of stress proteins are molecular chaperones, most of which have been identified as HSPs. The thylakoid membranes of the chloroplasts are most important for scaling heat sensitivity, so one critical aspect of heat tolerance in plants is the continual maintenance of photosynthesis. Consequently, one of the first signs of high temperature stress is a reduction in photosynthetic activity. Recent research has proved that many HSPs are transported into the chloroplasts, where they promote the heat tolerance of the photosynthetic system. Genetic evidence has established that the Hsp100 family proteins are essential for the acquisition of thermotolerance in plants. Loss-of-function mutants of Hsp101 in *Arabidopsis* (Hong et al. 2003) and maize (Nieto-Sotelo et al. 1999) are unable to acquire thermotolerance at several different growth stages.

2.4.2 Exogenous Salicylic Acid-Induced Thermotolerance

Evidence show that SA may be involved in heat stress responses in plants. Thermotolerance can be induced in potato plants by treatment with an acetyl SA spray at low concentrations (0.01–0.1 mM). Both treatment with SA and hardening at 45 °C for 1 h led to an increase in H_2O_2 level and a reduction in CAT activity (Dat et al. 1998a). In a similar manner, 0.01 mM acetyl SA was able to improve the heat tolerance of potato in tissue culture, by increasing the endogenous H_2O_2 level in the plants. This induced thermotolerance was extremely long-lasting (Lopez-Delgado et al. 1998, 2004). Foliar SA treatment was also found to reduce the oxidative damage caused by heat stress in *Arabidopsis* plants (Larkindale and Knight 2002) and in creeping bent-grass (Larkindale and Huang, 2004). The long-term thermotolerance induced by spraying SA onto the leaves of grape plants is thought to involve both Ca^{2+} homeostasis and antioxidant systems (Wang and Li 2006). Sulphosalicylic acid, a derivative of SA, also increased heat tolerance in cucumber when sprayed at 1 mM concentration (Shi et al. 2006), while foliar application (60 nl l^{-1}) of another derivative of SA, methyl salicylate reduced the thermotolerance of holm oak (Llusia et al. 2005). Both results were explained by alterations in the antioxidant defence mechanism.

Several studies demonstrated that the application of SA caused changes in the activity of antioxidant enzymes. In creeping bent-grass SA pre-treatment had no effect on POD activity whereas the CAT activity was lower than in the control plants, but SA increased the APX activity (Larkindale and Huang 2004). In

contrast, increased SOD and CAT activities were observed in Kentucky blue grass during heat stress after SA application (He et al. 2005). The efficacy of heat acclimation and SA, applied as a 0.1 mM foliar spray, in inducing thermotolerance was also tested in *Cicer arietinum* L. plants (Chakraborty and Tongden 2005). Both SA treatment and heat-acclimation resulted in an increase in the protein and proline contents over the control seedlings, and led to the induction of POD and APX, while there was a reduction in CAT activity.

Other studies investigate the effect of a lack of SA in transgenic plants or in plants with a mutation affecting SA biosynthesis. *NahG* transgenic plants carry a salicylate-hydroxylase gene of bacterial (*Pseudomonas putida*) origin, which prevents them from accumulating SA, as the salicylate-hydroxylase enzyme converts SA into catechol (Gaffney et al. 1993). It was found that after heat stress *NahG Arabidopsis* plants became more sensitive to the oxidative damage caused by high temperature than non-transformed plants with a normal level of SA. Furthermore, exogenous SA pre-treatment for 1 h enhanced the survival of *Arabidopsis* plants after heat treatment and reduced the levels of thiobarbituric acid-reactive substances (TBARS), the indicator of oxidative damage to membranes, (Larkindale and Knight 2002). However, it has also been shown that catechol, the product of SA degradation in *NahG* plants, induces susceptibility to pathogens in wild-type *Arabidopsis* plants, most probably due to catechol-mediated H_2O_2 production (van Wees and Glazebrook 2003). When evaluating the experiments it is thus important to check, that the observed changes were not caused by the catechol produced during the decomposition of SA.

Later it was found that SA pretreatment at 10 or 20 μM for 2 h prior to heat shock was just as effective in imparting thermoprotection at the seedling stage in *Brassica* species as heat acclimation for 3 h at sublethal temperature. SA pretreatment helped the seedlings to recover from heat stress by increasing total soluble sugars and fresh/dry weight and by stimulating the activity of the enzymes invertase, CAT and POD. Furthermore SDS-PAGE revealed the enhanced expression of new proteins including heat shock proteins (HSPs) after both pre-treatments (Kaur et al. 2009). In a recent study it was found that spraying 100 μM SA onto the leaves of grapevine did not affect the level of HSP21before heat stress, but the HSP21 immune signal increased in both SA-treated and control plants during heat stress. During the recovery period, the HSP21 levels remained high until the end of the experiment in the SA-treated leaves, but decreased in the control (Wang et al. 2011).

2.4.3 Involvement of Endogenous Salicylic Acid in Heat Tolerance

The role of SA in the signal transduction process involved in heat tolerance development is also confirmed by the increase in the level of endogenous bound and free SA during the heat acclimatisation of mustard plants (Dat et al. 1998b). The endogenous glucosylated SA content was enhanced in the shoots of plants grown on 0.01 or 0.1 mM SA, while free SA was also enhanced in those grown on 0.1 mM SA (Dat et al. 2000). In heat-treated grape plants, both the endogenous SA

level and the activity of phenylalanine ammonia-lyase (an enzyme also involved in SA biosynthesis) rapidly increased during the first hour, and then declined (Wang et al. 2005). The determination of the SA and ABA contents during heat acclimation in the presence of SA and/or ABA biosynthesis inhibitors revealed an interesting relationship between the endogenous SA and ABA levels. An SA biosynthesis-related benzoic acid-2-hydroxylase enzyme inhibitor was unable to influence the maximum peak of free SA content, but the peak of SA-2-O-b-D-glucose (SAG) was missing, while treatment with an ABA biosynthesis inhibitor resulted in the disappearance of the free SA peak during heat acclimation. The ABA peak induced by heat treatment disappeared in the presence of ABA biosynthesis inhibitor, rather than in that of the SA synthesis inhibitor (Liu et al. 2006). It was concluded that the inhibition of ABA biosynthesis did not lead to as great a loss of thermotolerance as that of SA biosynthesis. It was also found that the importance of conjugated SA in the development of thermotolerance was analogous to that of free SA or heat acclimation.

However, the use of *Arabidopsis* genotypes with modified SA signalling showed that SA-dependent signalling plays a role in the maintenance of basal thermotolerance: 0.5 or 1 mM SA pre-treatment promoted basal thermotolerance in 3-week-old *Arabidopsis* plants (Clarke et al. 2004).The level of endogenous SA correlated with basal thermotolerance. It was also shown that SA is not essential for acquired thermotolerance: all the genotypes could be heat acclimated irrespective of their endogenous SA content. It may be that SA is dispensable because heat acclimation is always initiated by some other key factor(s), or because SA is only one of multiple alternative acclimation signals. If SA was a potential mediator of a heat-induced acclimation response, the SA levels should be heat-inducible. A moderate, transient increase in glucosylated SA during heat treatment was also detected in wild-type *Arabidopsis* plants. Given the significant basal levels of SA in *Arabidopsis* and mustard, the extent to which such changes in the SA metabolism provide additional thermotolerance is uncertain, but they suggest that despite the metabolic stress caused by heat treatment, the plants actively maintained the biosynthesis of this hormone. In plants, SA is subject to glucosylation, making it more suitable for translocation (Seo et al. 1995) or vacuolar localization (Dean et al. 2003). Heat-induced increases in SA were not apparent in the other *Arabidopsis* genotypes, although this might be because of their altered SA metabolism or signalling (Clarke et al. 2004).

2.5 Cold Stress

2.5.1 Low Temperature and Cold Tolerance

Each plant has a unique temperature requirement, which is optimum for proper growth and development. For crops, low temperature is one of the most important factors restricting cultivation in a given area. Plants are said to be cold-sensitive

(e.g. maize) if they die or suffer severe damage at temperatures between 0 and 15 °C (chilling stress). On the other hand, cold-tolerant plants (e.g. winter wheat) are still able to grow near freezing point and are capable of surviving temperatures as low as 10–15 °C below zero (freezing stress). Apart from genetic factors, cold sensitivity also depends on the stage of development and the level of metabolic activity. Plants are more sensitive to cold in the early phases of development, during the day, in bright light, under dry conditions and in the case of potassium deficiency, for example.

Besides the various phenotypic symptoms caused by chilling stress, which include reduced leaf expansion, wilting, chlorosis, necrosis and the inhibition of reproductive development, chilling also results in oxidative stress, leading to loss of membrane integrity, and the reduction or impairment of photosynthesis and general metabolic processes. Low-molecular-weight osmolytes, including glycinebetaine, proline and organic acids, have a crucial role in sustaining cellular function during chilling stress. Plant growth substances such as SA, gibberellic acid and ABA also modify the response of plants to chilling stress. Polyamines and several enzymes act as antioxidants and reduce the damaging effects of low temperature (Farooq et al. 2009). The major effect of freezing is severe membrane damage, which is largely due to ice formation rather than to the acute dehydration and cell damage induced by low temperature (Mahajan and Tuteja 2005). Certain plants are able to increase their freezing tolerance by cold acclimation (exposure to low non-freezing temperatures). During cold acclimation the proportion of unsaturated fatty acids increases leading to the stabilization of the membranes against freeze injury by decreasing the transition temperature. Besides the alteration in the amount of unsaturated fatty acids, the composition of glycerolipids, proteins and carbohydrates also changes. The most important way of avoiding freezing is the accumulation of osmolytes (sugars, polyalcohols, amino acids, polyamines, quaternary ammonium compounds, etc.), which decrease the freezing point within the cell and prevent the dehydration of the cytoplasm. Plants such as perennials and conifers, on the other hand can tolerate frost through extracellular ice formation in the apoplasts, where it does not have the lethal consequences it would have within the cell. Recent research has proved that the antifreeze proteins demonstrated in many plant species are capable of protecting the cell by preventing the formation of large crystals.

2.5.2   Cold Tolerance Induced by SA and Relative Compounds

SA and other phenol derivatives are known to improve the cold tolerance of plants. It was first shown that the addition of 0.5 mM SA to the hydroponic growth solution of young maize plants under normal growth conditions provided protection against subsequent low-temperature stress. Besides the obvious visual symptoms, this observation was confirmed by chlorophyll fluorescence parameters and electrolyte leakage measurements from the leaves (Janda et al. 1997, 1999). Among the antioxidant enzymes, the activity of CAT decreased, while that of

glutathione reductase (GR) and guaiacol peroxidase (G-POD) increased as a result of preliminary treatment with SA. These changes might explain the increased cold tolerance. Further studies proved that not only SA, but also related compounds, such as BA, aspirin or coumaric acid, may have a protective role against chilling stress in young maize plants (Janda et al. 1998, 2000; Horváth et al. 2002). It should also be mentioned, however, that these compounds may cause severe damage to the leaves and roots of maize when added to the nutrition solution above a certain concentration at normal growth temperature (Janda et al. 1998, 2000; Pál et al. 2002). SA treatment resulted in many changes in the ultrastructure of banana cells, such as the separation of the cells from palisade parenchymas, the appearance of crevices in the cell walls, the swelling of grana and stromal thylakoids, and a reduction in the number of starch granules (Kang et al. 2007).These results also demostrated that SA treatment at normal conditions could act as a stress factor.

In contrast to this, the foliar application of SA or aspirin enhanced stomatal conductance, transpiration and photosynthetic rates in both soybean and maize (Khan et al. 2003). Other stimulating effects of SA were reported in embryogenic cell suspension cultures of *Coffea arabica* L. treated with picomolar concentrations of SA (Quiroz-Figueroa et al. 2001). Seed treatment with 100 μM SA significantly improved the germination percentage, germination rate and seedling criteria, compared with control seeds under optimal and low temperature stress conditions (Gharib and Hegazi 2010). Furthermore, seed priming with SA improved seedling emergence, root and shoot length, seedling fresh and dry weights, and leaf and root scores compared to the control both at optimal and chilling temperatures in maize (Farooq et al. 2008). The cell ultrastructure of banana seedlings pre-treated with 0.5 mM SA in the form of foliar spray or root irrigation showed less deterioration than that of control seedlings after chilling stress (Kang et al. 2007).

The chilling tolerance of leaves or hypocotyls was significantly increased by the application of 0.5 mM SA not only in maize, but also in cucumber and rice (Kang and Saltveit 2002), but this improved chilling tolerance was only observed in the leaves of rice and in the hypocotyl of cucumber, as exposure to SA caused a significant reduction in the growth of the radicle. The inhibitory effect of SA on radicle growth did not appear if SA application started at germination, suggesting that seedlings may become acclimated to SA (Kang and Saltveit 2002). Pretreatment with 0.5, 1.0 and 2.0 mM SA for 24 h before chilling at 5 °C for 1 d decreased the chilling tolerance of rice (Wang et al. 2009b), while a 2 mM concentration of SA was highly effective in reducing chilling injury in pomegranate fruit (Sayyari et al. 2009). SA was able to regulate the leaf photosynthetic functions of cucumber seedlings and enhance seedling resistance to low temperature and light intensity. The optimum concentration of SA for foliar-spraying was 1 mM (Liu et al. 2009).These results clearly showed that there is a need to investigate the best method of SA application and the dose giving maximum protection, as a function of plant species, the growth stage and the plant organ, treated.

Pre-treatment of the leaves of chilling-sensitive banana seedlings with 0.5 mM SA solution by spraying the foliage or irrigating the roots for 1 day induced an increase in chilling tolerance during subsequent 5 °C chilling stress, which was accompanied by achange in the activity of antioxidant enzymes (Kang et al. 2003a). Furthermore, although SA pre-treatment at 30/22 °C resulted in H_2O_2 accumulation but reduced H_2O_2 overproduction during the subsequent 5 °C chilling stress (Kang et al. 2003b). SA was able to reduce lipid peroxidation through the inhibition of lipoxygenase activity and a decline in the H_2O_2 content, leading to the maintenance of cellular membranes integrity under stress conditions (Lapenna et al. 2009). The effect of SA in alleviating chilling injury during cold storage may be attributed to its ability to induce antioxidant systems (including enzymatic and non-enzymatic compounds) as demonstrated in peach (Wang et al. 2006). In contrast, it was recently demonstrated that rice pre-treated with 0.5, 1.0 and 2.0 mM SA for 24 h before chilling at 5 °C for 1 d had decreased chilling tolerance and that the down-regulation of the antioxidant defence system might be involved in the reduction of chilling tolerance in plants pre-treated with SA (Wang et al. 2009b).

Soaking the seeds before sowing could also be an effective way of improving cold tolerance. In tomato and bean plants, 0.1 and 0.5 mM concentrations of both SA and acetyl SA proved effective not only against heat and drought stress, but also against low temperature stress (Senaratna et al. 2000). Certain salicylates, such as 2,6-dihydroxy BA, aspirin and SA, hastened the germination of carrot seeds at 5 °C (Rajasekaran et al. 2002). Priming pepper (*Capsicum annuum* L.) seeds in solution containing aspirin at a concentration range of 0.05–0.5 mM resulted in an increase in the germination percentage, and the germination at low temperature became faster and better synchronised (Korkmaz 2005). It was also shown that soaking pea seeds in SA solution before sowing may increase productivity; however, it has been demonstrated that the increase in the endogenous level of SA in various tissues does not originate from the exogenous SA, but mainly from *de novo* synthesis (Fig. 1) (Szalai et al. 2011).

Another related compound, methyl SA, used at a final vapour concentration of 10^{-4} M for 1 day at room temperature, increased resistance to chilling injury in freshly harvested green bell pepper (*Capsicum annuum* L. cv. Century). This increase may result from increased heat evolution, especially in chilling-tolerant species. The increased capacity to produce respiratory heat after exposure to chilling temperatures may contribute to the cold-acclimation process (Moynihan et al. 1995). The alternative respiratory pathway may moderate chilling injury by keeping the production of ROS in balance with the levels of antioxidants and active oxygen-scavenging enzyme systems. The expression patterns of alternative oxidase (AOX) and of several other genes involved in the defence against oxidative stress before and during the early chilling period suggested that the pre-treatment of pepper fruit with methyl SA vapours preferentially increased the transcript levels of AOX and that this increase was correlated with chilling tolerance.

The positive effect of SA was shown not only during chilling but also under freezing conditions. Exogenous SA caused an increase in ice nucleation activity under cold and control conditions, similarly to cold acclimation in winter wheat
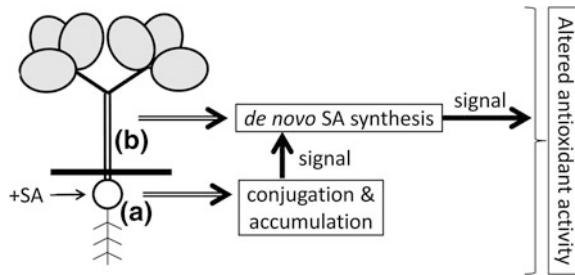
Fig. 1 Changes induced in one-week-old pea plants by soaking seeds in salicylic acid (SA) before sowing. The applied SA remained mainly in the seeds but in conjugated form (a). An increase was found in the free SA fraction in the epicotyl but the use of labelled SA and gene expression measurements showed that this increase was derived from de novo synthesis (b). Furthermore, altered antioxidant activity was detected in the whole plant (Szalai et al. 2011)

leaves. These results show that SA can increase freezing tolerance by affecting apoplastic proteins (Taşgín et al. 2003).TheSA-induced increase in the activity of certain antioxidant enzymes during the cold hardening period has also been described in wheat plants (Sasheva et al. 2010). In rye, however, the apoplastic proteins induced by salicylic acid did not have antifreeze activity (Yu et al. 2001), and only treatment with ethylene induced antifreeze activity in winter rye leaves. Cryo preservation is actually a more valuable technique for the long-term *in vitro* conservation of plant germplasm, since, when frozen in liquid nitrogen, the metabolism ceases to function, tissues are maintained without growth and genetic alterations do not take place even during a very long period of storage. SA also significantly enhanced the viability percentage of encapsulated embryonic axes of Persian lilac (*Melia azedarach* L.) when they were subjected either to the cryo preservation technique, involving dehydration and freezing in liquid nitrogen, or to cold preservation by storing the alginate beads in empty petri dishes at 4 °C (Bernard et al. 2002).

A SA analogue 4-hydroxybenzoic acid (4-HBA) ameliorated the freezing tolerance of the spring wheat Chinese Spring (Horváth et al. 2007). It is hypothesized that 4-HBA increases the impermeability of the cell wall, leading to increased resistance to pathogen infection. The reinforcement of the cell wall by 4-HBA may also contribute to increased tolerance against freezing stress.

2.5.3 Role of Endogenous SA (Free and Bound Forms) During Cold Stress

Chilling-induced SA accumulation, particularly that of conjugated SA was reported in both the leaves and roots of rice cultivars. When the endogenous SA content was monitored after SA treatment in the leaves and crowns of two wheat cultivars differing in cold tolerance, SA was found to accumulate in a

concentration-dependent manner in the roots of both cultivars, conjugated SA accounting for most of the increase (Wang et al. 2006). After the plants were transfered to the cold, the SA level significantly decreased in the leaves of winter wheat (Samanta). This was followed by a pronounced increase in SA, with a maximum after 7 days. After prolonged cold treatment, a gradual decrease in SA occurred. In the leaves of spring wheat (Sandra), the SA profile basically followed the same trend, but the change in SA levels was less pronounced. A decrease in the SA level was observed in Samanta crowns after 1 day, followed by a peak between 3 and 7 days. Prolonged cold treatment was associated with a decrease in SA. In Sandra crowns, the down regulation of SA was much more pronounced than in Samanta, with a minimum being detected after 3 days, when a gradual elevation of SA began. This latter increase might be related to the function of SA in regulating ROS evolution, which is an important part of plant stress defences (Kosová et al. 2012). These data are in accordance with a report by Janda et al. (2007), who found increases in both free and bound SA and in its precursor *o*HCA in winter wheat. Although, cold acclimation increased frost tolerance in androgenic genotypes of *Festulolium*, resistant genotypes were characterized by higher ABA concentrations and lower concentrations of SA than susceptible genotypes during the first 54 h of cold acclimation (Pociecha et al. 2009).

   The use of mutants and transgenic plants in which the synthesis, accumulation or translocation of SA is modified could help to clarify its role under stress conditions. The growth of *Arabidopsis* plants under chilling conditions could be related to their SA levels (Scott et al. 2004). *NahG* and wild type *Arabidopsis* plants grew at similar rates at 23 °C, and the growth of both genotypes was slowed by transfer to 5 °C. However, at 5 °C, *NahG* plants displayed relative growth rates about one-third greater than the wild type. This resulted primarily from greater cell expansion in *NahG* rosette leaves. Net assimilation rates were similar in the two genotypes at 23 °C, but higher in *NahG* at 5 °C. At 5 °C wild-type shoots accumulated SA, particularly in glucosylated form. A similar tendency was observed in *NahG* shoots at 5 °C, but at greatly depleted levels. Growth and SA levels were also examined in SA-signalling and metabolism mutants at 5 °C. The partially SA-insensitive *npr1* mutant (non-expresser of SA-inducible PR genes) displayed growth intermediate between that of *NahG* and the wild type, while the *eds5* mutant (inhibited SA transport from the chloroplast) behaved like *NahG*. In contrast, the *cpr1* mutant (constitutive expresser of PR genes) accumulated very high levels of SA at 5 °C and its growth was much more inhibited than that of the wild type. At both temperatures, *cpr1* was the only SA-responsive genotype in which oxidative damage was significantly different from that of the wild type. In another experiment, freezing tests on *Arabidopsis* transgenic lines and mutants revealed that after cold-hardening *NahG* and the wild type showed similar survival rates during recovery after 1d at −10 °C, while the SA-deficient mutants, *eds5* and *sid2* (chloroplastic SA synthesis deficient), had higher rates (Majláth et al. 2011). Under control condition *eds5*, *sid2* and *NahG* had lower levels of bound SA than the wild type, while a significant increase in bound SA was observed in all the genotypes after cold

treatment. These results suggested that there is a strong negative correlation between the growth rate under cold conditions and the levels or perception of SA.

In conclusion, SA has a contradictory role in cold stress tolerance. Although exogenous SA treatment can protect plants against stress-induced injury, the treatment itself causes stress to the control plants. Furthermore, SA treatment may alter the endogenous SA metabolism, either by inducing *de novo* synthesis (Szalai et al. 2011) or by decreasing and disturbing it (Rakhmankulova et al. 2010). Although cold stress induces an increase in endogenous SA, it seems it is better if the SA level remains at a low level.Thus,the effect of SA in plants is related to its initial endogenous content (Yang et al. 2004), to changes in this and possibly to redistribution between free and conjugated forms.

2.6 Other Stresses

2.6.1 Ozone Stress

Ozone is formed through photochemical reactions between nitrogen oxides, carbon monoxide and hydrocarbons released, primarily, through the burning of fossil fuels in urban areas (Mauzerall and Wang 2001). Ozone production is particularly favoured in summer months by strong sunlight, high temperature and stagnant high-pressure systems, and concentrations therefore tend to be at their highest during the growing season of most of the world's crop plants. Ozone is toxic to plants and animals because it is a powerful oxidizing agent, which is able to react directly with lipids and proteins. Such reactions and the decomposition of ozone in aqueous environments such as the plant apoplast can lead to the production of other ROS such as the hydroxyl radical, singlet oxygen and H_2O_2 (Kanofsky and Sima 1991; Mehlhorn et al. 1990; Evans et al. 2005). The primary site of ozone interaction with plant cells is the extracellular matrix, where ozone challenges the antioxidant protection of the cells (Baier et al. 2005). Long-term chronic exposure to ozone can lead to a reduction in growth and crop yield, resulting from the inhibition of photosynthesis, premature senescence, altered biomass partitioning and changes to reproductive processes (Black et al. 2000; Pell et al. 1997; Saitanis and Karandinos 2002; Sandermann 1996). Ozone is also able to act as an abiotic elicitor of plant defence reactions and acute exposure can result in the appearance of small necrotic hypersensitive response (HR)-like lesions on foliage (Rao and Davis 2001).

Ozone treatment led to the accumulation of SA, the synthesis of PR protein and the development of virus resistance in tobacco (Yalpani et al. 1994). The role of SA in counteracting ozone stress was also demonstrated in *Arabidopsis* plants, where *NahG* plants were more sensitive to the damaging effect of ozone. The synthesis of some ozone-induced mRNAs is SA-dependent, so only a few were found in transgenic plants. Other authors reported that both a deficiency and an excess of SA caused greater ozone sensitivity (Rao and Davis 1999).

SA biosynthesis has also been investigated during ozone fumigation. The Cvi-0 *Arabidopsis* genotype, which accumulates SA, is ozone-sensitive, since the large quantity of SA induces oxidative processes during ozone stress, leading to cell death similar to that caused by the hypersensitive reaction. In *NahG* plants, however, which are incapable of accumulating SA, the lack of a satisfactory antioxidant response led to increased ozone sensitivity (Rao and Davis 1999). In the Cvi-0:*NahG* genotype the lack of SA reduced the level of ozone-induced cell death. Exposure of *Arabidopsis* to O_3 enhanced the accumulation of SA and the activity of isochorismate synthase (ICS) but did not affect PAL activity. In *sid*2 mutants, which have a defect in ICS1, the level of SA and the activity of ICS did not increase in response to O_3 exposure. These results suggest that SA is mainly synthesized from isochorismate in *Arabidopsis*. Furthermore, the level of ICS1 expression and the activity of ICS during O_3 exposure were elevated in plants deficient for SA signalling (*npr1* and *eds5* mutants and *NahG* transgenics) (Ogawa et al. 2007). When C^{14}-labelled BA was applied to ozone-exposed tobacco leaves, it was efficiently metabolized to SA (Ogawa et al. 2005). However, there was no increase in the activity or mRNA level of ICS. In contrast, the activity of this enzyme was increased in ozone-exposed *Arabidopsis*. These results suggest that SA is synthesized via BA from phenylalanine in ozone-exposed tobacco leaves but via isochorismate in *Arabidopsis*. Ozone increased the activity of phenylalanine ammonia-lyase and the transcript levels of the chorismate mutase and phenylalanine ammonialyase genes in wild-type tobacco.

2.6.2 Ultraviolet Radiation

The depletion of the stratospheric ozone layer may result in an increase in the level of potentially harmful ultraviolet (UV) radiation reaching the surface of the earth. UV radiation is traditionally divided into UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–280 nm) wavelength ranges, which have increasing levels of energy and harmful effects. Plants, which use direct sunlight for photosynthesis, are unable to avoid UV radiation, so mechanisms which may protect them from the harmful effects of UV radiation are of particular interest (Hollósy 2002)

It has been shown that the foliar application of SA at 150 mg m^{-2} may alleviate UV-B damage by upregulating plant defence systems in the grass species *Poa pretensis* L. (Ervin et al. 2004). Endogenous α-tocopherol, SOD and CAT were reduced by UV-B stress. The anthocyanin content increased in the first 5 days then decreased during continuous UV-B irradiation. The application of SA enhanced the photochemical efficiency after UV-B treatment. In addition, the application of SA increased the α-tocopherol concentration, the SOD and CAT activity and the anthocyanin content compared to the control 10 days after UV-B initiation, leading to improved plant growth under UV-B stress. These results suggest that the application of SA may alleviate the decline in photochemical efficiency and turf quality associated with increased UV-B light levels during the summer. Exogenous

treatment with SA also attenuated the UV-B radiation stress in soybean seedlings (Zhang and Li 2012). The concentration of UV-absorbing substances was increased by increasing levels of SA in maize (Gunes et al. 2007).

Like ozone, UV radiation was shown to induce the accumulation of SA, while also stimulating PR-protein synthesis and inducing virus resistance in tobacco plants (Yalpani et al. 1994). Increase in SA level was accompanied by the accumulation of SA conjugate and an increase in the activity of BA 2-hydroxylase, which catalyses SA biosynthesis.

2.6.3 Various Light Conditions

Light is one of the most strongly fluctuating environmental parameters. Tests were made to discover how the varying light conditions found in shade affected the endogenous SA content, and the possible role of SA in shade avoidance by sunflower (*Helianthus annuus* L.) hypocotyls was examined. A logarithmic increase in PAR (photosynthetically active radiation) levels caused a roughly 10-fold increase in endogenous SA levels. Of the far-red (FR), red (R) and blue wavelengths that make up PAR, only FR light had a significant and positive effect on endogenous SA levels. Furthermore, a low R/FR ratio significantly increased the endogenous SA content in hypocotyls compared with normal and high R/FR ratios. Uncoupling the effect of the R/FR ratio and PAR irradiance on the endogenous SA content demonstrated that PAR irradiance is a much stronger signal than FR lightenrichment. Thus, while a low R/FR ratio increases the SA content in sunflower hypocotyls, low PAR, the other component of canopy shade, decreases the SA content much more effectively than a low R/FR ratio increases it. Therefore, it appears that SA probably has no direct role in shade avoidance effects (Kurepin et al. 2010). It was reported that exogenous SA could protect the photosynthetic apparatus against strong light-induced photo-damage in the leaves of Satsuma mandarin tree (*Citrus unshiu* Marc.) (Qiu et al. 2011), and that SA is able to overcome low irradiance stress in rice (Maibangsa et al. 2000). Furthermore, in *Arabidopsis* the dwarf phenotype displayed by mutants with high SA content (*cpr1-1, cpr5-1, cpr6-1* and *dnd1-1*) was less pronounced when these plants were grown in strong light, suggesting that the inhibitory effect of SA on growth was partly overcome at higher light intensities. On the other hand, mutants with low foliar SA content were less able to become acclimated to transient exposure to strong light and thus predisposed to oxidative stress.These observations implied an essential role of SA in light acclimation processes (Mateo et al. 2006).

2.6.4 Hypoxia

Higher plants are aerobic organisms requiring oxygen for growth and metabolism. However, low oxygen concentration may occur in the root zone in regions with higher rainfall due to soil compaction and soil water-logging. It was concluded that

SA could alleviate the detrimental effects of hypoxia stress on plant growth and of oxidative stress in the leaves of *Malus robusta* Rehd.by enhancing the antioxidant defence system (Bai et al. 2009).

3 Salicylic Acid In Relation to Other Plant Hormones

Various hormones involved in plant defence mechanisms cross talk with SA where both negative and positive interactions have been reported. Endogenous ABA levels rise in several species when they are exposed to stress conditions (Veisz et al. 1996; Lee et al. 1997; Janowiak et al. 2002). The modes of cross talk between ABA and SA are the subject of intensive research. SA treatment caused ABA accumulation in the leaves of *Hordeum spontaneum* (Bandurska and Stroinski 2005) and tomato plants (Szepesi et al. 2009). It was also found that SA treatment caused the accumulation of both ABA and indoleacetic acid (IAA), but did not influence the cytokinin (CK) content, while diminishing the changes in phytohormone levels in wheat seedlings under salinity (Sakhabutdinova et al. 2003). In *Panicum virgatum*, moderate water stress treatment decreased the endogenous SA contents, accompanied by an increase in the ABA content (Yasuda et al. 2008). Salt stress caused an increase in the ABA and jasmonate (JA) content in *Iris hexagona*, while the levels of IAA and SA declined (Wang et al. 2001). In salt-stressed soybean, the endogenous gibberellic acid (GA$_3$) and free SA contents decreased, while a significant increase was observed in the endogenous ABA and JA contents (Hamayun et al. 2010). Furthermore, both heat and SA treatment caused a rapid increase in the level of ABA, although this declined sharply, shortly afterwards (Wang et al. 2005).The addition of SA or even that of ethylene (ET) precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or ABA to plants may also protect them from heat-induced oxidative damage. It was demonstrated that the inhibition of ABA biosynthesis caused a less serious loss of thermotolerance than the inhibition of SA biosynthesis (Liu et al. 2006). In addition, the ethylene-insensitive mutant *etr-1*, the ABA-insensitive mutant *abi-1* and a transgenic line expressing *nahG* showed increased susceptibility to heat (Larkindale and Knight 2002). Although, it was suggested that the induction of several heat shock proteins (HSPs) by ABA may be one mechanism whereby it confers thermotolerance (Pareek et al. 1998), it was later reported that ABA and SA are involved in acquired thermotolerance separately from heat shock protein induction (Larkindale et al. 2005).

An SA analogue, benzothiadiazole S-methylester (BTH), repressed NaCl-induced ABA biosynthesis, while ABA or NaCl treatment antagonized BTH-induced resistance to *Pseudomonas syringae* in *Arabidopsis* (Yasuda et al. 2008). ABA signalling was also positively correlated with the susceptibility of *Arabidopsis* to *P. syringae* (de Torres-Zabala et al. 2007). These results suggested an antagonistic interaction between these two hormones, probably as a result of sharing common intermediaries in the signalling pathway. Responses to both biotic and abiotic stresses require significant amounts of energy for gene expression and metabolic

changes, so plants need to effectively regulate the strength of these responses to survive (Dietrich et al. 2005).

Most of the papers published on the crosstalk between ABA and SA deal with the effect of SA treatment on the ABA content during abiotic stress and with the relationship between ABA and SA-mediated SAR. In a recent publication the effect of ABA treatment on the SA content, under normal and cold stress conditions was examined (Pál et al. 2011). Under normal growth conditions an increased level of bound SA was observed in the leaves during ABA treatment, but when it was followed by cold treatment the SA levels decreased, in contrast to that in unchilled plants. Although the exact mechanism of the cross-talk between ABA and SA signalling pathways is unclear, it has been demonstrated that ABA may inhibit the activity of SA glucosyl transferase, thus increasing the level of free SA in pea plants (Liu et al. 2006).

Complex phytohormone responses have been investigated during plant hormone treatment or abiotic stress. Seed treatment with SA not only significantly improved the germination percentage, germination rate and seedling criteria compared with those of control seeds under optimal and low temperature stress conditions, but also increased the content of IAA, GA_3 and ABA in six bean varieties at 15 °C, the greatest increase being observed in the case of GA_3. The steep increase in the GA_3/ABA ratio seemed to correlate with the pattern obtained for germination and subsequent seedling growth (Gharib and Hegazi 2010). GA_3, a plant hormone closely associated with seed germination, also reverses the inhibitory effects of high salinity on seed germination and seedling establishment (Lee and Park 2010). The exogenous application of GA_3 was also able to reverse the inhibitory effect of heat stress in the germination and seedling establishment of *Arabidopsis* plants. This effect was accompanied by an increase in endogenous SA levels. Furthermore, GA_3 treatment induced an increase in the expression levels of the ICS1 and NPR1 (non-expressor of PR genes) genes, which are involved in SA biosynthesis and action, respectively (Alonso-Ramírez et al. 2009).

In a recent study it was detected that ABA, ACC, SA and JA had differential temperature responses in three *Labiatae* species (rosemary, sage and lemon balm). The ABA level increased at high temperatures in all the species investigated, while a significant increase in the ACC level was only found in one species. The JA level decreased slightly with increasing temperature in all three species, while the SA level remained unchanged (Asensi-Fabado et al. 2012). Since it was earlier shown that the chilling-induced level of ACC is negatively correlated with the cold tolerance of maize plants (Janowiak and Dörffling 1996), an investigation was also made on changes in ACC after chilling stress. Chilling at 5 °C caused an increase in ACC content; however, this increase was less pronounced in cold-acclimated plants and in those pre-treated with SA for 1 d before cold stress (Szalai et al. 2000). SA was reported to be an effective inhibitor of ethylene biosynthesis due to its inhibitory effect on the conversion of ACC to ethylene in pear cell suspension cultures (Leslie and Romani 1988). Furthermore, SA has been shown to inhibit the wound-induced transcription of ACC synthase and its activity in tomato fruit (Li et al. 1992), and ACC oxidase activity in apple fruit (Fan et al. 1996).

The interactions between ethylene, JA and SA induced by ozone, and the inhibition of the ethylene or SA pathways by JA have also been investigated. Pretreatment with methyl JA hindered the accumulation of SA or H_2O_2, thus preventing ozone-induced necrosis (Rao et al. 2000). It was also shown that ROS-mediated SA biosynthesis and that the SA signalling pathway in an ozone-sensitive ecotype was influenced by the JA pathway (Rao et al. 2000). The results of a cDNA microarray assay indicated that ozone-induced defence gene expression was mainly regulated by ethylene and JA, and that the SA pathway acted as a strong antagonist to gene expression induced by ethylene and JA signalling (Tamaoki et al. 2003). During ozone exposure, transgenic plants with reduced ozone-induced ethylene production accumulated less SA than wild-type plants. Ozone increased the activity of phenylalanine ammonia-lyase and the transcript levels of the chorismate mutase and phenylalanine ammonialyase genes in wild-type tobacco, but their induction was suppressed in transgenic plants. These results indicate that ethylene promotes SA accumulation by regulating the expression of chorismate mutase and phenylalanine ammonialyase genes in tobacco plants, exposed to ozone (Ogawa et al. 2005). Contrary to these results, the *NahG* transgenic line and the *npr1Arabidopsis* mutant failed to produce ethylene in response to ozone, indicating that the SA pathway is required for ethylene signalling (Rao et al. 2002).

During the investigation of complex phytohormone responses during the cold acclimation of two wheat cultivars differing in cold tolerance, three phases were distinguished (Kosová et al. 2012). The alarm phase was characterized by an increase in ABA and in the content of ACC, and by a decrease in CK, GA_3 and IAA. The upregulation of the ABA content coincided with the rapid down regulation of SA and JA. During the acclimation phase the ABA level was down regulated, while the levels of CK, GA_3 and IAA exhibited a transient maximum. The decrease in ABA during the acclimation phase coincided with the elevation of ACC and SA. The plants become resistant after 21 days of cold treatment. The ABA content was maintained at a higher level and the SA level exhibited a gradual decrease in winter wheat leaves, but it was still significantly higher than that in spring wheat. The JA content was also higher in the leaves of the tolerant genotype, the increase occurring after 7–21 days of cold treatment. As JA inhibits cell division and growth in a similar manner to ABA, it is possible that JA may replace ABA in suppressing growth during later stage of cold stress. The ACC levels did not differ between the genotypes. Parallel to this, lower levels of the growth-promoting hormones CK, GA_3 and IAA were observed in tolerant genotypes, while relatively high CK levels were maintained in the sensitive genotypes with a mild elevation in the IAA levels. These results indicated that the cross-talk between the different plant hormones results in synergetic or antagonic interactions that play a crucial role in the response of plants to cold stress, as in the case of other abiotic stresses.

In another study on *Pinus radiate* a decrease in CK level was found to be the primary signal of drought, while IAA and ABA accumulation represented a secondary signal. While the CK level further decreased, the ABA and IAA levels doubled. At the end of the drought period, less tolerant genotypes exhibited an over

10-fold increase in IAA compared with the control. External damage also caused JA accumulation, which in turn induced the accumulation of SA as a defence mechanism (De Diego et al. 2012). ABA may indirectly affect SA signalling via its effect on JA signalling and/or vice versa (Adie et al. 2007). In contrast, investigations on ET- and JA-insensitive mutants showed that the suppressive effect of ABA on SAR is regulated by unknown mechanisms, independent of the JA/ET-mediated signalling pathway (Yasuda et al. 2008).

Not only are synergistic and antagonistic effects demonstrated between plant hormones, but each also has an impact on the biosynthesis of the other. SA inhibits JA biosynthesis and/or JA-dependent gene expression (Gupta et al. 2000). Conversely, JA inhibits the SA pathway in response to wounding (Niki et al. 1998). It has been discovered that IAA promotes GA biosynthesis; on the other hand, GA_3 application enhances the catabolism of ABA (Javid et al. 2011).GA_3 stimulates SA biosynthesis by inducing the *SID2* gene. SA also induces genes encoding the enzymes involved in GA_3 biosynthesis (Lee and Park 2010). In another study it was found that ABA negatively regulated SA synthesis by the transcriptional regulation of at least ICS (de Torres Zabala et al. 2009).

Based on these results, there is clearly a relationship between SA and other plant hormones. SA is primarily antagonistic to JA/ET and to ABA signalling. JA/ET and ABA, in turn, repress SA signalling, while SA and GA_3 are in close positive correlation with each other.

4 Relationship Between Biotic and Abiotic Stress Factors

One of the earliest responses observed after pathogen attack is the oxidative burst, which involves the generation of ROS (Torres 2010). The relationship between SA and ROS is complicated and bidirectional (Vlot et al. 2009), but the role of SA in signalling disease resistance is well documented, both by studies on SA-induced pathogen resistance and by reports on altered endogenous SA levels after pathogen attack. There is more and more evidence showing that biotic and abiotic signalling pathways cross-communicate by each other (Fujita et al. 2006; Atkinson and Urwin 2012), but it is not yet clear how they interact if plants are exposed simultaneously to both biotic and abiotic stress (Mittler 2002).

Phytohormones have a central role in abiotic and biotic stress signalling. SA is essential for the activation of defence responses against biotrophic and hemi-biotrophic pathogens as well as for the establishment of systemic acquired resistance. By contrast, JA and ET are usually associated with defence against necrotrophic pathogens and herbivorous insects. However, in the last decade evidence has accumulated to suggest that ABA is also involved in biotic stress signalling, In *Arabidopsis* bacterial effectors rapidly activate ABA biosynthesis (de Torres-Zabala et al. 2009). It is becoming increasingly clear that ET, JA and SA are also involved in the response to abiotic stress (see Sect. 3).

The occurrence of simultaneous biotic and abiotic stressors presents an added degree of complexity, as the responses to these are largely controlled by different hormone signalling pathways that may interact with or inhibit one another. The exposure of plants to pathogens often increases the effects of abiotic stress, while long-term abiotic stress may weaken plant defences and cause enhanced pathogen susceptibility (Atkinson and Urwin 2012). On the other hand, one stressor may cause an acclimation response, possibly be resulting in cross-tolerance to a second stressor. Studies on the joint effect of an abiotic stress and pathogens have revealed both positive and negative interactions depending on the timing, nature and severity of each stress.

It was demonstrated that pre-exposure to a mild dose of Cd increased plant resistance to viral or fungus infection (Ghoshroy et al. 1998; Mittra et al. 2004), suggesting that heavy metals may induce defence pathways and increase resistance to biotic stress. The cross-talk between pathogens and heavy metal stress is also supported by the finding that various heavy metal ions induce ethylene and JA accumulation, and that JA and heavy metals have a similar effect on gene expression (Maksymiec 2007). Externally applied methyl JA had a protective effect against excess Cd and Cu in *Arabidopsis* (Maksymiec and Krupa 2002).

Plants exposed to ozone exhibited enhanced resistance to virulent *Pseudomonas syringae* strains. The results indicate that there is overlapping between the development of ozone- and pathogen-induced resistance and that both are SA-dependent (Sharma et al. 1996). Like ozone, UV radiation was shown to induce the accumulation of SA, while also stimulating PR-protein synthesis and inducing virus resistance in tobacco plants. The results suggest that UV light, ozone fumigation and tobacco mosaic virus activate a common signal transduction pathway that leads to SA and PR-protein accumulation and increased disease resistance (Yalpani et al. 1994). In barley, increasing levels of salt-induced osmotic stress were directly correlated with resistance to powdery mildew (Wiese et al. 2004), while drought stress can enhance resistance to the fungus *Botrytis cinerea* in tomato (Achuo et al. 2006). Infection with viruses can actually provide protection against drought stress (Xu et al. 2008). Futhermore, many types of bacteria and arbuscular mycorrhizal fungi are known to enhance stress tolerance in a range of crop species by producing antioxidants, suppressing ethylene production, stabilizing the soil structure, increasing osmolyte production, and improving ABA regulation (Atkinson and Urwin 2012).

Drought stress resulted in a two-fold increase in endogenous ABA as well as a 50 % reduction in *B. cinerea* infection and a significant suppression of *Oidium neolycopersici* on tomato cv. Money maker. Although salt stress did not affect *B. cinerea* infection, it significantly reduced infection by *O. neolycopersici*, but with no obvious increase in endogenous ABA. Compared with the wild type, the ABA-deficient *sitiens* mutant was more resistant to *O. neolycopersici* and *B. cinerea*. Exogenous ABA resulted in increased susceptibility of the *sitiens* mutant to both pathogens, but did not increase the basal susceptibility of wild-type tomato plants (Achuo et al. 2006). It can be concluded that drought and salt stress stimulate

different, but possibly overlapping, pathogen-defence pathways in tomato, which may not necessarily involve ABA. Although, a high endogenous ABA level suppressed the resistance of tomato to *O. neolycopersici* and *B. cinerea*, exogenous application of ABA did not increase susceptibility to these pathogens.

In contrast, abiotic stress may also interact negatively with pathogen stress. An increase in temperature may lead to a negative interaction by lowering resistance to bacterial, viral, fungal and nematode pathogens: in wheat, higher mean temperatures over a 6 year experimental period correlated with heightened susceptibility to the fungus *Cochliobolus sativus* (Sharma et al. 2007). In tobacco and *Arabidopsis*, the hypersensitive response (HR)- and R-gene-mediated defence responses to *Pseudomonas syringae* and viral elicitors are compromised at high temperatures, allowing these pathogens to multiply (Wang et al. 2009a).

Certain gene products are crucial to both biotic and abiotic stress signalling, and may therefore control the specificity of the response to multiple stresses (Atkinson and Urwin 2012). Several transcription factors were found to be associated with the control of both biotic and abiotic stress responses. For example, MYC2 is a positive regulator of specifically JA-induced defence genes, but represses the genes induced by combined JA/ET signalling. It also acts as a key repressor of the SA pathway, and MYC2 has also been found to be activated by ABA. Therefore MYC2 may act as a central regulator by which ABA controls biotic stress signalling pathways (Pieterse et al. 2009). The partial synergy between ABA and JA signalling may explain how pathogenresistance can be enhanced by abiotic stress (Atkinson and Urwin 2012), as found in barley, where abiotic stress increased resistance to *Blumeria graminis,* and the resistance was in positive correlation with the salt concentrations applied (Wiese et al. 2004).

Mitogen-activated protein kinase (MAPK) cascades are crucial in eukaryotes for transducing the perception of environmental stimuli into internal signalling pathways. A recent publication reviewed a list of MAPKs involved in abiotic stresses signalling in various plant sources (Sinha et al. 2011). MAPK cascade-mediated signalling is also an essential step in the establishment of resistance to pathogens. The best-characterized MAPKs are MPK3, MPK4 and MPK6, all of which are activated by various abiotic stresses, pathogens and also by oxidative stress (Pitzschke et al. 2009). MPK4 and MPK6 were activated by cold, salt stress, osmotic stress, touch and wounding (Ichimura et al. 2000), while Cd treatment activated both MPK3 and MPK6 through the accumulation of ROS (Liu et al. 2010). *mpk4* mutants were shown to constitutively express SA-dependent stress genes, and this was found to be due to elevated levels of SA, as the dwarfed phenotype of these mutants could be rescued by the expression of the bacterial *nahG* gene (Petersen et al. 2000). MPK4 acts as a negative regulator of SA-mediated defence against biotrophic pathogens, while it is an essential component in the JA- and ET-mediated defence against necrotrophic pathogens (Brodersen et al. 2006).

Another response common to biotic and abiotic stress is ROS (Fujita et al. 2006). ROS accumulation increases during abiotic stress conditions such as

osmotic stress, salinity and strong light. The production of ROS damages plant cells, so their removal is essential to minimize damage, and plants are capable of producing antioxidants and ROS-scavenging enzymes for this purpose (Apel and Hirt 2004). In contrast, plants actively generate ROS following pathogen infection, in a process known as the oxidative burst to limit pathogen spread by contributing to the hypersensitive response and cell death, in this case requiring the coordinated down regulation of ROS-scavenging mechanisms (Torres 2010). However, it is also evident that ROS are important signals mediating the activation of defence genes (Levine et al. 1994). So, ROS have additional regulatory functions in defence mechanisms in conjunction with other plant signalling molecules, such as SA, ABA and JA (Robert-Seilaniantz et al. 2011). ROS and SA are part of a positive feedback loop (Torres et al. 2006) (see also Sect. 5).

Another interesting example of common pathways for the regulation of protective mechanisms against biotic and abiotic stresses was detected using the *edr1* (enhanced disease resistance 1) *Arabidopsis* mutant, which displays enhanced stress responses and spontaneous necrotic lesions under drought conditions in the absence of pathogens, suggesting that EDR1, which encodes a CTR-1 like kinase, is also involved in stress response signalling and cell death regulation. Double mutant analyses showed that the *edr1*-mediated growth inhibition and cell death phenotypes are also SA-dependent (Tang et al. 2005). An *Arabidopsis* mutant, designated *adr1* (activated disease resistance1), constitutively expressed SA-dependent defence genes and was resistant to a broad spectrum of virulent pathogens (Grant et al. 2003). ADR1 was found to encode a coiled-coil/nucleotide-binding site/leucine-rich repeat protein, which has homologous domains with serine/threonine protein kinases. It was also shown that both the constitutive and conditional enhanced expression of ADR1 conferred significant drought tolerance. This was not a general feature of defence-related mutants because *cir* (constitutive induced resistance)*1*, *cir2* and *cpr* (constitutive expressor of PR genes)*1*, which constitutively express systemic acquired resistance, failed to exhibit this phenotype. The increased drought tolerance of *adr1* was not indicative of cross-tolerance, because it did not show improved tolerance to other stress factors; in fact the plants showed increased sensitivity to heat and salinity stress. Furthermore, neither hemizygous nor homozygous *adr1* plants exhibited either increased tolerance or sensitivity to heavy metal stress. In a similar fashion, *adr1* plants failed to exhibit any significant tolerance to freezing (Chini et al. 2004). Similar observations were made when a P4-chitinase genomic sequence was isolated from a bean genomic library using a P4-ch cDNA. Various stress conditions, such as wounding, SA and NaCl treatments, heat and cold stress were applied to bean (*Phaseolus vulgaris* L.) plants. Whereas wounding, NaCl treatment and cold stress were ineffective, the transcription of P4-chitinase mRNA was induced by SA treatment and, surprisingly, in response to heat stress (Margispinheiro et al. 1994). Hence, *adr1*-activated signalling may antagonise some stress responses. The Northern analysis of abiotic marker genes revealed that the dehydration-responsive element DREB2A was expressed in adr1 plant lines, but not DREB1A, RD(response to dehydration) 29A
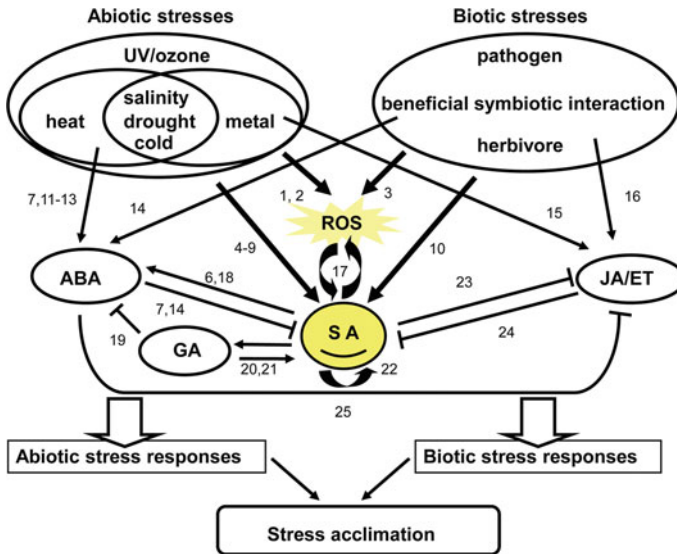
Fig. 2 Relationship between salicylic acid and other plant hormones under stress conditions. For details, see text. Numbers indicate references: 1. Smirnoff 1993; 2. Kocsy et al. 2011; 3. Torres 2010; 4. Horváth et al., 2007; 5. Pál et al. 2005; 6.Bandurska and Stroinski 2005; 7. Liu et al 2006; 8. Janda et al. 2007; 9. Ogawa et al. 2007; 10. Enyedi et al. 1992; 11. Aimar et al. 2011; 12. Zhang et al. 2006; 13. Veisz et al. 1996; 14. de Torres-Zabala et al. 2009; 15. Maksymiec 2007; 16. Fujita et al. 2006; 17. Vlot et al. 2009; 18. Szepesi et al. 2009; 19. Javid et al. 2011; 20. Lee and Park 2010; 21. Alonso-Ramírez et al. 2009; 22. Szalai et al. 2011; 23. Gupta et al. 2000; 24. Niki et al. 1998; 25. Pieterse et al. 2009.

or RD22. Furthermore, DREB2A expression was SA-dependent but NPR1-independent. In the double mutants *adr1*/ADR1 *NahG*, *adr1*/ADR1 *eds* (enhanced disease susceptibility)1 and *adr1*/ADR1 *abi1* (ABA insensitive), drought tolerance was significantly reduced. The microarray analysis of plants containing a conditional adr1 allele demonstrated that a significant number of the up-regulated genes were previously implicated in responses to dehydration (Chini et al. 2004).

These results clearly demonstrated that biotic and abiotic stress signalling cannot be separated into two distinct pathways. There is cross talk between these pathways at most levels bringing about a complex signalling network when the plants have to cope with various biotic and abiotic stresses, or a combination of these stresses. In conclusion, ABA, JA and SA appear to be the plant hormones most involved in the regulation of responses to biotic and abiotic stresses (Tuteja and Sopory 2008; Singh et al. 2011). Interactions between these plant hormones in relation to biotic and abiotic stresses are represented in Fig. 2, with SA in the central position.

5 Possible Salicylic Acid Action Mechanisms and Signalling

5.1 Action Mechanisms and Signalling at the Molecular Level

5.1.1 SA in Relation To Oxidative Stress

It is a fact that most stress factors, whether biotic or abiotic, are usually associated with the oxidative burst, leading to oxidative stress, a secondary phenomenon which is a common component of several types of stress. The generation of ROS causes rapid cell damage by triggering chain reactions. Cells have evolved a complex system of enzymatic and non-enzymatic antioxidants to scavenge these harmful molecules (Ahmad et al. 2010; Kocsy et al. 2011). In many cases increased tolerance can be attributed to the efficiency with which the plant is capable of neutralising ROS (Gill and Tuteja 2010). The rapidity of ROS production and the ability of H_2O_2 to freely diffuse across membranes suggested that at low concentration ROS may play a central role in the complex signalling network of cells under normal and stress conditions (Sharma et al. 2012). The level of ROS is controlled both by production and by removal through various scavengers. SA may influence both of these processes, as being involved in the fine-tuning of signalling pathways.

In most studies on the role of exogenous SA during abiotic stress, it was demonstrated that the protective effect of SA treatment was accompanied by transient oxidative stress, which induced the antioxidant defence system and the synthesis of certain stabilising substances (see Sect. 2). Thus, the application of SA can act as a hardening process, leading to a decrease in oxidative injury. Recent results also show that while NaCl induces cell death mainly by ET-induced ROS production, but ROS generated by SA was not controlled by ET in tomato cell suspension (Poór et al. 2012b).

As a stress acclimatisation process, SA treatment at a suitable concentration may decrease the catalase activity and in turn increase the level of H_2O_2 in several plant species (Dat et al. 1998a; Janda et al. 1999; Taşgin et al. 2006). Furthermore, SA has been shown to bind to the catalase enzyme, resulting in the inhibition of its activity (Chen et al. 1993; Conrath et al. 1995; Horváth et al. 2002). SA treatment decreased the catalase activity in wheat (Taşgín et al. 2006) and in tomato (Szepesi et al. 2005), but had no effect in *Brassica napus* L. (Haddadchi and Gerivani, 2009). The decrease in catalase activity in relation to an increase in SA content was also investigated in rice, wheat and cucumber seedlings exposed to oxidative stress (Shim et al. 2003). Although, SA initially decreased the catalase activity in bermudagrass, its inhibitory effect disappeared 12 days after the treatment (Zhang et al. 2009). In contrast, some authors reported on SA-induced increase in catalase activity, for example in wheat (Agarwal et al. 2005a,b), maize (Ahmad et al. 2012)

and soybean (Simaei et al. 2011). Contradictory results for the same species suggested that the biochemical effect of SA depends not only on the species, but on several other factors, such as the mode of application, the concentration, the environmental conditions, etc. Different SA concentrations had different effects on the enzyme activity in pepper (*Capsicum annuum* L.). Concentrations of 0.7, 1.5 and 3 mM SA decreased catalase activity, but concentrations of 6 and 9 mM increased it (Mahdavian et al. 2007). These contradictory results can be explained not so much by differences in the SA concentrations applied, but by differences in the binding of SA to various catalase isoenzymes in different plant species. In tobacco all the catalase isoenzymes are inhibited by SA (Durner and Klessig, 1996), while in maize and rice differences were found between the catalase iso-enzymes in their sensitivity to SA. A substantial level of non-competitive inhi-bition was caused by 2 mM SA in the activity of the CAT1 isoenzyme of maize, while in the case of CAT2 the inhibition was competitive and weak (Horváth et al. 2002). In rice SA inhibited the activity of the CATb isoenzyme, but not that of CATa (Chen et al. 1997). The CAT1 isoenzyme of maize and the CATb isoen-zyme of rice, both of which are sensitive to SA, exhibited considerable sequence homology with tobacco catalase, which is also inhibited to a great extent by SA. The tissue-specific expression of various catalase isoenzymes may lead to differ-ences in the effect of SA on the given tissue if catalase does indeed play a role in transmitting the effect of SA.

There is evidence that not only may SA cause a rise in the quantity of ROS in the cell, but ROS may also lead to the accumulation of SA (León et al. 1995, Enyedi 1999). This observation suggested the existence of a self-induced SA-H_2O_2 cycle, resulting in the accumulation of ROS and the death of the cell (Van Camp et al. 1998). As H_2O_2 treatment alone did not cause such a great extent of oxidative damage, and as dimethyl-thiourea treatment reduced the damaging effect of SA treatment (Rao et al. 1997; Luo et al. 2001) by reducing the H_2O_2 level, it could be assumed that the effect of SA is only mediated in part by H_2O_2. In fact, it is thought that SA might act as an electron donor, diverting catalase to the slower peroxidative pathway. At low levels of H_2O_2 this is manifested as inhibition, while at damaging levels of H_2O_2 it protects the enzyme (Durner and Klessig 1996). During SA binding to catalase, SA itself was also converted into a free radical leading to lipid peroxidation. Both the higher H_2O_2 level caused by catalase inhibition and the lipid peroxidation arising in the course of inhibition are thought to be involved in the signal transduction process leading to SA-dependent resis-tance (Anderson et al. 1998). SA also influences the activity of other antioxidant enzymes. In some cases SA stimulates the activity of the Cu- and Zn-SOD enzymes, which again may contribute to a rise in the H_2O_2 level (Krantev et al. 2008; Sahu and Sabat 2011).

Besides CAT, the H_2O_2 level is also regulated by G-POD, which was found to exhibit increased activity after SA application in various plant species (Ananieve et al. 2004; Taşgín et al. 2006; Mahdavian et al. 2007; Ahmad et al. 2012). Although the total activity did not increase substantially in maize treated with SA, a new peroxidase isoform was detected (Janda et al. 1999). It should also be

mentioned that the treatment of *Ficus carica* leaves with SA by submerging the leaves into SA solution did not cause any significant increase in the mRNA level of peroxidase (Kim et al. 2003a).

The enzymes involved in the ascorbate–glutathione cycle also play an important role in scavenging stress-induced ROS. These antioxidant enzymes showed increased activity in response to several abiotic and biotic stresses (Tuteja 2010; Bhattacharjee 2005). Furthermore, the overexpression of these enzymes improved tolerance to various stresses (Sharma et al. 2012). APX, which is part of this cycle, is thought to play the most important role in scavenging ROS, as it has higher affinity for H_2O_2 than catalase. Other components of the cycle are monodehydroascorbate reductase (MDHAR) and GR. MDHAR plays a role in the transformation of de-hydro-ascorbate (created when H_2O_2 is reduced to H_2O) to ascorbate. GR catalyses the reduction of GSSG to GSH in a NADPH-dependent reaction, thus maintaining the GSH pool. Increased APX (Agarwal et al. 2005a, b; Krantev et al. 2008) and GR activity (Agarwal et al. 2005a) was found after SA treatment in several plants. Although the APX and GR activity declined in pepper after treatment with less than 3 mM SA, application a concentration of 6 or 9 mM led to an increase (Mahdavian et al. 2007). Tobacco plants growing *in vitro* in the presence of 0.01 or 0.1 mM SA also showed increased glutathione reductase and dehydroascorbate reductase activity in the shoots, although there was no significant effect on APX. SA at 0.1 mM also increased the MDHAR activity (Dat et al. 2000). In another study it was found that the mRNA level of *BcMdhar* (encoding a polypeptide showing a high level of identity to cytosolic MDHAR) increased in response to the oxidative stress invoked by H_2O_2, SA, paraquat or ozone (Yoon et al. 2004).

GPXs have broad substrate specificities and are able to use H_2O_2 as a substrate. The expression of GPX genes showed a strong response to abiotic stress and was also affected by several plant hormones, including SA (Borsani et al. 2001; Milla et al. 2003). Interestingly, chloroplastic GPX depletion appears to causes compensatory changes in antioxidant levels in order to cope with the stress, as the accumulation of ascorbate, glutathione and SA could be detected in *Arabidopsis* (Chang et al. 2009).

Glutathione S-transferases (GST) form a large family of non-photosynthetic enzymes known to function in the detoxification of xenobiotics. Exposure to heavy metal and salt stress caused a substantial increase in GST activity (Halušková et al. 2009). The effect of SA is ambiguous in the case of the GST enzyme. The in vitro activity of the enzyme is inhibited non-competitively by SA (Watahiki et al. 1995), which however, stimulates its expression. In rice seedling roots the expression of the *osgstu4* and *osgstu3* genes, which are induced by various stresses (PEG, heavy metal, salt, H_2O_2), can also be triggered by plant hormones, such as ABA, JA, auxin and SA (Moons, 2003). Similarly, SA caused an increase in the expression of the *Gnt35* gene coding for GST in tobacco cells. Furthermore, the SA-responsive component, *as*-1 was identified in the promoter region of some GST genes, which is activated not only by SA but also by auxin and methyl JA via ROS (Garretón et al. 2002). In *Arabidopsis*, it was found that GSTs exhibit class-specific responses to SA treatment, suggesting that several mechanisms act to

induce GSTs upon SA treatment and hinting at class-specific functions for this gene family (Sappl et al. 2004).

Another pathway which may help to balance the production of ROS with the levels of antioxidants and ROS-scavenging enzyme systems is cyanide-resistant or alternative respiration chain, which has been mentioned in connection with thermogenesis and chilling tolerance (see Sect. 2). In tobacco the alternative respiration activity initially increased drastically during SA treatment after which it declined to a steady level. This early enhancement in response to SA was correlated with the expression of the AOX gene (*aox1*), as well as with the reduced, non-covalently linked state of AOX (Lei et al. 2008). Furthermore, during low temperature stress, the alternative pathway capacity in cucumber was enhanced as AOX expression increased in seedlings pre-treated with SA. These results indicated that the special protective role of the alternative pathway and AOX were related to SA-mediated plant resistance to environmental stresses (Lei et al. 2010).

5.1.2 Protein Kinases

The role of protein kinases, one of the most important components linking signal perception to the final cell response, in signal transduction pathways has been extensively studied. Mitogen-activated protein kinase (MAPK) cascades consist of a MAP kinase kinase kinase (MAPKKK) that phosphorylates a MAP kinase kinase (MAPKK), which in turn phosphorylates a MAP kinase (MAPK). MAPKs can phosphorylate a variety of substrates, including transcription factors, transcription regulators, splicing factors, receptors, histones and other protein kinases (Mishra et al. 2006). The outcome of MAPK activation depends on the duration of the activation. The length of time that a MAPK remains active depends on upstream specific regulation mechanisms, such as scaffolding, negative regulation by phosphatases and lipid signalling, which can initiate MAPK cascades (Opdenakker et al. 2012). In plants it has been reported that the two classes of stress-activated protein kinases, mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs), do not function independently, but that the concerted activation of both pathways controls the response specificity to biotic and abiotic stress (Ludwig et al. 2005; Li et al. 2008; Sinha et al. 2011).

A connection between SA and plant MAPKs was first reported when SA-induced protein kinase (SIPK) was purified and characterized in tobacco (Zhang and Klessig 1998). Later, besides SIPK, wound-induced protein kinase (WIPK) was also identified in tobacco, while the *Arabidopsis* orthologues AtMPK6 and AtMPK3, were shown to play an important role in responses to biotic stresses and abiotic stimuli, such as wounding, cold, drought, osmolarity, UV irradiation and ozone (Zhang and Klessig 1998; Jonak et al. 2002). The best-characterized MAPKs are MPK3, MPK4 and MPK6, all of which are activated by various abiotic stresses, pathogens and also by oxidative stress (Pitzschke et al. 2009). MPK4 and MPK6 were activated by cold, salt stress, osmotic stress, touch and wounding (Ichimura et al. 2000).

Two protein kinases with molecular masses of 48 and 40 kD are activated in tobacco cells exposed to NaCl (Hoyos and Zhang 2000). The 48-kD protein kinase was identified as SIPK. The activation of the 40-kD protein kinase is rapid and dose-dependent. Other osmolytes such as Pro and sorbitol activate these two kinases with similar kinetics. The activation of 40-kD protein kinase is specific for hyperosmotic stress, as hypotonic stress does not activate it. Therefore, this 40-kD kinase was named HOSAK (high osmotic stress-activated kinase). HOSAK is a Ca^{2+}-independent kinase. It has been also established that SIPK and 40-kD HOSAK are both components of a Ca^{2+}- and ABA-independent pathway that may lead to plant adaptation to hyperosmotic stress.

It was found that the UV-C-induced activation of SIPK was ROS-dependent, since free radical scavengers completely abolished the activation of MAPK by UV-C radiation in tobacco cell-suspension cultures (Miles et al. 2002), while the activation of AtMPK6, the *Arabidopsis* homologue of SIPK, required both ROS and Ca^{2+}-influx (Miles et al. 2004). Cd treatment activated both MPK3 and MPK6 through the accumulation of ROS (Liu et al. 2010).

As shown in tobacco plants, ozone may also induce the rapid activation of SIPK (Samuel and Ellisoverexpression lines 2002). Transgenic manipulation previously showed that the overexpression of SIPK leads to enhanced ozone-induced lesion formation with the concomitant accumulation of ROS. Ozone treatment strongly induced ethylene formation in sensitive SIPK-overexpressing plants at ozone concentrations that failed to elicit stress ethylene release in wild-type plants. By contrast, SIPK-overexpressing plants displayed no ozone-induced SA accumulation, whereas wild-type plants accumulated SA upon ozone exposure. The epistatic analysis of SIPK-overexpressing function suggests that the ozone-induced cell death observed in SIPK-overexpressing plants is either independent, or upstream, of SA accumulation (Samuel et al. 2005). The initial activation of AtMPK6 and AtMPK3 by ozone in *Arabidopsis* was also independent or upstream of SA, ethylene or JA signalling, since the activation pattern was similar in mutants deficient for the respective cascades. However, hormones are intertwined with MAPKs, since the activation of AtMPK6 was prolonged and AtMPK3 activation delayed in the ethylene-insensitive *etr1* mutant. Additionally, the basal expression level of AtMPK3 was only half the wild-type levels in SA-insensitive and -deficient accessions, and this lower level of expression was also reflected in AtMPK3 activity (Ahlfors et al. 2004). AtMPK6/SIPK and AtMPK3/WIPK are induced, among other stresses, by ozone, and are also activated by H_2O_2 and superoxide. Although both AtMPK6 and AtMPK3 are rapidly activated by ozone, the regulation of their activity by ozone is different. AtMPK3 was up-regulated by ozone at the transcriptional, translational and on post-translational levels, whereas only the post-translational activation of kinase activity was detected for AtMPK6. In addition, the activation of AtMPK3 lasted longer than that of AtMPK6. Plant ozone sensitivity and the expression of antioxidant genes is affected by these kinase classes, since both suppression and overexpression of the tobacco SIPK led to increased ozone sensitivity and changes in the expression of APX and GST (Kangasjärvi et al. 2005). In ozone-stressed transgenic plants of tobacco SIPK seems to regulate the activity of WIPK (the

AtMPK3 orthologue), since the WIPK activity induced by ozone was significantly reduced in SIPK overexpression lines, whereas the opposite was true for the SIPK suppression line (Samuel and Ellis 2002). Moreover, the suppression of OsSIPK (OsMAPK6) in rice was shown to increase the abundance of OsMAPK5a transcripts (an orthologue of NtWIPK) (Reyna and Yang 2006). These findings indicate that SIPK negatively regulates WIPK activation in response to ozone and other abiotic stresses. It is highly likely that such regulation is conserved in plants under diverse environmental conditions (Cho et al. 2009).

Over the past years, various MAPKs have been identified and characterized in several plant species. SIPK and its orthologue in other plants are of particular interest, as a large body of evidence demonstrated their involvement in the fine-tuned regulation of plant responses to ozone, wounding, SA and JA. Their function also appears to be conserved across reference plants, such as rice, tobacco and *Arabidopsis* (Cho et al. 2009). The first observation of the OsSIPK response to JA was the transcriptional upregulation of OsSIPK by JA treatment (Rakwal and Agrawal 2003). It should be noted that the JA-induced OsSIPK transcript was significantly stronger than that induced by wounding. So far, most MAPKs reported were found to be JA-responsive. It was also shown that a rice gene encoding an MAPK kinase kinase, OsEDR1, is constitutively expressed in seedling leaves and is up-regulated within a few minutes upon wounding, or treatment with JA, SA, ethylene, ABA or H_2O_2 (Kim et al. 2003b). Two other rice (*Oryza sativa* L.) MAPKs, OsMSRMK3 (multiple stress responsive) and OsWJUMK1 (wound- and JA-uninducible), which most likely exist as single copy genes in the genome, were also isolated (Agrawal et al. 2003). The steady state mRNA analysis of these MAPKs revealed that OsMSRMK3 was up-regulated by wounding, JA, SA, ethylene, ABA, H_2O_2, protein phosphatase inhibitors, chitosan, high salt/sugar and heavy metals, whereas OsWJUMK1 was not induced by either wounding, JA or SA, and only showed up-regulation as a result of H_2O_2, heavy metals and cold stress.

MPK4 also acts as a negative regulator of SA-mediated defence against biotrophic pathogens, while it is essential for the JA- and ET-mediated defence against necrotrophic pathogens (Brodersen et al. 2006).It has been shown that *mpk4* mutants constitutively express SA-dependent stress genes, and this was found to be due to elevated levels of SA, as the dwarfed phenotype of these mutants could be rescued by the expression of the bacterial *nahG* gene (Petersen et al. 2000).

MAPK signalling cascades were reported to be induced by cold/salt, drought, O_3-induced ROS, osmotic stress and wounding or ABA-induced H_2O_2 production. Besides abiotic-induced functions, a number of studies have also revealed the role of MAPK pathways in other processes, including stomatal patterning and auxin signalling (Colcombet and Hirt 2008).

Oxidative stress also causes Ca^{2+} influx into the cytoplasm from the extracellular environment and from the endoplasmic reticulum or sarcoplasmic reticulum (Ermak and Davies 2001). Ca^{2+} may control the activity of plant protein kinases throughindirect or direct interaction with the enzymes. Indirect interactions involve calmodulin, a calcium-binding protein. Six *Arabidopsis* genes designated as AtSR1-6, related to a tobacco early ethylene-responsive gene encoding a

Fig. 3 Complexity of salicylic acid signalling at the protein kinases level. For details, see text. Numbers indicate references: 1. Horváth et al. 2007; 2. Smirnoff 1993; 3. Torres 2010; 4. Li et al. 2008; 5. Vlot et al. 2009; 6. Ermak and Davies 2001; 7.Chung et al. 2004; 8. Pitzschke et al. 2009; 9. Ichimura et al. 2000; 10. Miles et al. 2002; 11. Miles et al. 2004; 12. Samuel et al. 2005; 13. Samuel and Ellis 2002; 14. Reyna and Yang 2006.
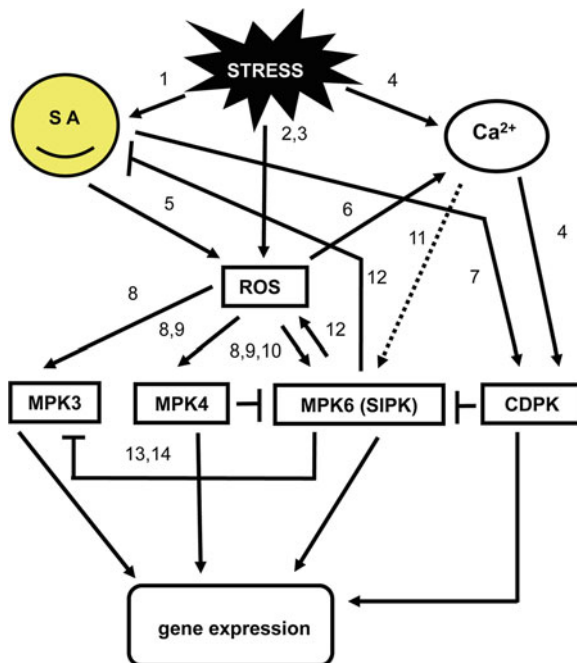
calmodulin-binding protein, were also shown to be rapidly and differentially induced by environmental signals such as temperature extremes, UV-B, salt and wounding, by hormones such as ethylene and ABA, and by signal molecules such as methyl JA, H_2O_2 and SA (Yang and Poovaiah 2002). Calcium-dependent protein kinases (CDPKs) sense the Ca^{2+} concentration changes in plant cells and play important roles in signalling pathways for disease resistance and various stress responses as indicated by emerging evidences (Li et al. 2008).The *Capsicum annuum* CaCDPK3 was rapidly induced in response to various osmotic stress factors, and CaCDPK3 RNA expression was also induced by an incompatible pathogen and by plant defence-related chemicals such as ethephon, SA and JA. It is assumed that CaCDPK3 is implicated in biotic and abiotic stresses in pepper plants (Chung et al. 2004). A cDNA clone (LeCRK1), encoding a novel isoform of calcium-dependent protein kinase (CDPK), was isolated by screening a tomato cDNA library (Leclercq et al. 2005). LeCRK1 transcript levels are low in unstressed leaves, but increase in response to wounding and cold treatment. Gene expression was slightly induced by ethylene, by spraying the leaves with a 4 mM solution of SA, and by mechanical wounding or cold treatment. The CDPK-mediated inhibition of SIPK and WIPK activation takes place via through ethylene. Negative communication between these distinct pathways may enable the plant to attenuate and switch off responses once the original triggering stimulus has been removed (Ludwig et al. 2005).

In Fig. 3 the complexity of salicylic acid signalling at the level of protein kinases was demonstrated.

5.2 Action Mechanisms at the Gene Expression Level

SA has long been known to induce both local resistance to TMV and the accumulation of pathogenesis-related (PR) proteins. Furthermore, SA treatment induces the expression of the same set of genes as TMV and the high-level expression of these genes correlates well with the onset of a resistant state (Ward et al. 1991). Several proteins, members of different classes of PRs, were identified,each having different functions.These included β-1,3-glucanase, chitinase, proteinase-inhibitor, endo-proteinase, peroxidase, thaumatin-like, ribonuclease-like, defensin, thionin and lipid-transfer protein (Van Loon and Van Strien 1999). The *Arabidopsis* PR-1 and tobacco PR-1a promoters, which are used as model systems to study SA-induced transcriptional regulation, each contain an activator sequence-1(*as*-1)-like element that is important for SA-inducible gene expression (Lebel et al. 1998; Strompen et al. 1998). Studies on the expression of PR genes also revealed the central role of protein non-expressor of PR genes1 (NPR1). NPR1 interacts with TGA transcription factors (a subgroup of the basic leucine zipper transcription factor family) that can bind to the *as*-1-like elements of PR-1 genes (Durrant and Dong 2004; Katagiri et al. 1989). The basic model of SA action is that SA accumulation stimulates the translocation of NPR1 into the nucleus, where it interacts with members of the TGA transcription factor family and enhances the binding of these factors to SA response elements in the promoters of PR genes, thus ultimately affecting the transcription of numerous genes in the SAR pathway. SA can modulate the redox state of TGA1 (reduction of its disulphide bridges) while NPR1 interacts specifically with the reduced form of TGA1 (Després et al. 2003; Echardt 2003; Kesarwani et al. 2007). Apart from the role of NPR in regulating SAR in the nucleus, the cytosolic function of NPR1 in cross-communication between SA- and JA-dependent defence signalling pathways has also been identified (Pieterse and Van Loon 2004). The existence of crosstalk between gibberellins and SA signalling in *Arabidopsis* seeds was suggested, because GA treatment or the overexpression of a GA-responsive gene were able to increase not only endogenous levels of SA, but also the expression of the ICS1 and NPR1genes (Alonso-Ramírez et al. 2009). Thus, NPR1 plays multiple roles in regulating SA-mediated defence, acting as a positive SA signal transducer and a positive and a negative regulator of SA accumulation (Wang et al. 2011). A negative feedback loop was demonstrated, which involves NPR1 and regulates SA accumulation, as SA accumulation after inoculattion with pathogen was higher in *npr1Arabidopsis* mutants, than in wild plants (Lu 2009). The expression of SID2 was also greater in pathogen-inoculated *npr1* plants than in wild plants (Wildermuth et al. 2001).

The expressions of OsBIERF1 to OsBIERF4 [*Oryza sativa* benzothiadiazole (BTH)-induced ethylene responsive transcriptional factor (ERF)] rice genes were analysed in response to rice diseases and under various abiotic stress conditions. It was found that OsBIERF3 was able to bind specifically to the GCC box sequence, while the expression of OsBIERF1, OsBIERF3 and OsBIERF4 was induced by treatment with BTH and salicylic acid (Cao et al. 2006).

Several TGA factors were reported to be essential activators of PR-1 expression (Zhang et al. 2003; Kesarwani et al. 2007; Blanco et al. 2009). In addition to TGA, the Myb protein has also been shown to bind to the PR-1a promoter in tobacco, where the Myb1 protein is preferentially bound to the MBSII sequence in the PR-1a promoter (Yang and Klessig 1996). The tomato Pti4 protein, which functions as a transcription factor, specifically binds the GCC-box cis element, which is present in the promoter region of many pathogenesis-related (PR) genes. The expression of the Pti4 gene in tomato leaves was rapidly induced by ethylene and by infection with *Pseudomonas syringae*pv.*tomato*, and this induction preceded the expression of GCC-box-containing PR genes. Although SA also induced Pti4 gene expression, it did not induce GCC-box PR genes. In fact, SA antagonized the ethylene-mediated expression of GCC-box PR genes (Gu et al. 2000).

The expression of the WRKY genes was strongly induced by TMV infection (Ülker and Somssich 2004). Like that of PR-1a, the expression of the NtWRKY12 gene was strongly induced by SA treatment. Accumulating evidence indicate that WRKY proteins are involved in differential responses to biotic stresses, either as transcriptional activators or as repressors, and WRKY proteins bind to the W box (TTGAC[C/T]) in promoters of various pathogen-responsive genes.This is in close proximity to binding sites in the PR-1a promoter for transcription factors TGA1a (as-1 box) and Myb1 (MBSII box) (van Verk et al. 2008). The results indicated that NtWRKY12 acts synergistically with TGA1a in the SA-mediated and pathogen-associated molecular pattern (PAMP)-mediated expression of the PR-1a gene. It was also demonstrated that signal transduction via the MAPK cascade MEKK1–MKK4/MKK5–MPK3/MPK6 leads to the activation of downstream WRKY22 and WRKY29 (Asai et al. 2002), while another MAPK cascade (MEKK1–MEK1/MKK2–MAPK4), induced by challenge inoculation with *Ps. syringae* or treatment with flg22 (a peptide corresponding to the most conserved domain of flagellin), leads to the phosphorylation of MAPK substrate 1 (MKS1), through which WRKY33 and possibly WRKY25 are bound to MAPK4. Upon phosphorylation of MKS1, WRKY33 is released in the nucleus to initiate the positive regulation of JA-induced defence genes and the negative regulation of SA-related defence genes (Pandey and Somssich 2009).

Multiple forms of cytochrome P450-dependent monooxygenases catalyse the in-chain hydroxylation, end-terminal hydroxylation and epoxidation of medium- and long-chain fatty acids. In plants, fatty acid hydroxylases are particularly important in the synthesis of plant cuticles and signalling molecules derived from fatty acids. Some members of the *Arabidopsis thaliana* CYP86A and CYP94B cytochrome P450 monooxygenase subfamilies have been functionally defined as being fatty acid omega-hydroxylases. Due to this activity, these and other fatty acid hydroxylases have a potential role in the synthesis of cutin, the production of signalling molecules, and the prevention of the accumulation of toxic levels of free fatty acids. The constitutive and stress-inducible patterns of the five *Arabidopsis* CYP86A subfamily members have now been defined (Duan and Schuler 2005). The differences observed in inducible expression suggest that, in addition to their roles in normal growth and development, each of these P450 s has a particular role

in stress responses, although the results also suggest that the activation of these CYP86A genes does not involve SA- or MeJA-dependent pathways (Duan and Schuler 2005). The expression patterns of cytochrome P450 genes were also analysed using a full-length cDNA microarray after various treatments, such as SA, pathogen inoculation, paraquat, UV-C, heavy metal stress, wounding, drought, high salinity and low temperature. The expression of several cytochrome P450 genes was induced by biotic stress,while that of some genes was also induced by abiotic stress, suggesting crosstalk between abiotic and biotic stresses. The promoter sequences and cis-acting elements of each gene were studied on the basis of full-length cDNA sequences. Most cytochrome P450 genes induced by both abiotic and biotic stresses contained recognition sites fortranscription factors MYB, MYC, ACGT-core sequence, TGA-box and W-box for WRKY in their promoters (Narusaka et al. 2004).

The expression of the *Arabidopsis* phospholipase A IIA (AtPLA IIA) gene, which is a member of the patatin-related PLA gene family, was induced by various treatments such as pathogen inoculation, cold, high salinity, ABA, SA, methyl JA, ethephon, paraquat, rose bengal, UV-C and $CuSO_4$ (Narusaka et al. 2003). The sequences of putative cis-acting elements were found in the promoter region of the AtPLAIIA gene. This gene has two ACGT-sequences and a TGA-box in the promoter, which are known to be the core sequences of activation sequence-1(Lam et al. 1989), which acts as an oxidativestress-responsive element. Therefore, these sequences may function as cis-acting elements in ROS-responsiv epromoters. The AtPLA IIA gene was also induced by both osmotic stress and ABA treatment. Although the signalling factors involved in the induction of AtPLAIIA are unclear, it may play an important role in plant defence responses. The expression of lipoxygenase genes is also known to increase in response to abiotic stress. Chloroplastic lipoxygenase, CPRD46, a single copy gene isolated from dehydrated cowpea (*Vigna unguiculata*) plants, was also shown to be induced by high-salinity stress and exogenous ABA, heat stress, methyl JA and SA, but not by cold stress (Iuchi et al. 1996). It was also found that isocitrate lyase, a key enzyme involved in lipid metabolism during seed germination, is induced by SA in the seeds of *Arabidopsis* (Rajjou et al. 2006).

In a recent study SA-induced genes were identified from the SA-treated leaves of *Mitragyna speciosa* using suppression subtractive hybridization and semi-quantitative RT-PCR. The results showed that the cDNA clones obtained represented stress related genes upregulated in response to SA treatment; furthermore, most genes responding to acute SA treatment were related to stress and signalling pathways which eventually led to cell death. These included genes encoding chaperone, heat shock proteins (HSPs), antioxidants and genes involved in secondary metabolite biosynthesis, such as sinapyl alcohol dehydrogenase, cinnamyl alcohol dehydrogenase and cytochrome P450. Overexpression of othergenes, those responsible for the MAPK, sarcosine oxidase (involved in the glycine, serine and threonine metabolism), transglutaminase (involved in many protein crosslinking reactions) and glycolate oxidase were also found in SA-treated plants (Jumali et al. 2011). The SA-induced synthesis of HSPs in tomato was also shown in an earlier

study, and it was found that SA was able to induce Hsp70/Hsc70 expression at higher, cytotoxic concentrations (1 mM) (Cronje and Bornman 1999).

The same technique was used to determine the transcript profile of tomato plants (*Lycopersicon esculentum* Mill.) after the foliar application of SA. In the SA library, 39 clones encoded proteins with no similarity, 46 were similar to putative proteins with unknown roles, 34 represented genes involved in biotic and abiotic defence mechanisms and 24 were associated with cell maintenance and plant development. Among the genes with known roles, those encoding phenylalanine ammonia lyase (PAL), chitinase, late embryogenesis abundant protein, bZip transcription factor, zinc finger domain protein, and I-2 disease resistance protein with leucine-rich repeat, are related to defence mechanisms, while those encoding cytochrome, ubiquitin, photosystem II apoprotein, manganese SOD and ribosomal 60S protein are related to cell metabolism (Amaral et al. 2008). SA also induced the expression of PAL gene in grape berry (Wen et al. 2008). The overexpression of genes with a role in cell maintenance and plant development indicated the activation of primary metabolism pathways, together with the induction of several signalling pathways leading to the expression of defence-related genes.

Analysis of the early genetic responses to SA in wild type and *npr1-1* mutant *Arabidopsis* seedlings, using a Complete Arabidopsis Transcriptome MicroArray (CATMAv2) chip, revealed that 217 genes were rapidly induced by SA (early SAIGs). These genes can be divided into two groups based on the activation pathway: NPR1-dependent (193) and NPR1-independent (24). These two groups of genes also differed in their functional classification, expression profiles and over-representation of *cis*-elements, supporting differential pathways for their activation. Examination of the expression patterns of these genes indicated that their activation by SA required the TGA2/5/6 subclass of transcription factors (Blanco et al. 2009).

Two rubber particle protein genes and one latex gene were isolated from fig trees (*Ficus carica*) and their expression was investigated after various abiotic stress and hormone treatments. Two major proteins closely associated with catalytically active rubber particles were identified, namely peroxidase (POD) and trypsin inhibitor (TRI). A cDNA encoding a basic class I chitinase (CHI) was also isolated from the fig tree latex. Wounding treatment strongly induced the expression of the three stress-related genes. Among the abiotic stresses drought treatment greatly induced the expression of POD, whereas the expression of CHI and TRI decreased after the same treatment. Cold treatment slightly reduced the transcript levels of the three genes, and NaCl marginally reduced the expression of CHI. The expression of POX, CHI and TRI was induced by jasmonic acid or abscisic acid. These differences in the expression of the stress-related genes following various abiotic stress or plant hormone treatments suggest the existence of crosstalk between the signal transduction pathways elicited by abiotic stresses and hormones in plants (Kim et al. 2003a). The induction of tcI 7, a gene encoding an L-subunit of proteasome, which has a function in protein degradation and is also required for the activation of proteins via the processing of inactive precursors, was found in tobacco plants treated with SA (Etienne et al. 2000)

In investigations on cold stress responses, 18 maize cold-induced (ZmCOI) genes were characterized, the majority of which share similarities with proteins with known function in signal transduction and photosynthesis regulation. RT–PCR was performed on ZmCOI6.1, ZmACA1, ZmDREB2A and ZmERF3, and their expression was found to be strongly induced by low temperature stress and other abiotic stresses such as drought and high salt concentration, but also by stress signalling molecules such as JA, SA and ABA. These results suggest that this group of genes is involved in a general response to abiotic stresses (Nguyen et al. 2009).

The transcript of a vacuolar ATPase C gene was found to be upregulated by SA in *Pennisetum* and its promoter was found to bind nuclear factors to TGA cis elements (Tyagi et al. 2005).

Receptor-like protein kinases (RLKs) are encoded by a divergent multigene family and their functions have been implicated in a wide range of signal transduction pathways. Examinations on the effect of SA on the expression of RLK genes in *Arabidopsis* revealed that transcripts of RKC1 and a number of its homologue, whose translation products contain C- $\times$ 8-C-X2-C motifs in the putative extracellular domain, accumulated to a higher level in response to plants treated with SA (Ohtake et al. 2000).

The expression of the BnBDC1 gene, which was isolated from oilseed rape (*Brassica napus*) following drought treatment, is upregulated by mannitol, NaCl and ABA, and downregulated by UV irradiation and SA. The expression level of BnBDC1 was significantly reduced after SA treatment and the effect was prolonged beyond 2 days, indicating that BnBDC1 is one of the target genes in the SA signalling pathway. Although the precise function of BnBDC1 is unknown, it is probably involved in multiple cell signalling pathways, and may play an important role in response to osmotic stress and plant pathogen infection in *Brassica napus* (Shunwu et al. 2004).

SA also induced the OsGGT gene, which was cloned from submerged plants of a submergence-tolerant rice cultivar (Qi et al. 2005), and its deduced amino acid sequence was homologous with that of glycogenin glucosyl transferase. The expression of this gene increased during submergence in the submergence-tolerant rice cultivar, but decreased in a submergence-intolerant cultivar. The accumulation of OsGGT mRNA also increased in response to ethylene, gibberellin, ABA, drought and salt treatment, but methyl JA treatment and cold stress had no effect. These results suggest that the OsGGT gene could be related to submergence stress and associated with a general defensive response to various environmental stresses.

The SAR8.2 genes of pepper (*Capsicum annuum* L.), designated CASAR82A, B and C, which are induced by all biotic and abiotic stresses, are not constitutively expressed in any of the organs of healthy pepper plants. Besides induction by pathogens, strong induction of the CASAR82A gene was found in pepper leaves treated with ethylene, methyl JA, indole-3-acetic acid, ABA, SA, benzothiadiazole, DL-beta-n-amino butyric acid or H_2O_2. Interestingly, the transcription of the CASAR82A gene was rapidly triggered by high salinity, drought or low-temperature stress, but not by mechanical wounding (Lee and Hwang 2003). Not only did CASAR82A overexpression enhance resistance to infection by various

pathogen species, such as *Ps. syringae*, *Fusarium oxysporum* or *Botrytis cinerea*, but the transgenic plants also exhibited increased salt, drought, or methyl viologen-induced oxidative stress tolerance in *Arabidopsis* plants (Lee and Hwang 2006).

In *Arabidopsis*, the cytosolic, patatin-related phospholipase A enzymes (PLA) comprise a family of ten genes, designated AtPLAs and thought to be involved in auxin and pathogen signalling (Holk et al. 2002). Their synthesis is up-regulated after treatment with SA or its analogue benzo[1,2,3]thiadiazole-7-carbothionic acid-S-methylester (Bion), and by wounding, ACC and JA. The properties of thismember of the patatin-related phospholipase A gene family suggest that it is a defence, iron-stress and phosphate-stress gene, being transcriptionally up-regulated within hours or days (Rietz et al. 2004).

In addition to its osmoprotectant properties, mannitol, one of the best known sugar alcohols, is an antioxidant and may have a significant role in plant-pathogen interactions. The catabolic enzyme mannitol dehydrogenase is a prime modulator of mannitol accumulation in plants. Up-regulation by SA and down-regulation by salt, osmotic stress and ABA was detected for its *mtd* gene in *Arabidopsis* plants (Zamski et al. 2001). A cDNA clone encoding another osmoprotectant osmotin in *Petunia hybrida* (PhOSM), was induced in response to octadecanoid pathway intermediates and treatment with aspirin or SA. These results indicate that PhOSM is developmentally regulated as well as being involved in stress signal transduction (Kim et al. 2002).

Investigations on the expression of the SbPRP gene, encoding a soybean pro-line-rich protein (PRP) showed that its mRNA was also expressed in response to SA and virus infection (He et al. 2002). In addition, SbPRP gene transcription was regulated by the circadian rhythm, salt stress, drought stress and plant hormones. These results indicate that the SbPRP gene might play a role in plant responses to multiple internal and external factors. The expression of another PRP, encoded by the PvSR1 (*Phaseolus vulgaris* stress-related protein) gene, was greatly enhanced in leaf tissue not only by alfalfa mosaic virus infection, wounding, heat shock, UV, drought and salt stress, but also by SA and H_2O_2. The precise biological role of PvSR1 is still unknown, but the expression of PvSR1 genes in various forms of stress suggests that PvSR1 may play an important role in maintaining cellular integrity during the stress, by forming strong linkages with the cell wall (Chai and Zhang 1999).

The effects of exogenous SA on the protein expression level in the leavesof *Baphicacanthus cusia* (Nees) Bremek were investigated using the two-dimensional electrophoresis method. A total of 20 significantly different protein spots were obtained, among which eight were identified including ATP synthase, alpha tubulin, cell division protein, glyceraldehydephosphate dehydrogenase and ACC oxidase, respectively. The expression abundance of all the proteins identified was up-regulated, except for that of ACC oxidase, which was down-regulated.

The above results clearly demonstrate that SA signalling leads to the repro-gramming of gene expression and protein synthesis. The SA-inducible proteins identified to date can be divided into the following functional groups; (a) proteins of the signalling systems, such as CAT, SOD, MAPK, transcription factors and

receptor-like kinase, (b) PR-proteins, heat shock proteins and proteins with a direct anti-pathogenic effect, such as chitinases and β-1,3-glucanase, (c) antioxidant enzymes,enzymes neutralizing toxic compounds and ozmoprotectants, (d) the enzymes of respiration and photosynthesis, such as AOX, isocitrate lyase and ATP-synthase, (e) proteolysis-regulating proteins, such as proteasome, (f) cyto-skeleton proteins, like tubulin and plant cell wall proteins, such as PRP, and (g) complexing proteins, such as glycine-enriched RNA-binding protein (Tarchevsky et al. 2010).

6 Conclusions

Based on the above, SA could be useful as a protective compound improving the abiotic stress tolerance of plants. Exogenous SA treatment at a suitable concen-tration is able to protect plants against stress-induced injury; however, the treat-ment itself may also cause stress to the plants. Furthermore, SA treatment may also alter the endogenous SA metabolism, either by inducing *de novo* synthesis or by disturbing it. The effect of SA depends on several factors, such as the mode of application, the concentration, environmental conditions, species and organ, etc. Although a number of questions remain unanswered, it can be seen that SA exerts an effect at several levels. Both endogenous and exogenous SA have a local, direct effect at molecular level, for example in the control of ROS production. SA is also involved in general stress responses, in a complex relationship with other plant hormones, leading to the regulation of gene expression.

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Chapter 11
Signaling Role of Salicylic Acid in Abiotic Stress Responses in Plants

Tomonori Kawano, Takuya Hiramatsu and François Bouteau

Abstract It is well known that salicylic acid (SA) is a natural signaling molecule involved in plant defense response against pathogen infection. In addition to plant responses to biological enermies, evidence on the involvement of SA in the plant abiotic responses has been recently provided. This chapter covers the recent progress in our understanding of the SA signaling pathways and mechanisms by which SA performs its role as the mediator of stress responses. In the upper half, history and progress in reactive oxygen species (ROS) and calcium signaling-related researches are covered, as both ROS and calcium are now considered to act downstream of SA action during both the plant defense against pathogenic microbes and cellular response to various abiotic stimuli. In the lower half of the chapter, plant cell responses to abiotic stresses in the surrounding environment including exposures of plant cells to photochemical oxidants chiefly ozone, radiation by ultraviolet light, and toxic metal ions such as ions of copper and aluminum are discussed.

Keywords Abiotic stress · Aluminum toxicity · Calcium · Reactive oxygen species · Ozone · Salicylic acid · Signal transduction

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1 Introduction

Living plants must respond to and combat a variety of stressful stimuli in the surrounding environments, which are often threatening the life of plants. Therefore, plants are naturally equipped with the systems for sensing of, adaptation to, and tolerance against both the biotic and abiotic stresses. Recent studies have elucidated that plant responses to different stresses are highly complex and involve the changes at transcriptome, cellular, and physiological levels (Atkinson and Urwin 2012).

As biotic factors threatening the plants include animal and insect herbivores and a wide range of pathogenic microbes such as viruses, bacteria, and fungi (Kerchev et al. 2012; Kangasjärvi et al. 2012). On the other hand, abiotic stimuli threatening the plants include physical stimuli such as transient and/or chronic exposures to lights (Yokawa et al. 2011a, b), water stresses (Jiang and Zhang 2002), mechanical challenges (Mori et al. 2004; Sukharev et al. 1993), high (Lin et al. 2006) and low (Lin et al. 2005, 2007) temperatures; and chemical stimuli such as exposure to toxic metal ions in the soils (Kagenishi et al. 2009) and atmospheric oxidants in both the natural and industry-affected environments (Yukihiro et al. 2012; Kadono et al. 2010).

Salicylic acid (SA) is one of the key factors determining the fate of plants exposed to such stressful conditions (Vlot et al. 2009). SA is a signaling molecule naturally found in plants and shown to be involved in the plant defense-related actions against infection by various pathogens. The name of SA and related compounds originally came from the *Salix helix* (willow) tree, since they were discovered as the major components in the extracts from the tree barks of willow and poplar, which had been used as natural anti-inflammatory drugs over centuries until 18th century (Rainsford 1984; Weissman 1991).

Acetylsalicylic acid which is widely known as aspirin is the world first synthetic drug that had been produced in 1897 by Bayer Company as an anti-inflammatory agent by mimicking the actions of the ancient medicine from the willow tree (Weissman 1991). Studies relating salicylates with plant disease resistance was initiated in 1970s when the application of aspirin against a plant virus in growing leaves was shown to be effective (White 1979). It is interesting that whereas aspirin, an active analog of SA, acts as an anti-inflammatory agent in animal system, SA in plants shows the heat-generating activity by targeting the mitochondrial alternative oxidase in some plant species (Voodoo lilies) and the heat generation reportedly results in induction of flowering (Rhoads and McIntosh 1992). Thus SA occasionally behaves as a flowering hormone in specific plant species. Among the diversified actions of SA in plants, the most important role for SA may be being an endogenous inducer of plant defense mechanism against the attacks by pathogens as discussed below.

The first reports focusing on the role of salicylates as disease resistance-inducing chemicals has described that injection of aspirin into tobacco leaves enhanced the resistance to subsequent infection by tobacco mosaic virus (White 1979; Antoniw and White 1980). Later studies have shown that aspirin and SA

induces the accumulation of pathogenesis-related (PR) proteins in plants (Kessmann and Ryals 1993; Malamy et al. 1990; Métraux et al. 1990).

2 Roles of Reactive Oxygen Species (ROS) and Calcium in Plant Defense Biology

2.1 ROS as Signaling Chemicals

The production of ROS members such as superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$) at the cell surface well known as the "oxidative burst" is one of the earliest events detectable during the incompatible interactions between plants and pathogens. As recently reviewed (Yoshioka et al. 2008), Doke, a plant pathologist in Nagoya University (Nagoya, Japan) made the first report on the involvement of ROS in the plant-pathogen interaction ca. 30 years ago, in which he stated that the infection by *Phytophthora infestans* (late blight pathogen of potato) in potato tubers causes the generation of $O_2^{\bullet-}$ at the host cells' plasma membrane (PM), only in the incompatible interactions (Doke 1983a). A series of his works demonstrated that the members of ROS possibly function as the chemical signals required for induction of hypersensitive response (HR) as typified by host cell death; now often referred to as plant apoptosis (Coll et al. 2011; De Pinto et al. 2012). Among Russian biologists, there is a popular view that plant apoptosis can be a model for the concept of genome-programmed cell death referred to as phenoptosis which is widespread in the kingdoms of bacteria, protozoa, fungus, plants and animals (Skulachev et al. 2012).

Apart from photosynthetic or photochemical reactions, above works were the first examples on the ROS generating activity in plants, which is specifically responsive to the attacks by pathogenic microorganisms. Doke further reported that the treatment of potato tuber protoplasts with the cell wall preparation from *P. infestans* readily induces the ROS production, suggesting that chemical components derived from pathogenic microorganisms (elicitors) trigger the burst of ROS production in order to stimulate the plant defense mechanisms (Doke 1983b).

In 1985, Doke and his coworkers have discovered that the membrane fractions isolated from the potato tubers inoculated with *P. infestans* produce the $O_2^{\bullet-}$ in an NADPH-dependent manner, and thus suggested that the enzyme for the ROS production is the NADPH oxidase, closely resembling those known to be operated in the activated neutrophils (Doke 1985; Doke and Chai 1985; Doke and Miura 1995). Plant NADPH oxidases, also known as respiratory burst oxidase homologues (RBOHs), are now considered as the most thoroughly studied enzymatic ROS-generating systems and thus serving as important molecular 'hubs' during ROS-mediated signaling in plants (Marino et al. 2012). Our understanding of the diversified roles for the ROS producers chiefly RBOHs in various plant processes covering the cell growth, plant development and plant response to abiotic

environmental constraints and biotic interactions, both pathogenic and symbiotic, has increased considerably in recent years (Marino et al. 2012).

Early reports from the Doke's team also provided temporal profile of oxidative burst after inoculation with an avirulent isolate of *P. infestans* onto potato leaves, which was biphasic oxidative bursts, consisted of earlier phase peaking at initial 3 h followed by a massive continuous phase of ROS production (Chai and Doke 1987a). In the same year, the data correlating the systemic induction of resistance to *P. infestans* and the systemic regulation of ROS, *i.e.* activation of both the pro-oxidative ($O_2^{\bullet-}$ generating reaction) and anti-oxidative mechanisms involving superoxide dismutase (SOD) and peroxidases (POXs) in potato plants were reported (Chai and Doke 1987b, c). The oxidative burst induced by pathogen-derived signals may play a key role in development of systemic immunity known as systemic acquired resistance (SAR) by activation of defense responses throughout the plants (Fobert and Després 2005). To date, multiple roles of ROS have been proposed in direct microbicidal actions, strengthening of cell wall through oxidative cross-linking of glycoproteins, induction of intracellular signaling pathway such as the synthesis of SA and activation of mitogen-activated protein kinase (MAPK) cascade, or activation of SAR associated with systemic propagation of the oxidative burst (Yoshioka et al. 2008; Swanson et al. 2011).

Nowadays, a number of teams working on plant ROS biology are distributed worldwide and their studies concern numerous aspects of the plant physiology throughout the plants' life cycle (Yoshioka et al. 2008). ROS production is actually recognized as common denominator not only to biotic stress but also abiotic environmental stressful conditions such as high salinity, drought, high intensity light and low or high temperature stresses that cause major crop losses worldwide. ROS are in fact, inevitably produced as by-products from a consequence of normal metabolic reactions including mitochondrial respiration, photosynthetic processes and fatty acid metabolism (Møller 2001; Baker et al. 2006; Noctor et al. 2007). A common property of all ROS types is that they can cause oxidative damage to cellular components such as proteins, DNA, and membranes (Møller et al. 2007). However, they have the potential to be beneficial to living organisms in addition to their harmful action, depending on the conditions (Apel and Hirt 2004). The specificity of the biological response of living plant cells to ROS depends on the chemical identity of ROS, intensity of the signal, sites of production and developmental stages (Del Río et al. 2002).

Exposures to environmental stresses increase intra- and intercellular levels of H_2O_2 by modulating the finely elaborated ROS-detoxification and regeneration networks, composed of ROS-producing enzymes, antioxidant enzymes, and biosynthetic pathways for low molecular antioxidants, all responsible for maintaining the homeostasis of ROS levels under tight control (Yoshioka et al. 2008; Kawano 2003; Del Río et al. 2002; Kotchoni and Gachomo 2006; Bolwell et al. 2002). This allows ROS to serve as signaling molecules in regulation of plant metabolism and cellular signal transduction pathways activated in response to environmental stresses (Gechev et al. 2006; Mittler et al. 2011). Accumulated pieces of evidence suggested that hormonal signaling pathways leading to development of SAR are

regulated through ROS production as observed for SA, abscisic acid (ABA), jasmonic acid (JA) and ethylene (ET) (Gaupels et al. 2011). Such ROS-mediated hormonal regulations play key roles in the crosstalk between biotic and abiotic stress signaling (Kawano 2003; Ströher and Dietz 2006; Mori and Schroeder 2004). Although many components of the ROS signaling networks have recently been identified, the mechanisms for orchestrated controls of the diversified ROS production mechanisms at different cellular sites through fine tuning of ROS feedback control to meet the physiological requirements such as plant growth, development, stress adaptation and programmed cell death (PCD) are now actively studied (Coll et al. 2011).

2.2 Calcium Signaling Events Downstream and Upstream of ROS

Land plants often show a variety of plasticity and behavior to adopt and conceive the environmental stresses such as drought, salinity and attacks by pathogens or insects. When plant cells are exposed to environmental stresses or perceive signaling molecules involved in growth and development, ion channels are transiently activated to convert these stimuli into intracellular events (Furuichi et al. 2007). When ion channels are activated by given stimuli, transient changes in cytosolic concentration of the specific ion(s) and membrane potential are induced by rapid activation of ion channels coupled to receptors, or themselves behaving as specific receptors. Since the activities of some sort of the channels are known to be regulated by the membrane potential, the initial activation of ion channels may further cause the sequential activation of other channels to relay and enhance the signals. As above, the regulation of ion channel is inevitable for the signal transductions and determination of plant behaviors at the end.

Among the ions taken up by the plant cells, calcium ion (Ca^{2+}) plays an essential role as an intracellular secondary messenger in plants and thereby the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is strictly regulated (Muto 1993; Knight et al. 1991; Sanders et al. 2002). In the steady state, $[Ca^{2+}]_c$ is constantly maintained to be at low levels by homeostatic mechanisms involving continuous export of Ca^{2+} by Ca^{2+}-ATPase and H^+/Ca^{2+} antiporter. When Ca^{2+}-permeable channels are activated in response to a variety of biotic and abiotic stimuli, the induced pulse of channel-mediated Ca^{2+} influx causes a drastic increase in the $[Ca^{2+}]_c$.

Since Muto and Miyachi (1977) discovered the presence of calmodulin (CaM) from pea seedlings, the first calcium-binding protein found in plants, a growing number of CaM-related proteins have been identified in plants. Recent studies have confirmed that CaM members are likely involved in SA-mediated plant defense signaling (Du et al. 2009). After the action of calcium channels, Ca^{2+} entered into the cells may bind and interact with Ca^{2+}-regulated signaling proteins such as CaMs and calcium-dependent protein kinases (CDPKs) which constitute a

large multigene family in various plant species, by modifying their activities or affinities to bind the specific sets of downstream targets (Shang et al. 2005; Asano et al. 2012). Recently, functional analysis using gain-of-function and loss-of-function mutants has revealed that several CDPKs are essential factors in abiotic stress tolerance, positively or negatively regulating stress tolerance by lowering the accumulation of ROS (Asano et al. 2012).

Above signaling events mediated by transient or spontaneous changes in the $[Ca^{2+}]_c$, termed Ca^{2+} signaling, are mostly initiated by the activation of Ca^{2+}-permeable channels, and a large efforts in the electrophysiological studies elucidated that plants have several Ca^{2+} channels belonging to distinct types differing in their gating mechanisms, namely ligand-gated, voltage-controlled and stretch-activated (mechanosensitive) channels, and many corresponding genes have been isolated (Piñeros and Tester 1997; Furuichi et al. 2007). In the last decade, a variety of plant genes encoding Ca^{2+}-permeable channels have been identified by the efforts of genome sequencing projects for certain plants and some of them are functionally characterized. They mostly differ from the animal types of channels, and many members of these plant channels permeate not only Ca^{2+}, but also K^+, Na^+ and other cations. However, it has been revealed that the regulation of expression levels of some such genes markedly affect the Ca^{2+} homeostasis in plants, and thus in case of some plant species studied, so-called Ca^{2+}-permeable channels surely play important roles in Ca^{2+}-signaling (Furuichi et al. 2007).

2.3 Early Signaling Models for SA: Oxidative and Calcium Signaling Events

In early 90's, involvement of ROS (Chen et al. 1993a, b) and intracellular calcium ion (Raz and Fluhr 1992; Schneider-Müller et al. 1994) during SA actions leading to activation of SAR have been proposed based on the model enzyme demonstrations and calcium depletion model experiments, respectively. By that time, a number of studies have indicated that SA is an oxidative signal inducer which is essentially involved in development of SAR against various pathogens with various natures. However, knowledge on the oxidative SA signal transduction mechanism was largely lacked. It has been proposed that SA signal transduction leading to SAR may be mediated by ROS derived from H_2O_2, since SA binds and inhibits catalase (CAT) in vitro (Chen et al. 1993b). While the CAT inhibition model mearly explains a passive mechanism supporting the increases in ROS, models with active mechanisms involving both POX (Kawano et al. 1998; Kawano and Muto 2000) and RBOHs (Yoshioka et al. 2001, 2003) have been reported.

In addition to ROS, Ca^{2+} is another possible mediator of SA signals as discussed above, and certain number of reports indicated that Ca^{2+} is essential for the action of SA during plant defense. For instance, removal of Ca^{2+} or blockade of Ca^{2+} uptake inhibits the induction of SA-inducible chitinase accumulation in

tobacco cells and leaves (Raz and Fluhr 1992), and carrot cell culture (Schneider-Müller et al. 1994).

The first report connecting ROS and Ca^{2+} in plant SA signaling (Kawano et al. 1998) reported the direct measurements of the SA-induced $O_2^{\bullet-}$ and the SA-induced increase in $[Ca^{2+}]_c$ in aequorin-expressing tobacco BY-2 cells. Addition of SA to tobacco BY-2 cells reportedly resulted in rapid and transient generation of ROS chiefly $O_2^{\bullet-}$ (Kawano et al. 1998) and H_2O_2 (Kawano and Muto 2000), and also a transient increase in $[Ca^{2+}]_c$ (Kawano et al. 1998). There, ROS actively triggers the influx of Ca^{2+} into the cells and this early oxidative burst by SA was shown to be an extracellular event catalyzed by extracellular free and cell wall-bound POXs (Kawano 2003).

Action of SA mediated by both the cell wall POX-dependent ROS production and Ca^{2+} influx was also observed in the cell suspension-cultured *Arabidopsis thaliana* (Kadono et al. 2010) and *Vicia faba* epidermis (Mori et al. 2001). Both SA-induced $O_2^{\bullet-}$ and chemically generated $O_2^{\bullet-}$ were shown to induce the closure of stomata, which is known as a Ca^{2+}-dependently regulated event studied in *Commelina communis* L. (Lee 1998), *Vicia faba* L. (Manthe et al. 1992; Mori et al. 2001) and *Arabidopsis thaliana* (Khokon et al. 2011).

The SA-induced stomatal closure in *Vicia faba* and *Arabidopsis thaliana* was reportedly inhibited by pre-treatment with ROS scavengers such as CAT and SOD, and an inhibitor of POX suggesting the involvement of POX-mediated production of extracellular ROS during the action of SA leading to stomatal closure (Mori et al. 2001; Khokon et al. 2011). It is noteworthy that pharmacological inhibition of RBOHs nor mutations of *atrbohD* and *atrbohF* showed no inhibition on the SA-induced stomatal closures (Khokon et al. 2011), suggesting that the POX-dependent mechanism is solely responsible for the rapid stomatal regulation by SA. As the stomata is the major path connecting the internal environment in the plants and the aerial environment surrounding plants through gas exchange, it is tempting to relate the perception of air quality by plants and the action of SA leading to stomatal closure.

3 Plant Responses to Air Pollutants

3.1 Ozone (O₃)-Induced Cell Death

O_3 produced by a complex series of photochemical reactions via primary precursor emissions of nitrogen oxides and volatile organic compounds, is a major secondary air pollutant often reaching high concentrations in urban areas under strong daylight, and studies are now suggesting that a steep increase in global background concentrations of O_3 is in progress and thus the impact of atmospheric O_3 to plants including valuable crops might be severer in the future world (Ashmore 2005). Despite of great efforts to identify the physiological and biochemical elements of

O_3 tolerance in plants, the pictures obtained are not clear enough (Fiscus et al. 2005). The most widely accepted model describing the nature of O_3 toxicity/tolerance is the oxidative stress model in which generation of ROS and release of oxidation products are involved in the generation and propagation of toxic compounds throughout the plants (Fiscus et al. 2005).

Several studies have shown that exposure to O_3 elicits the production of ROS in plants. In order to understand the oxidative signaling, the approaches to identify the chemical types of ROS and the subcellular site of ROS generation are of great importance (Baier et al. 2005). In 1990, an electron spin resonance (ESR) study has provided the first evidence for the presence of radical species in the leaves of O_3-exposed plants such as pea (*Pisum sativum* L.) and bean (*Phaseolus vulgaris* L.) using *N-t*-butyl-α-phenylnitrone as a spin trap (Mehlhorn et al. 1990). However, the nature of the trapped radicals could not be identified. Later ESR study with replicated ESR signal subtraction methods using 1,1-diphenyl-2-picrylhydrazyl as a spin trap has shown that a signal with characteristics of $O_2^{\bullet-}$ could be generated in the leaves of bluegrass (*Poa pratensis* L.), ryegrass (*Lolium perenne* L.) and radish (*Raphanus sativus* L.) after being exposed to high concentrations (ca. 10–25 ppm) of O_3 (Runeckles and Vaartnou 1997). Since the detection of such signal was light-dependent, a role for chloroplasts as the sites of photochemical $O_2^{\bullet-}$-generation was suggested. Based on these ESR results, a model for O_3 toxicity in plants has been proposed (Runeckles and Vaartnou 1997). According to this model, O_3 approaches and penetrates into the cells across the PM and further reaches to the photosynthetic apparatus in the chloroplasts to participate in the photochemical reactions leading to generation of $O_2^{\bullet-}$. As parts of the protective mechanism, the entry of O_3 could be blocked by ascorbate in the apoplast and the released $O_2^{\bullet-}$ could be quenched by the chloroplastic SOD-POX system. Thus only when excess, O_3 can damage the cells via this pathway. However, actual role for $O_2^{\bullet-}$ in the O_3-induced cell death has not been examined.

In general, a short exposure to high concentration of O_3 causes the formation of cell death lesions on the leaves of sensitive plants (Overmyer et al. 2005). Such localized cell death is a common feature of O_3 phytotoxicity and is generally thought to be initiated by strong oxidizing action of O_3 itself as well as by O_3-derived ROS intermediates (Schraudner et al. 1998).

Chronic expositions to low O_3 concentrations often show a negative impact on crop yields by reducing photosynthesis and growth, and inducing premature leaf senescence in sensitive plants (Pell et al. 1997). On the other hand, acute and transient exposures to O_3 results in development of cell death, which is visible and known as O_3 lesions in the leaves (Kangasjärvi et al. 1994). The lesions induced by O_3 highly resembles the PCD that takes place in HR in plant-pathogen interactions (Kangasjärvi et al. 2005; Overmyer et al. 2005; Pasqualini et al. 2003).

Since early 1960s, Bel-W3, a tobacco (*Nicotiana tabacum* L.) variety hypersensitive to O_3 which readily produces visually recognizable symptoms on leaves, has been used in many countries as an indicator of the presence of phytotoxic concentrations of O_3 (Heggestad 1991). Bel-B tobacco is also widely used as the reference plant since this variety is highly tolerant to O_3 (Heggestad 1991). By

focusing on the induction of cell death, Kadono et al. (2006) have examined the O_3-induced cell death in two suspension-cultured cell lines of tobacco derived from Bel-W3 (hyper-sensitive to O_3) and Bel-B (highly tolerant to O_3). Upon exposure to the pulse of ozonized air, the freshly prepared cell lines showed difference in O_3 sensitivity as observed in their original plants, depending on the exposure time. As addition of several ROS scavengers and chelators inhibited the cell death induced by O_3, involvements of singlet oxygen ($^1O_2$), H_2O_2, $HO^\bullet$ and redox-active metals such as Fe^{2+} play central roles in O_3-induced acute damages to the cells were suggested. As expected, much higher production of $^1O_2$ and H_2O_2 in the O_3-treated Bel-W3 cells than Bel-B cells was observed (Kadono et al. 2006). Addition of some ROS scavengers after the pulse of O_3 effectively rescued the cells from the induced cell death, confirming the O_3-simulated progress of ROS production and development of cell death mechanism.

3.2 Involvements of SA and Stress-Related Hormones During O_3-Induced PCD

Upon exposure to O_3, a rapid increase in $[Ca^{2+}]_c$ occurs in the plant cells (Clayton et al. 1999; Evans et al. 2005, Kadono et al. 2006). Increases in $[Ca^{2+}]_c$ was sensitive to Ca^{2+} chelators, ion channel blockers, and ROS scavengers suggesting that Ca^{2+} fluxed in from the apoplast acts as a secondary messenger initiating the oxidative cell death (Overmyer et al. 2005; Kadono et al. 2006), in addition to the rapid changes of the protein phosphorylation pattern (Baier et al. 2005). The production of ROS finally leads to PCD characterized by early release of cytochrome c from mitochondria, activation of protease, DNA fragmentation, electrolyte leakage and ultrastructural changes characteristic of PCD (Pasqualini et al. 2003; Kangasjärvi et al. 2005; Overmyer et al. 2005; Kadono et al. 2006). Interestingly, early studies have pointed the similarity between the defense activation mechanism and O_3 response in plants (Sandermann et al. 1998). In fact, the sequence of the events starting from ROS generation and induction of calcium signalling finally reading to cellular response resembles the SA-induced phenomena such as cell death, stomatal closure and SAR-related gene expression (Kawano et al. 1998, 2004a; Mori et al. 2001).

The responses that follow the pulse of O_3 also include the changes in gene expression patterns and synthesis, and accumulation of the plant stress hormones, such as ET, ABA, JA and SA (Sharma and Davis 1997; Sandermann et al. 1998; Rao and Davis 2001; Tamaoki et al. 2003; Overmyer et al. 2000, 2005), finally regulating the induction and spreading of oxidative stress symptoms (Rao et al. 2000; Vahala et al. 2003; Moeder et al. 2002; Kangasjärvi et al. 2005). Treatment with O_3 also induced a rapid accumulation of nitric oxide (NO), which could coincide with the formation of HR-like lesions, suggesting that NO is also an important signalling molecule in the plant response to O_3 (Ahlfors et al. 2009).

Recently, Kadono et al. (2010) have demonstrated that O_3 challenge induces the activation of PM anion channel which is an early prerequisite of the O_3-induced cell death in *A. thaliana*. As the additions of ROS scavengers or iron/copper chelators to the cells of *A. thaliana* effectively lowered the level of cell death induced by O_3 (Kadono et al. 2010), the central roles for ROS members, chiefly $^1O_2$ and H_2O_2 (and derived $HO^\bullet$), in O_3-induced acute damages observed in the earlier study using tobacco cells was further confirmed (Kadono et al. 2006). In *A. thaliana*, the obtained data further suggested the interplay between anion channel activation, Ca^{2+} influx and ROS generation by RBOHs in mediating the oxidative cell death (Kadono et al. 2010). This interplay might be fuelled by several ways in addition to direct ROS generation by O_3, namely, increase in anion channel activity by oxalate generated via ascorbate degradation by O_3 (Tran et al. 2013), and H_2O_2 generation by SA and ABA.

Since NO and plant hormones SA, JA, ABA and ET are involved in determining the duration and extent of O_3-induced cell death propagation (Baier et al. 2005; Kangasjärvi et al. 2005; Tamaoki 2008; Ahlfors et al. 2009), their impact in the newly proposed model was further examined (Kadono et al. 2010). Mutant lines and pharmacological analysis indicated that JA, ET and NO are not the major factors in the signalling pathways leading to cell death in *Arabidopsis* cultured cells. On the contrary, ABA synthesis would take place in response to O_3 since pre-treatment on cells with fluridon was able to counteract the O_3 effect.

In the same manner, involvement of SA in O_3-induced cell death was assessed using the suspension-cultured cells derived from *NahG*, *cpr5* and *npr1* plants, which are impaired in SA signalling, and *sid2* mutant, which are impaired in SA biosynthesis through the isochorismate pathway (Fig. 1). The extent of O_3-induced cell death in *NahG, cpr5* and *npr1* cell lines showed significant decreases, revealing that O_3-induced death depends on SA signalling in *Arabidopsis* cells. Interestingly, *Sid2* cell line presented the same extent of cell death compared to the level recorded for O_3 treatment in wild type cells (col), suggesting that SA synthesis is not required for this acute cell death induction in model cells. If this is the case, how the SA signalling pathway could be activated? As SA could be rapidly released in the apoplast from the storage form of SA, salicylic acid β-glucoside (SAG) (Hennig et al. 1993; Kawano et al. 2004b), SAG pool may be serving as the source of O_3-dependently acting SA. This view must be testified in the future studies.

As the possible involvement of SA in O_3 action was suggested, we have also tested the putative impact of SA on anion channel activation. As shown in Fig. 2, SA at 200 μM, a physiological concentration, induced a rapid but slight hyperpolarisation of the cells followed by a larger depolarisation event within a few minutes. The temporal changes in the PM potential following addition of SA was compared with changes in anion channel activity. As the delayed depolarization being correlated with an increase in anion channel activity, SA could not be responsible for the early depolarization induced by O_3 but SA could also fuel the generation of H_2O_2 and Ca^{2+} influx involved in O_3-induced cell death. The SA-induced generation of H_2O_2 via stimulation of peroxidise and/or NADPH-oxidase is known to lead to Ca^{2+} influx (Kawano and Muto 2000; Kawano et al. 2004a, b, c) which could explain the

Fig. 1 Involvement of SA in the O_3-induced cell death in suspension cultured cells of *Arabidopsis thaliana*, demonstrated by Kadono et al. (2010). **a** Microscopic images of O_3-induced cell death in wild type cells (col) visualized after staining with Evans blue and Neutral red. **b** Effect of SA-related mutations on the O_3-induced cell death
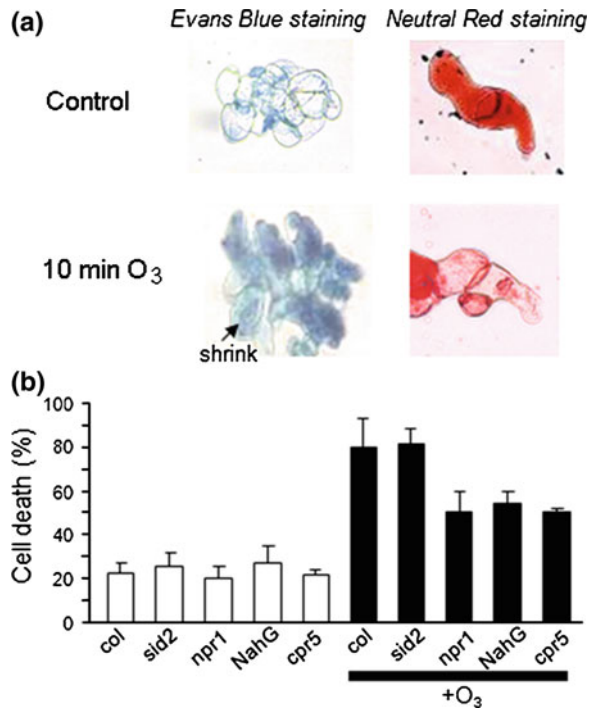
delayed increase in anion currents observed in response to exogenous application of free SA. Upon stimulation, SA could be release from apoplastic SAG pool through the action of SAGase (Kawano et al. 2004a, b). This might explain the fact that the cell death extents recorded after O_3 challenge in the cell lines in sid2 mutant impaired in SA synthesis was severer compared to the cell lines impaired in SA signalling (*NahG*, *cpr5* and *npr1*).

3.3 Peroxyacetyl Nitrate (Pan)-Induced Oxidative and Calcium Signaling Events Leading to Cell Death in Ozone-Sensitive Tobacco Cell-Line

It has long been concerned that some secondary air pollutants such as smog components, O_3 and PAN, are highly phytotoxic even at low concentrations (Barret et al. 1998). Compared with the biology of O_3, we largely lack the information on the toxicity model for PAN at the cellular signaling levels. Recently, we have studied the cell-damaging impact of PAN using suspension culture of smog-sensitive Bel-W3 tobacco cells (Yukihiro et al. 2012). The cells were exposed to freshly synthesized PAN and the induced cell death was assessed. Involvement of ROS in PAN toxicity was suggested by PAN-dependently
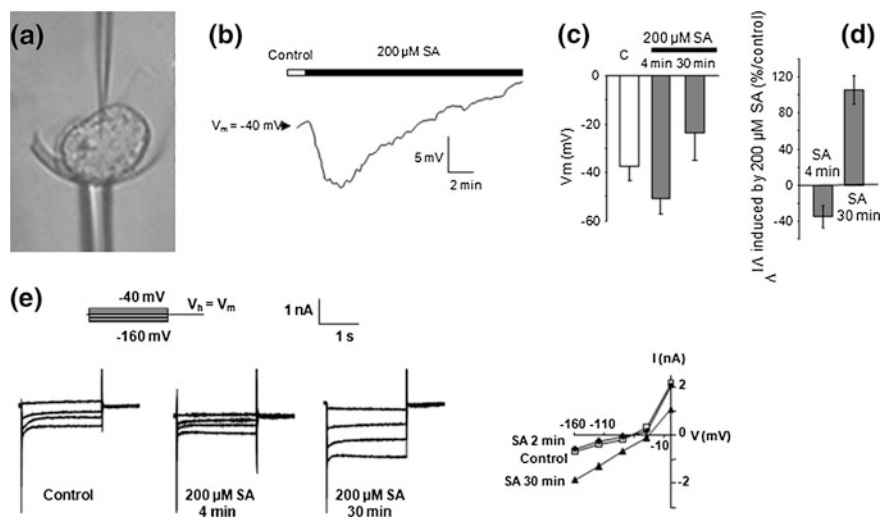
Fig. 2 Effects of SA on PM potential and anion current in *A. thaliana* cells (adopted and modified from Kadono et al. 2010). **a** A single cell of *A. thaliana* under voltage clumping. **b** SA-induced temporal variation in PM potential. **c** Mean values of PM potential recorded 4 and 30 min after SA addition. **d** Mean current variations after SA addition relative to the control level prior to SA addition. Data correspond to mean values $\pm$S.D. ($n \geq 6$). **e** Actual anion currents measured under control conditions, 4 and 30 min after addition of SA

increased intracellular H_2O_2 and also by the cell-protective effects of ROS scavengers and related inhibitors. As Ca^{2+} chelator lowered the level of PAN-induced cell death, an involvement of Ca^{2+} as a key mediator of induced cell death was suggested. Using a transgenic cell line expressing aequorin, an increase in $[Ca^{2+}]_c$ responsive to the pulse of PAN, but sensitive to Ca^{2+} channel blockers, was recorded, confirming that Ca^{2+} channels are activated by PAN or PAN-derived signals. Above data tell us a similarity between the signaling mechanisms responsive to O_3 and PAN.

3.4 Orchestrated Cellular Events Upon Exposure to Smog Oxidants

To date, our knowledge on the impacts of O_3, as a major smog-related oxidant, to living plants, at the molecular biological, cell biological and even at proteomic levels are rapidly increasing (Renaut et al. 2009). Our group has also participated to the study on the behavior of plant cells (leading to cell death) in responses to O_3 using cell suspension cultures of *Arabidopsis thaliana* (Kadono et al. 2010) and O_3-sensitive and O_3-tolerant tobacco (Kadono et al. 2006), revealing that secondarily produced ROS, calcium signaling events, and anion channel regulations are all involved in the O_3-stimulated signaling leading to PCD.

One of key finding is that SA is involved in cell death development through regulation of signaling cascade including oxidative signaling and calcium signaling mechanisms. Involvement of SA in O_3 response in living plants have been also suggested elsewhere (Yalpani et al. 1994; Ogawa et al. 2005; Tamaoki 2008). In addition to SA, its glucoside is also reportedly produced in plants in response to the presence of O_3 (Scott et al. 1999). While SA acts as a fast signal for plant defence, development, its glucoside acts as a slow inducer of oxidative burst in tobacco cells (Kawano et al. 2004b). Lately, involvement of SA glucosyltransferase which converts SA to its glucoside in chemical induction of defence mechanism has been demonstrated in rice plants (Umemura et al. 2009). At gene expression level (examining the SA-responsive gene expression with micro-array) and molecular genetic level (using SA-related mutant cell lines), involvement of SA signal transduction pathway in O_3 response in *Arabidopsis thaliana* have been proven (Sun and Kang 2003; Yoshida et al. 2009).

In contrast, we still largely lack the information on the cellular toxicity model for PAN in plants. Therefore, in our recent study (Yukihiro et al. 2012), we examined the extent and mechanism of the cell-damaging impact of PAN in O_3-sensitive cell line of tobacco maintained as suspension culture (Bel-W3 cells), by focusing on the cell death-mediating cellular signaling events. Our data suggested that response of the cells and induced signalling path leading to cell death induction was almost identical with the O_3 toxicity model. We realized that obtained data are suggesting the similarity between PAN-dependent signaling and plant responses to atmospheric O_3 where SA is involved as an intermediate signal (Kadono et al. 2006, 2010). In this context, SA acts as one of key signaling molecules that stimulate the ROS production followed by calcium signaling finally leading to HR represented by PCD increase, and development of SAR represented by expression of PR genes active against pathogen invasion such as PR-1a.

Reportedly, both calcium signaling and ROS signaling were required for cell death development in response to ozone (Kadono et al. 2006, 2010). To confirm above view that PAN-response is somewhat similar to SA -mediated O_3 responses, we have tested the effect of PAN on induction of PR-1a expression by RT-PCR. Among the gene examined, PR-1a was shown to be highly inducible by exposure to PAN while no signal was observed in the absence of PAN (Yukihiro et al. 2012). The model for PAN must be evaluated through further experiments.

In Fig. 3, hypothetical scheme summarizes the action of SA during O_3-induced signaling finally leading to PCD in *A. thaliana* cells. Upon exposure to O_3, apoplastically released SA likely interacts with (1) ion channels, directly with anion channel and indirectly via ROS production with calcium channel, and (2) ROS-generating enzymes, directly with cell wall-bound POX and indirectly with PM-localized RBOHs upon exposure to O_3, finally leading to PCD in *A. thaliana* cells.
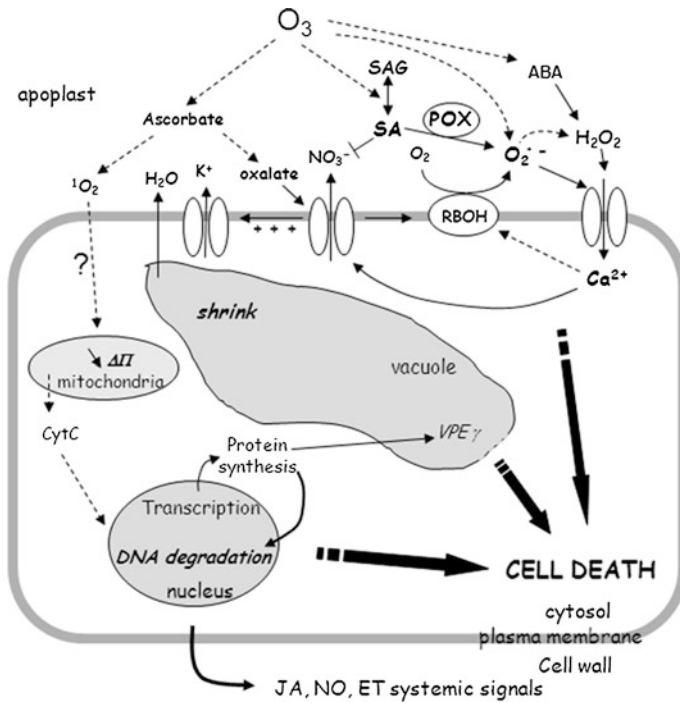
Fig. 3 Hypothetical scheme for the action of SA interacting with ion channels and ROS-generating enzymes upon exposure to O_3, finally leading to PCD in *A. thaliana* cells

4 Metal Toxicity

4.1 Role of Trace Elements During Environmental Oxidative Stress Responses

Induction of cell death by oxidative stresses accompanying the generation of ROS is often mediated by early signaling events such as Ca^{2+} influx via ROS-mediated activation of calcium channels on PM (see above sections). Our previous studies have revealed that various phytotoxic metals such as Al^{3+} and 15 rare earth elements induce an acute generation of ROS such as $O_2^{\bullet-}$ by stimulating the plant RBOH activity (Kawano et al. 2001, 2002, 2003) and as a consequence, ROS stimulates the opening of ROS-responsive calcium channels on the surface of plant cells (Kawano et al. 2003, 2004a, c).

Copper is known to be a phytotoxic metal which induces an increase in $[Ca^{2+}]_c$ in cultured tobacco cells (Inoue et al. 2005). However, the possible mechanism of acute copper action may differ from such metals stimulating the $O_2^{\bullet-}$ generation since production of $O_2^{\bullet-}$ could not be detected after addition of $CuSO_4$ to the plant cell suspension culture (Kawano and Muto 2000). On the other hand, copper may

catalyze the Fenton-type reaction forming HO$^\bullet$ (the most violent members of ROS) in the presence of both certain reducing agents (such as ascorbate) converting Cu(II) to Cu(I), and biologically supplied H_2O_2. Previously, monitoring of the Fenton-type reaction leading to the formation of HO$^\bullet$ by electron spin resonance spectroscopy was performed and this reaction was shown to proceed in a tobacco BY-2 cell suspension culture after addition of $CuSO_4$ (Kawano and Muto 2000).

In the earlier sections, we have described the studies using Bel-W3 cell-line which is highly sensitive to oxidative stresses. Actually, this cell line lacks the anti-oxidative enzyme activities (such as CAT), while Bel-B cell-line derived from Bel-B tobacco variety show tolerance to oxidative stresses by being active in removal of ROS (Kadono et al. 2006). Using those cell cultures, we have examined the roles for two Fenton reagents in cellular responses to environmental oxidative stresses namely, UV-C, O_3 and PAN (Fig. 4).

With Bel-W3 cells which are ROS-sensitive, we showed that the level of cell death induction is significantly higher than in Bel-B, ROS-tolerant control cell-line, when exposed to three different oxidative stresses, namely UV-C, O_3 and PAN (Fig. 4). Data shown here confirmed that cell death induction in responses to UV-C, O_3 and PAN require the involvement of trace metals such as Fe and Cu since chelators of these metals clearly blocked the induction of cell death.

Application of two chelators of iron and copper, 2$'$,2-bipyridyl (bipy) and o-phenanthroline (o-phen), resulted in strong inhibition of the cell death induction by UV-C and PAN in both cell lines (Fig. 2), as Kadono et al. (2006) have suggested that O_3-induced cell death may involve Fenton-type reactions reading to generation of HO$^\bullet$, Fenton catalysts (Fe and/or Cu) likely participate in the redox-responses in tobacco cells. In case of O_3-induced cell death, o-phen-treated cells showed stronger inhibition of cell death induction while the action of bipy was less obvious. Involvement of Fe^{2+} and/or Cu^+ in redox responses in tobacco cells was the likely conclusion from these demonstrations. Therefore, we have shifted to the tests for determining the impacts of directly added iron and copper in the redox sensitive tobacco cell line.

To analyze the toxic impacts of Fe and Cu in Bel-W3 cell suspension culture, Bel-W3 cells were treated with 0.5 mM Fe^{2+} and Cu^+ (Fig. 5). In addition, we tested the effect of 1 mM ascorbic acid (AsA) as a reducing agent. Combination of AsA and Fenton-type catalysts (both Cu^+ and Fe^{2+}) resulted in enhancement of cell death. Interestingly, the background AsA toxicity was shown to be blocked in the presence of two metal chelators, bipy and o-phen, suggesting that AsA toxicity requires the presence of trace metals such as copper.

Synergetic and damaging action of AsA and copper is a well known phenomenon. Oxidative damages to DNA and proteins are reportedly promoted in the presence of ROS such as HO$^\bullet$, which can be generated thorough the Fenton-type or Harbor-Weiss-type reactions in the presence of the ions of transition metals (chiefly copper and iron), AsA and/or H_2O_2 (Chiou 1983). Among the members of ROS, HO$^\bullet$ is the most highly reactive species oxidizing any neighboring
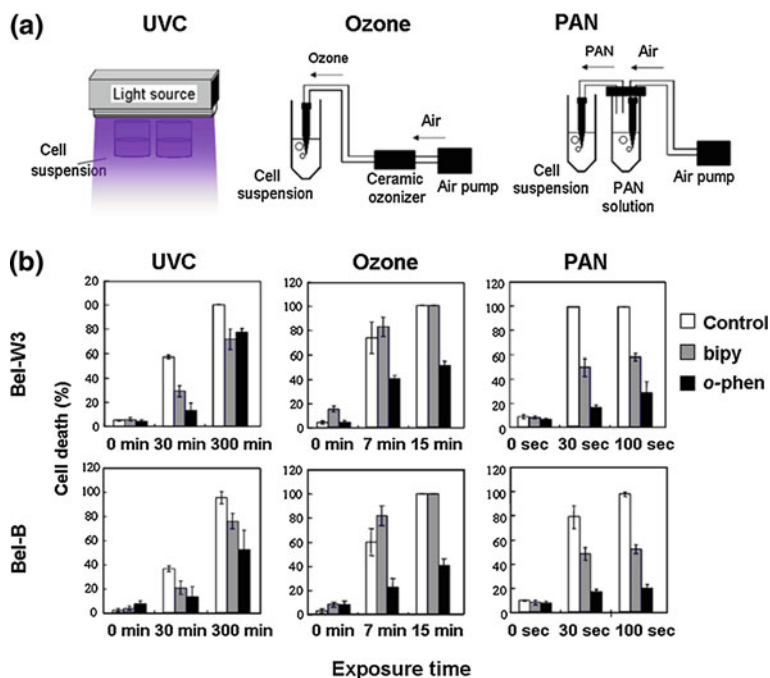
Fig. 4 Involvement of trace metals in the oxidative abiotic stress responses leading to cell death in tobacco cells. **a** Treatment of tobacco cells with three different environmental oxidative stresses, namely, UVC irradiation (wave length 254 nm, intensity 2.2 mW/cm^2), high dose O_3 exposure (concentration 150 ppm), and PAN exposure (0.75 $\pm$ 0.15 ppm). **b** Effects of two metal chelators against the cell death induction by environmental oxidative stresses. Each environmental stress was applied to Bel-W3 or Bel-B cell suspension for certain length of time as indicated and statically incubated for 2 h. Then these treated cells were stained with Evans blue for 1 h and dead cells were counted under microscopes. Evaluation of cell death was repeated for 4 times for each sample. Two chelators, 2 mM 2′,2-bipyridyl (bipy) and 2 mM o-phenanthroline (o-phen), were added to cell suspension cultures 5 min prior to oxidative treatments. (*Left*) Involvement of Fe/Cu under UV-C exposure. (*Middle*) Involvement of Fe/Cu in O_3-induced cell death. (*Right*) Involvement of Fe/Cu in PAN-induced cell death

molecules. Therefore, generation of HO$^\bullet$ in the biological systems results in immediate damages to DNA molecules and the subsequent DNA degradation may further leads to apoptotic reaction and carcinogenesis in the living cells. Such oxidative stress-mediated DNA fragmentation and chromosomal dysfunction play key roles in mammalian cell death mechanisms (Higuchi 2003). Therefore, it is important to seek for the ways to prevent or detoxify the Cu-mediated oxidative mechanism. Natural chelators of copper such as small peptides reportedly prevent the degradation of DNA (Yokawa et al. 2011a, b) and cell death and ROS-stimulated calcium signaling in tobacco cells (Kagenishi et al. 2009). Interestingly, SA might be one of such natural chelators.
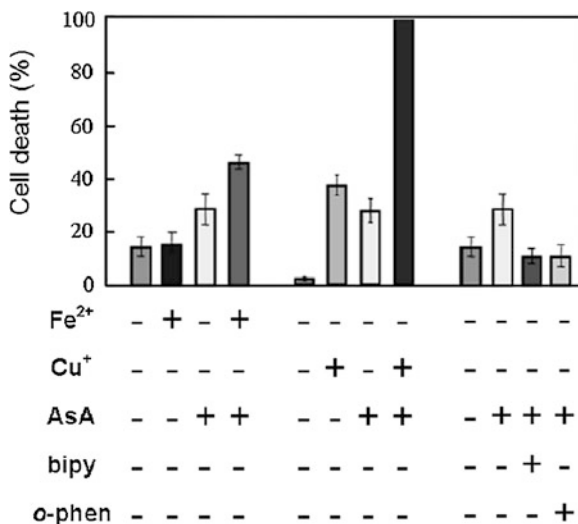
Fig. 5 Fe/Cu-induced cell death and effect of ROS scavengers and metal chelators in Bel-W3 cell suspension cultures. Bel-W3 cell suspensions were treated with Fe^{2+} or Cu^+ (1 mM) and incubated for 2 h. Then cells were harvested and stained with Evans blue for 1 h. Percentage of cell death induction was worked out by counting the dead cells (repeated for 4 times). To elucidate the involvements of ROS in Fe/Cu toxicity, 1 mM ascorbic acid (AsA) used as both a reducing agent and an antioxidant, 2 mM 2′,2-bipyridyl (bipy) used as a Fe chelator and 2 mM o-phenanthroline (o-phen) used as Cu chelator were added to the cell suspension cultures

4.2 SA Prevents the Action of Fenton-Type Catalysts

While generation of $O_2^{\bullet-}$ (Kawano et al. 1998) and H_2O_2 (Kawano and Muto 2000) are stimulated by pro-oxidative action of SA in plant cells, SA also behaves as an antioxidant. The addition of exogenous H_2O_2 to the suspension-cultured tobacco cells reportedly results in generation of $HO^{\bullet}$ (detectable with spin trapper-mediated ESR spectroscopy) by transition metal-catalyzed Fenton-type reaction. However, in the presence of SA, generation of $HO^{\bullet}$ becomes hardly detectable since SA can chelate the metals (Kawano and Muto 2000). In addition, SA also removes $HO^{\bullet}$ generated in the absence of the catalytic metals since SA acts as an efficient scavenger of $HO^{\bullet}$. As studies with animal systems have shown that exogenously administrated aspirin and SA can act as effective scavengers of $HO^{\bullet}$ in vivo (Aubin et al. 1998; Sagone and Husney 1987), SA is one of the strongest scavengers for $HO^{\bullet}$. The structures of likely products due to SA-$HO^{\bullet}$ interaction in vivo are 2,3- and 2,5-dehydroxybenzoic acids and catechol and therefore these products often reports the production of $HO^{\bullet}$ in vivo (Grootveld and Halliwell 1986; Halliwell et al. 1991).

As above, SA protects the cells from highly violent $HO^{\bullet}$, and therefore the involvement of $HO^{\bullet}$ in SA signal transduction could be eliminated (Kawano and

Muto 2000) if the SA-HO$^\bullet$ byproducts are all inert. However, the likely products from SA-HO$^\bullet$ interaction could be still active as we have reported that 2,3- and 2,5-dehydroxybenzoic acids, two major products of SA-HO$^\bullet$ reaction, has strong activity in induction of $O_2^{\bullet-}$ generation in tobacco cell suspension (Kawano et al. 2004b). This implies that SA actively sacrifies itself for elimination of HO$^\bullet$ without minimal loss of $O_2^{\bullet-}$ generating activity. Since the presence of SA does not allow the presence of HO$^\bullet$, we can propose that SA-induced $O_2^{\bullet-}$ and derived ROS such as H_2O_2 (but not HO$^\bullet$) function downstream of SA.

4.3 Aluminum-Responsive Oxidative Burst

To date, number of studies has documented the toxic impacts of Al ions on roots (Ma 2000), hypocotyls (Ma et al. 1999) and germinating pollens (Li et al. 2000). It has been proposed that early effects of Al toxicity at the root apex, such as those on cell division, cell extension or nutrient transport, involve the direct intervention of Al on cell function (Lazof 1994). In addition to intact plants, the cell suspension cultures derived from model plant species such as tobacco (*Nicotiana tabacum*) have been frequently employed for elucidating the molecular components involved in the mechanism of metal toxicity (Kawano et al. 2001, 2002) and Al phyto-toxicity (Lin et al. 2005).

According to the recent studies, one of key factors required for Al cytotoxicity is the generation of ROS as observed in various materials (Yamamoto et al. 2003). Our group found that Al^{3+} and other trivalent cations added to tobacco cells trigger the apoplastic generation of cytotoxic $O_2^{\bullet-}$ (Kawano et al. 2001, 2003). While Yamamoto's group has reported the involvement of mitochondrial oxidative burst as the source of Al-induced ROS (Yamamoto et al. 2002), our data were indicative of the involvement of RBOHs as the Al-induced $O_2^{\bullet-}$ has been shown to be sensitive to an inhibitor of NADPH oxidase (Kawano et al. 2003, 2004c; Lin et al. 2005), by analogy to the earlier works with various trivalent cations such as lanthanide ions chiefly, La^{3+} and Gd^{3+} (Kawano et al. 2001).

In many occasions in plant responses to biotic and abiotic stresses including plant–microbe interactions, the involvement of RBOHs in burst of ROS production has been documented (Yoshioka et al. 2008). It has been suggested that plant cells naturally respond to SA by inducing the RBOH-dependent oxidative burst (Yo-shioka et al. 2001). Such RBOH-dependent bursts in ROS production induced by SA is likely a late response that follows the earlier events involving apoplastic POX-dependent acute $O_2^{\bullet-}$ generation (Kawano et al. 1998, 2004a).

Recently, we showed the molecular genetic evidence that Al treatment results in expression of an NADPH oxidase-coding gene through SA signal transduction pathways, by employing the cell lines derived from *Arabidopsis thaliana* mutants lacking SA synthesis or SA-dependent signaling factors (Kunihiro et al. 2011). We observed that Al treatments of suspension-cultured cells of *Arabidopsis thaliana* resulted in biphasic $O_2^{\bullet-}$ generation consisted of immediate (maximal response

observed in few seconds) and long lasting gradual increase (spending hours), similarly to the tobacco model. Among six respiratory burst oxidase homologs (*atrbohs*) coding for plant NADPH oxidase, *AtrbohD* was shown to be the only gene responsive to Al. In the Al-treated cells, the induced $O_2^{\bullet-}$ generation, *AtrbohD* expression and cell death were all inhibited by NADPH oxidase inhibitor and SOD. According to the gene expression and oxidative burst profiles and effect of inhibitors, it is likely that the acute burst of $O_2^{\bullet-}$ generation could be catalyzed by pre-existing enzyme and slower response could be the consequence of long-lasting gene expression.

In *Arabidopsis thaliana*, six genes for RBOHs as well characterized *AtrbohA-F* are known (Ogasawara et al. 2008). Among *Atrbohs*, *AtrbohA*, *B* and *C* were shown to be expressed only in the roots, especially in the elongating regions. *AtrbohE* is reportedly expressed in seeds and roots. *AtrbohD* and *F* are known to be expressed systemically in the plants. As the expression of systemically distributable *AtrbohD* was shown to be rapid and long-lasting (1 min–24 h), we expect that Al response has similarity with SAR-inducing stimuli (Kunihiro et al. 2011).

4.4 Involvement of SA Signal Transduction in Aluminum Action in *Arabidopsis thaliana*

In order to examine the involvement of SA signaling pathways in the cells of *Arabidopsis thaliana*, the cell lines lacking SA synthesis (transgenic NahG cell line and *sid2* mutant cell line) and the cell lines with altered SA-dependent signaling factors (*npr1* mutant cell line and transgenic NRP1-overexpressing cell line, NPR-Ox) were used for comparison (Kunihiro et al. 2011). As shown in Fig. 6, no
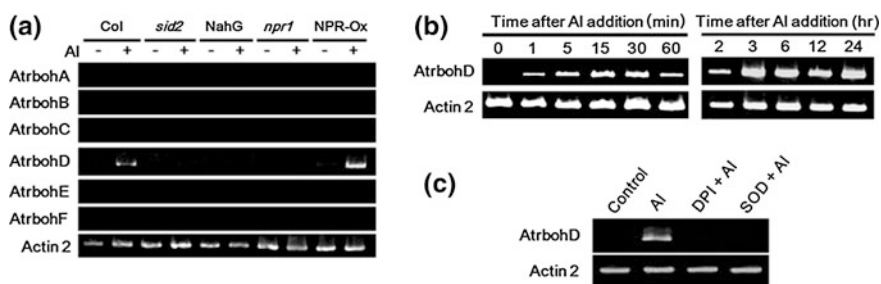
Fig. 6 Involvement of SA biosynthesis and signaling pathway in Al-induced expression of *Atrboh*s. **a** Suspension-cultured cells of *Arabidopsis thaliana* with ecotype Columbia background namely wild type (cell line, Col-0), mutants (cell lines, *sid2* and *npr1*) and transgenic cell lines (cell lines, *NahG*, over-expressing bacterial SA hydroxylase; *NPR-Ox*, over-expressing *NPR1* gene) were treated with 0.1 mM $AlCl_3$ for 3 h. **b** Al-induced expression of *AtrbohD* at different time points (0–60 min and 2–24 h). **c** Inhibition of Al-dependent expression of *AtrbohD* by 1 mM diphenyleneiodonium chloride (DPI) and 5,000 units/ml SOD (examined 3 h after treatment with Al). Data adapted from Kunihiro et al. 2011

Fig. 7 A model mechanism
for Al-induced cell death
signaling path involving SA.
SA signaling is activated in
downstream of ROS.
AtrbohD expression involves
the NADPH oxidase-
mediated oxidative burst and
SA-dependent signaling
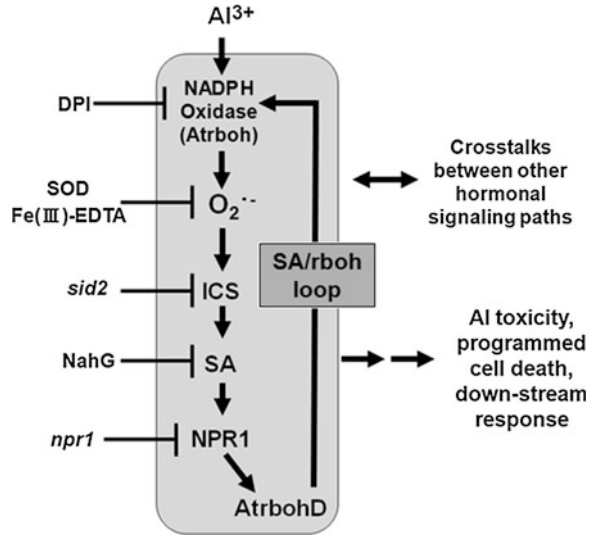
expression of *Atrboh* genes could be observed in *sid2*, NahG and *npr1* cells
regardless of the presence of Al. In contrast, in the NPR-ox cells, Al-induced
expression of *AtrbohD* was shown to be enhanced while no expression of other
Atrboh isoforms were observed. Above data clearly demonstrated that Al-induced
AtrbohD expression requires the presence of SA biosynthesis and SA signaling.

One of the likely consequences of Al-induced oxidative burst is induction of
cell death. Following Al treatment, increase in cell death can be observed in both
tobacco cells and *Arabidopsis* cells (Kawano et al. 2003; Kunihiro et al. 2011). As
addition of NADPH oxidase inhibitor and $O_2^{\bullet-}$-scavenger resulted in partial
inhibition of Al-induced cell death, it is likely that ROS production in the early
phase of Al response plays a key role in the Al-dependent induction of cell death.
Furthermore, in the *sid2* mutant cells and NahG transgenic cells both lacking the
accumulation of SA, the level of Al-induced cell death was partially but signifi-
cantly lower than that in wild-type cells, thus, confirming that, at least partially,
SA is involved in Al-induced cell death mechanism.

Taken together, there would be a loop of SA signaling and SA-dependent
expression of *rbohD* gene leading to prolonged ROS production in the Al-exposed
cells. Figure 7 shows the recently proposed model for Al-dependent signaling
path, which is a series of signaling events firstly initiated by acute ROS production
stimulated by Al, subsequently leading to the cell death and other downstream
responses, to be mediated through SA biosynthesis and signaling by inducing the
expression of *rbohD*. The SA-dependently activated or induced *rbohD* may further
contribute to the oxidative burst (SA/RBOH loop).

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Chapter 12
The Interplay Between Salicylic and Jasmonic Acid During Phytopathogenesis

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Abstract There is no doubt that the salicylic acid (SA) plays an important role in plant defence against pathogens attacks. According to the established opinion, SA induces the systemic acquired resistance (SAR) that is effective defense against numerous biotrophic pathogens that colonize living plant tissue from where they consume nutrients, suppressing their immune response. SAR is largely due to programmed cell death and early oxidative burst in the host cells. In contrast, necrotrophic pathogens do not suffer from cell death and salicylic acid–dependent defenses. SA-induced cell death can promote development of pathogenic structures. Mechanisms of defence against necrotrophs are regulated by another set of defense responses activated by jasmonic acid (JA) and so-called induced systemic resistance (ISR). Literature data indicate that the signals inducing SAR or ISR are strictly individual: SA can antagonize JA signaling and vice versa. Probably, crosstalks between SA and JA help the plant to minimize fitness costs and create a flexible signaling network that allow the plant to regulate its defense responses against invaders. However, there are some data evidencing certain synergy or additive effect of SA on processes attributed to ISR. This article is focused on some aspects of interplay of SA with JA during the establishment of plant resistance to pathogens with different type of nutrition and participation of peroxidases in this process.

Keywords Salicylic acid · Jasmonic acid · Plant defense · PGPR · Peroxidase · Reactive oxygen species

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1 SA in the Regulation of Reactive Oxygen Species Level During Pathogenesis

In contrast to the influence of abiotic stressors or growth conditions (humidity and temperature) pathogenic attacks aren't the regular factors. Plants have to balance the costs and potential benefits of investing in defense in an environment where enemy attack is variable. Because they are costly to produce, natural selection is presumed to favor the evolution of inducibility. It means that besides the basic, constitutively maintained level of the resistance to high variety of pathogens "permanent costs", plants evolved specific mechanisms of the resistance against the aggressive forms, triggered by the early detection of the enemy. This priming results in faster and stronger inducer of defense mechanisms after detecting pathogen attack, and this effect can be observed for a long time. In literature, there are reports of systemic acquired resistance (SAR), associated with localized priming during the contact with a broad spectrum of pathogenic (or symbiotic) organisms e.g. viruses, bacteria, fungi, oomycetes (Durrant and Dong 2004; Luna et al. 2012). It has been noted that SAR is effective against biotrophic pathogens and their components, primarily associated with hypersensitive responses of plant cells, triggered by salicylic acid (SA). Recent evidences suggest that plant immunity involves regulation by chromatin remodeling and DNA methylation. So, PstDC3000 induces DNA hypomethylation in Arabidopsis. Luna et al. (2012) suggested that transgenerational SAR is transmitted by hypomethylated genes that direct priming of SA-dependent defenses.

SA regulates the expression of PR-proteins and production of low-molecular antibiotic compounds at the infection site and in distant tissues (Liu et al. 2012). Moreover, SA was demonstrated not to be a translocable signal of SAR but its accumulation was indicated as an essential factor required for the expression of multiple modes of plant disease resistance.

Participation of SA in plant resistance to pathogens was first demonstrated in early 80s (Antoniw and White 1980). It was found that the treatment of tobacco leaves by SA increased their resistance to tobacco mosaic virus. During the penetration of pathogen in plant tissues was displayed with fast accumulation of SA together with local oxidative burst at the invasive site (Torres et al. 2006). The SA-induced disease resistance is limited not so much to its constituted endogenic contents in plants as by presence of specific receptors and, accordingly, signaling systems, sensitive to exogenic SA. This is confirmed by the fact that during penetration of *Stemphyllium vesicarium* in tissues of *Allium cepa* gradual increase of SA content wasn't influenced by pathogen development. However, if plants were pre-treated by exogenic SA, infection caused the rapid expansion of endogenic SA-content, correlated with peroxidase activity and, subsequently, with the resistance to this pathogen (Abo-Elyousr et al. 2009). So, plants have an effective mechanism of regulation of the defence reactions with participation of exo- and endogenic SA, mediated, possibly, by some components of pro-/antioxidant systems. Thus, there is a hypothesis that in the accumulation and regulation of SA

content the mitochondrial alternative oxidase participates (Chaturvedi and Shan 2007). Besides, it is also considered that the accumulation of H_2O_2 under the influence of SA is due to catalase inhibition (Chen et al. 1993) and SA binding activity to the heme group of ascorbate peroxidase (Vlot et al. 2009). Therefore, the relationship between H_2O_2 and SA isn't so simple, because H_2O_2 and SA are substrates for peroxidases and participate in signalling regulation of gene expression which have directs antibiotical effect on pathogens (Kawano and Furuichi 2007). The consequence of inhibitory activity of SA on reactive oxygen species scavengers in plants would be the elevated level of H_2O_2 in the immediate vicinity of the infection site and this level has been postulated to act as a second messenger of SA in the signal-transduction pathway leading to SAR and activation of genes encoding PR proteins (An and Mou 2011; Liu et al. 2010).

One of the evidences of the peroxidase regulation during pathogenesis is the inhibition of the oxidative burst induced by SA in plants treated by peroxidase inhibitors—salicylhydroxamic acid or monodehydroascorbate (Kawano and Muto 2000). Proteomic analysis of *Pisum sativum* apical meristem showed that ascorbate peroxidase neutralizing H_2O_2 in ascorbate–glutathione cycle was induced by SA. However, apoptosis-inducing concentration of SA (100 μM) reduced the content of this enzyme (Tarchevsky et al. 2010). Apparently, for triggering of SA-induced genes the oxidative burst caused by pathogen penetration is necessary. So, in SA-treated asparagus plants, infected by root rot pathogen the increase of peroxidase activity was found, but individual influence of SA or pathogen did not lead to this observation (He and Wolyn 2005).

A variety of class III peroxidase genes are SA-induced, although the majority of them are not SA-competent, in the strict sense. It is expected that sensibility to SA is a specific "marker" of peroxidases, participating in the defence reactions (Almagro et al. 2009). So, in our investigations in wheat plants under the influence of SA the activity of peroxidase with $pI \sim 3.5$ and $pI \sim 9.7$ in cytoplasmic fractions of proteins increased. Interestingly, these isoperoxidases can interact with some surface structures of pathogenic fungi, example, chitin and glucans (Maksimov et al. 2010). Recently we found that SA promoted transcription of genes encoding anionic peroxidase in wheat, infected by pathogenic fungus *Septoria nodorum* (Burchanova et al. 2007). The treatment of parsley calli by synthetic analogue of SA (benzothiodiasole) induced the expression of anionic isoperoxidase (Katz et al. 1998).

It should be noted that SA can stimulate the capacity of peroxidases to produce O_2^- and inhibit the antioxidant activity of that simultaneously (Kawano and Furuichi 2007; Almagro et al. 2009). It causes the SA-radicals generation and production of some phenolic compounds participating in antibiotics synthesis (phytoalexines, terpenes, alcaloides) (Okazaki et al. 2004; Reszka et al. 2005) and lignin polymerization. SA-radical (or SA) can take part in lipid peroxidation (Kawano and Furuichi 2007; Hatamzadeh et al. 2012) and SA-induced expression of PR1 gene was suppressed by diethyldithiocarbamate converting peroxy-derivatives of fatty acids in hydroxylic forms (Kawano et al. 2003).

SA-induction of the defence reaction in plants is based on direct mechanism or mediated by secondary messengers, in particular, ROS and products of their action. It was found that SA takes part in the regulation of NADP(H)-oxidase activity and NO level, and can activate lipoxygenase- and MAP-kinase systems (Karuppanapandian 2011; Robert-Seilaniantz et al. 2011). Furthermore, a number of researches propose to classify the defence reaction on SA-dependent and SA-independent pathways, influencing reciprocally on each other and provide an integrated network of regulatory cross-talks (Halim et al. 2009; Almagro et al. 2009).

2 JA as a Component of Signal Transduction System

Jasmonic acid, methyl jasmonate and related compounds are a class of plant hormones that play an important role in the regulation of many cellular processes, such as wound and defence responses (Liechti and Farmer 2002; Kuśnierczyk et al. 2011). Now the problem of participation of JA in processes, linking with root colonization by symbiotic microorganisms, such as nitrogen-fixing microorganisms and plant growth promoting bacteria has captured the attention of investigators (Gutjahr and Paszkowski 2009). Recently it was found that these organisms can prime induced systemic resistance (ISR) to the range of pathogens in plants where JA is one of the important components of this priming (Weller et al. 2012). The production of JA is a tightly regulated process, and its concentrations in unperturbed plant tissues are often very low (Kim et al. 2009). JA accumulates in wounded plants or in plants and cultured cells, treated with pathogen elicitors where it acts as a signal, activating the expression of various genes, such as proteinase inhibitors, thionin, and enzymes in phytoalexin metabolism (Gfeller et al. 2011). Therefore, in Arabidopsis about 200 genes are JA-dependent in the absence of stress factors, and about 800 genes participating in the defence reactions against *Brevicoryne brassicae* invasion that are suppressed in JA-deficient plants (Kuśnierczyk et al. 2011).

The treatment of plants by JA induced the synthesis of wound-induced proteins, such as proteinase inhibitors (PDF1.2 for example), β-1,3 glucanase, chitinase, thionine, napine, storage proteins, PAL, chalcone synthase, lipoxygenase (Wu and Bradford 2003), and rapid accumulation of ROS (Denness et al. 2011). Interesting to note that all the genes encoding the enzymes of JA biosynthesis are JA-induced and activated by exogenic JA (Wasternack 2007; Egusa et al. 2009).

JA, in contrast to SA is considered as a less specific signal, caused by each wounding of plant tissues. Accordingly, necrotrophic pathogens and phytophages are more susceptible to JA-dependent defence mechanisms (Ali and Agrawal 2012). Recently it was noted that defence reactions against these two groups of parasites are regulated by different transcription factors, belonging to JA-signaling pathway. Thus, eating of Arabidopsis leaves by *Pieris rapae* caterpillars caused the expression of transcription factor MYC2 and marker gene VSP2.

Simultaneously the suppression of active components of the defence system against necrotrophic fungi and bacteries, such as factor ORA59B and gene PDF1.2 was detected (Verhage et al. 2011).

All of the plant responses to JA, whether applied externally or released internally, appear to be correlated with alterations in gene expression. At least three major jasmonate effects have been reported:

1. the induction of novel abundant polypeptides, designated jasmonate-induced proteins (JIPs);
2. the selective repression and synthesis of several polypeptides that are present before jasmonate or stress treatment;
3. the temporally delayed general down-regulation of protein biosynthesis occurring in long-term MeJA treated or long-term stressed leaf tissues.

3   SA/JA Interplay

It's clear that SA and JA are signal molecules, able to regulate the resistance of plants to biotrophic and necrotrophic pathogens respectively by the regulation of contiguous molecular processes. This suggests the presence of some kind of antagonistic or synergistic interactions between specific reactions operated by SA and JA. The SA–JA crosstalk that often results in reciprocal antagonism between these two pathways has been interpreted as being an adaptive plant strategy, representing a cost-saving measure given that phenotypically different enemies are susceptible to distinct defense strategies (Thaler et al. 2012). Numerous facts of the strict antagonism between SA and JA were detected in a broad spectrum of investigations by using mutants with SA- or JA- deficient plants or plants having disruption of their signal transduction. Thus, mutations interrupting JA signal pathway (coi1) promoted the expression of SA-dependent gene encoding PR1, and, conversely, disruption of the SA-perception induced the expression of JA-dependent genes, such as a range of proteinase inhibitors (PDF1.2) (Kazan and Manners 2008).

As a result of negative cross-talk between SA and JA, activation of SA response renders a plant more susceptible to attackers that are resisted through JA-dependent defenses and vice versa. Indeed, many examples of trade-offs between SA-dependent resistance against biotrophic pathogens and JA-dependent defense against insect herbivores and necrotrophic pathogens have been reported (Smith et al. 2009). Conversely, silencing of *PAL* reduced SA accumulation and SAR, and enhanced herbivore-induced resistance against *H. virescens* (Felton et al. 1999). Application of SA analog, benzothiadiazole S-methyl ester (BTH) has been shown to reduce tomato resistance to corn earworm *Helicoverpa zea* (Stout et al. 1999). Treatment of tobacco by *Erwinia carotowora* elicitors led to increasing of JA level in plant tissues and to inhibition of the expression of SA-dependent genes (Chaturvedi and Shan 2007). Koornneef et al. (2008) reported that under the

combined influence of 1 mM SA and 0,1 mM JA in 18 cultivars of Arabidopsis grows in different climatic zones a significant decrease of transcription of JA-dependent gene, encoding *PDF1.2* was observed. However it's to be noted that in this work concentrations of these compounds were about 10-fold higher than the concentrations used for stimulation of potato defence reactions against *P. infestans* (Panina et al. 2005).

There are some explanations of these facts. So, high concentrations of SA, inhibiting conversion of 13-s hydroxylinolenic acid in 17-oxophytodienic, i.e. disrupted one of the steps of JA biosynthesis, in vitro (Pena-Cortes et al. 1993). But Leon-Reyes et al. (2009) reported that the antagonistic effect of SA on the expression of JA-marker gene PDF1.2 in different JA biosynthesis mutants with down-regulation of JA biosynthesis pathway is not essential for SA-mediated suppression of JA signaling. In mutant aos/dde2, where JA production is completely blocked, PDF1.2 and VSP2 were not expressed. However, exogenous application of MeJA rescued the JA-responsive phenotype in aos/dde2, but PDF1.2 transcription induced by MeJA could still be antagonized by SA. This indicates that SA-mediated suppression of JA-responsive gene expression functions downstream of the JA biosynthesis. Besides, there is a model of SA-JA interaction, according to this NPR1 protein constitutively present in Arabidopsis leaves is essential both for the expression of SA-dependent genes and for suppression of JA-dependent genes (Koornneef et al. 2008). It was found that NPR1 while in the nucleoplasm regulates mechanisms of SAR, mediated by SA, but for the development of PGPR- induced ISR against necrotrophic fungi, mediated by JA this protein is necessary to be located in cytoplasm. In Arabidopsis plants unable to accumulate SA (*NahG*) due to salicylate-hydroxylase (SL-H) action 25-fold increase of JA-content was observed, as well as intensifying of wound-induced proteins synthesis, compared with wild-type plants (defensine, lipoxygenase II) (Spoel 2007). However, in tobacco plants expressing SL-H gene, despite of significant decrease of SA content, this effect wasn't detected (Mur et al. 2006). It's possible that these differences were associated with the fact that the majority of the evidences of hard antagonism between SA and JA are based on the investigation of transgenic plants having a considerable dis-balance of phytohormone levels, as pointed out in Glazebrook (2005) paper.

According to another opinion, the relative proportion and concentration of various phytohormones is very important for their interaction. Next to antagonistic effects, the synergistic interplay between SA and JA has also been described. Microarray analysis performed by Schenk et al. (2000) revealed a number of genes that were induced or repressed by both SA and methyl jasmonate (MeJA), indicating coordinated regulation of SA- and JA-dependent defense responses. Therefore, treatment of *Pisum sativum* by JA activated phenylalanine ammonia lyase, enzyme which catalyzed initial steps of biosynthesis of a broad spectrum of phenolic compounds, in particular SA (Liu et al. 2008). Moreover, Wees et al. (2000) reported that exogenic JA wasn't influencing the transcription of SA-dependent gene encoding PR1 in Arabidopsis, besides, a combined treatment of tobacco by SA and JA mixture activated the accumulation of PR1 protein (Xu et al. 1994). This protein together

with proteinase inhibitor I accumulated in tobacco under the influence of TMV (Mur et al. 2006). It's to be noted that in Arabidopsis plants infected by necrotrophic pathogen *Fusarium graminearum* activation of transcription of JA-induced gene encoding PR6 protein was simultaneous with the accumulation of transcripts of PR1 encoding gene (Makandar et al. 2010).

Thus, practically all pathogens combined both necrotrophic and biotrophic stages in their life cycle and their ratio is determined by the prevailing terms and conditions. So, the resistance of tomato to necrotrophic fungus *Botritis cinerea* is regulated by SA-induced reactions, but the resistance to *B. cinerea* is mediated by JA/ET signaling (Achuo et al. 2004). However, pathogens with strictly combined types of trophicity attributed to the group called "haemibiotrops" the regulation of the defence reaction against the group invaders is much less understood. So, in cocoa plants infected by haemibiotrophic fungus (*Moniliphthora pernicola*) the increase of SA-level resulted in hypersensitive reaction and cells necrotisation at the infected sites. The pathogen, however, stayed alive and continued to feed on dead cells (Kilaru et al. 2004).

4   The Role of SA and JA in Signaling Regulation of Potato Resistance to the Late Blight Pathogen *Phytophthora Infestans*

Since *P. infestans* belongs to pathogens with mixed type of trophity (Hardham and shan 2009) the roles of SA and JA in the control of potato plant resistance to late blight could be ambiguous. In our investigations of the pathosystems of *Solanum tuberosum* – haemibiotrophic oomycete *P. infestans,* the plant treatment with SA and/or JA suppressed late blight development (Fig. 1). The leaves, however, at the later stage of the plants pretreated with SA or JA, the size of necrotic lesions increased.
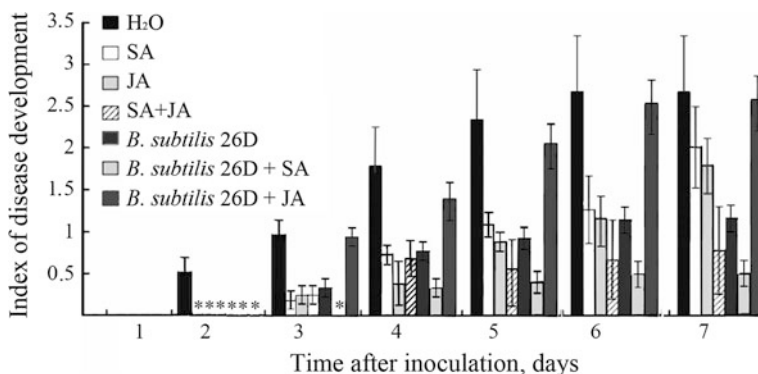
Fig. 1 Effects of *SA* and *JA* and their mixture on the late blight development on the leaves of tube-grown potato plants. *Disease symptoms were not detected

The smallest necrotic lesions were observed after treatment with the mixture of these acids. Thus, a mixture of SA and JA formed out to the most efficient inducer of resistance in potato plants to late blight. It is of importance that treatment with SA and JA did not exert any visual effect on the morphology of healthy, non-infected potato leaves. As potato plants are characterized by the constitutively high content of SA (Panina et al. 2005). Both SA and JA are involved in the regulation of potato resistance to late blight (Vasyukova et al. 2008) by increasing the level of H_2O_2 in infected tissues (Hung et al. 2006; Liu et al. 2008).

24 h after inoculation, peroxidase was activated locally at the site of contact between plant leaf epidermal cells and pathogen mycelium (Fig. 2a–d). It was stronger after plant pretreatment with SA and/or JA. In the last, color reaction was most intense in the zone of stomata in infected plants (Fig. 2d, e). The accumulation of phenolic compounds in cell walls of leaf epidermal cells in the zone of infection is known to be an important component of plant defense responses against pathogens. In our experiments, plant treatment with SA and JA induced this process (Fig. 2g–j). Phenolic compounds accumulated mostly after treatment with SA, which is evidently an important inducer of these compounds in potato plants. Similar changes have also been observed earlier after treatment with SA analog, benzothiodiazole (Hukkanen et al. 2007). In our experiments, accumulation of phenolic compounds in treated plants (Fig. 2f–j) was correlated with local peroxidase activation in the zone of infection (Fig. 2c, d).

Enzyme activity was detected not only in the space between stomatal guard cells but also on the pathogen cell walls (Fig. 2f). DAB did not stain *P. infestans* mycelium, grown on nutrient medium. Therefore, it may be suggested that local reaction in infected tissues is determined by the host extracellular peroxidases and interacting with the surface of *P. infestans* mycelium.

5 Affinity of Potato Isoperoxidases to *P. infestans* Cell Walls and Chitin

Some potato peroxidases are known to manifest ion-exchange affinity for chitin, the component of fungal cell walls. However, the cell walls of the late blight causal agent contains predominantly β-1,3-glucans (Hardham and Shan 2009). In our experiments, it was found that potato isoperoxidase with $pI \sim 9.3$ interacted mainly with *P. infestans* cell walls. As evident from Fig. 3 (lane g), this isoperoxidase was activated by JA treatment and infection.

In addition, such an increase in enzymatic activity could result from changes in the conformation of the enzymatic molecules due to the high electrostatic activity of polysaccharides (Dunand et al. 2002). It can be proposed that PO sorption on chitin could not be considered to be a classic ion exchange process because both the anionic and cationic isoforms of the plant POs interact with chitin (Maksimov et al. 2005). Additionally, it contains 3 high anionic POs (3.5, 3.7, 4.0) but only 2 of them (3.5 and 3.7) are adsorbed on chitin (Fig. 3a).
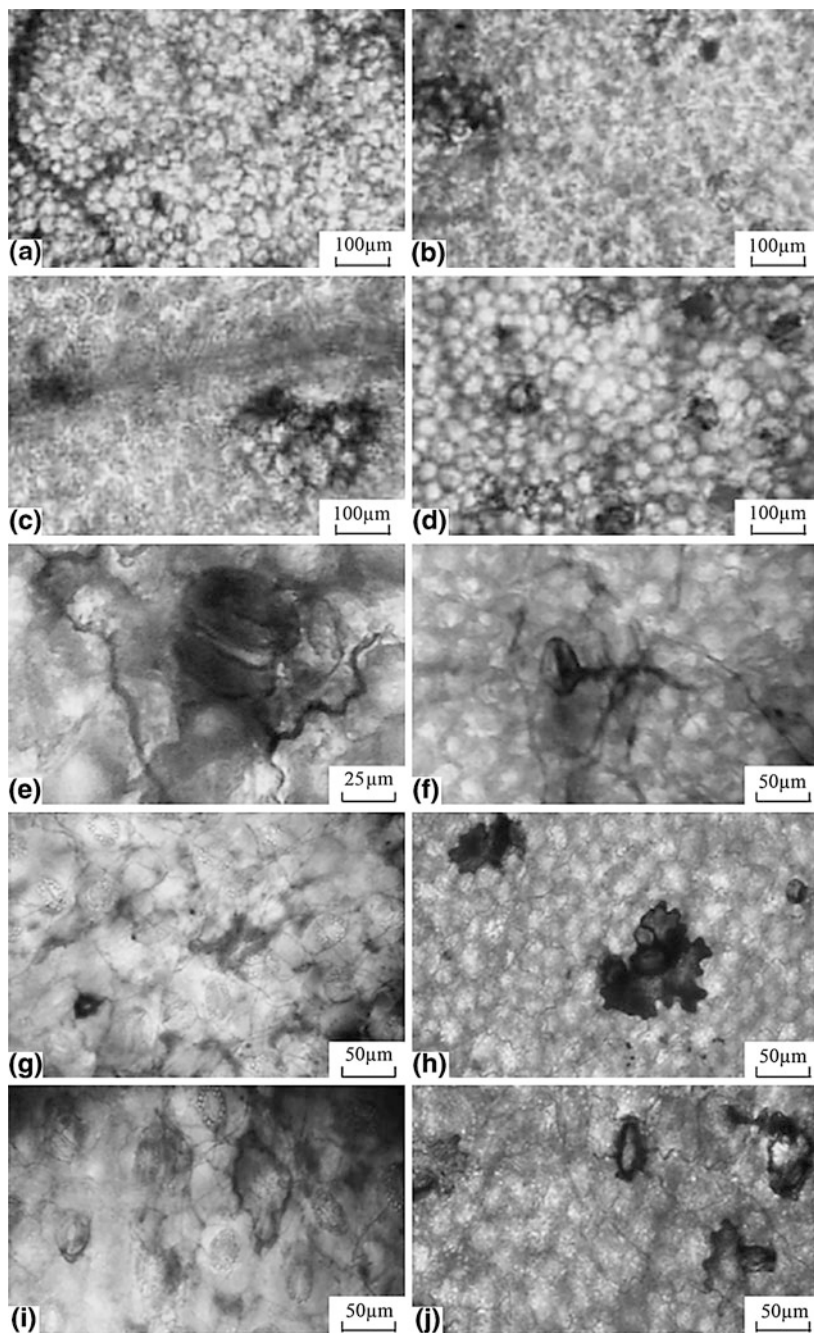
Fig. 2 Effects of SA and JA and their mixture on local activity of peroxidase (**a–f**) and accumulation of phenolic compounds (**g–j**) in the zone of infection with *P. infestans*. (**a**, **g**) Non-treated control; (**b**, **h**) SA; (**c**, **i**) JA; (**d**, **j**) SA + JA. Peroxidase activity in stomatal guard cells and intercellular spaces of adjoining epidermal leaf cells (**e**) and on the surface of mycelium contacting with stomata (**f**) in 48 h after inoculation
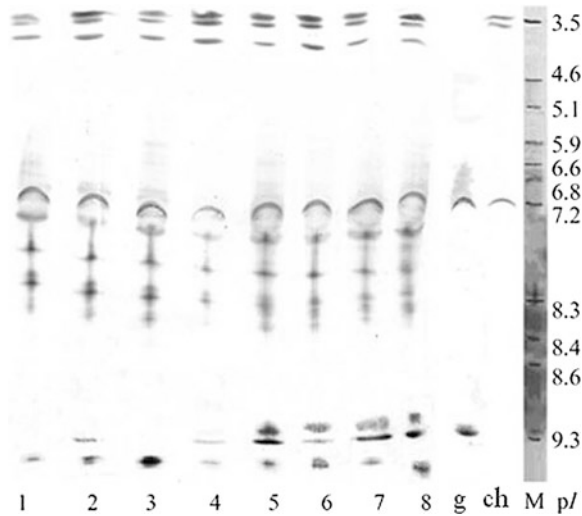
Fig. 3 Effects of infection with *P. infestans* and treatments with SA, JA, and their mixture on isoenzyme composition of isoperoxidases in tube-grown potato plants in 48 h after inoculation. (*1*) Nontreated control; (*2*) infection; (*3*) treatment with SA; (*4*) treatment with SA + infection; (*5*) treatment with JA; (*6*) treatment with JA + infection; (*7*) treatment with SA + JA; (*8*) treatment with SA + JA + infection; *g* - isoperoxidases interacting with *P. infestans* mycelium; *ch* - isoperoxidases interacting with chitin. *M* - marker proteins; p*I* - isoelectric poit

   In some cases, the anionic POs adsorbed on chitin have similar antigenic determinants, but the plants belonging to different families—and even members of the same family—could have polysaccharide-specific POs with different structures. Thus, the majority of investigated species had anionic chitin-specific peroxidases, and these isoforms from potato (*Solanaceae*) and horseradish (*Brassicaceae*) formed lines of precipitation with antibodies to wheat chitin-bound PO but not to anionic isoPO (Maksimov et al. 2010).

   Previously Roberts et al. (1988) defined the fragment of gene encoding one of potato anionic peroxidases M21334. We found that transcription of this gene in potato infected by *P. infestans* increased significantly in early stages of the defence reaction but decreased rapidly to the control level. In plants treated with SA and infected, transcription of M21334 was higher by 20–30 % and under the influence of JA–by 60–80 %.

   Treatment of plants by SA wasn't promote the accumulation of gene under study, however infecting of SA-treated plants by late blight pathogen led to a multifold increase of a number of transcripts. Possibly it isn't capable to induce an expression of *M21334* gene in potato plants but can sensitize genome to the perception of elicitor signal.
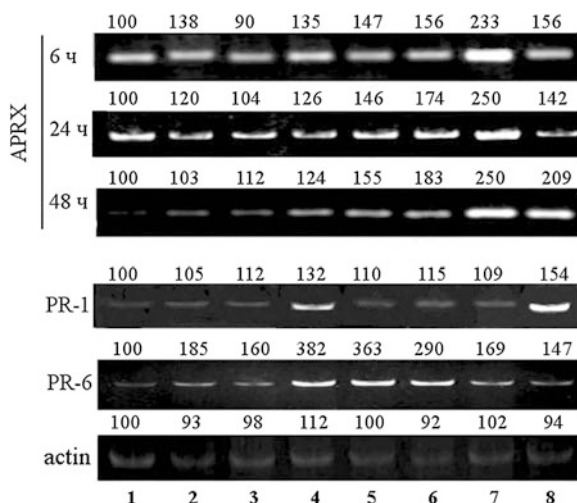
Fig. 4 Effects of infection with *P. infestans* and treatments with SA, JA, and their mixture on isoenzyme composition of isoperoxidases in tube-grown potato plants in 48 h after inoculation. (*1*) Non-treated control; (*2*) infection; (*3*) treatment with SA; (*4*) treatment with SA + infection; (*5*) treatment with JA; (*6*) treatment with JA + infection; (*7*) treatment with SA + JA; (*8*) treatment with SA + JA + infection; (*9*) isoperoxidases interacting with *P. infestans* mycelium. M—marker proteins, p*I*—isoelectric poit. Fig. 3a

Interestingly, the simultaneous influence of SA and JA led to maximal increase of transcriptional activity of this gene in non-infected plants (more than two times in comparison with control).

In literature, it is noted that simultaneous expression of genes encoding peroxidases depends on both SA and JA during the development of plant defence reaction against pathogens (Pieterse and van Loon 1999; Spoel et al. 2003). So, we can suppose that this isoform can be regulated both by SA and JA, but JA influences on its activity directly but for SA-mediated triggering requires the presence of some hypothetical elicitors during pathogen invading.

So, in infected plants treated with SA-JA mixture we observed rather high transcription activity of PR1 gene. However, expression of JA-inducible PR6 gene decreased by the addition of a mixture of SA and JA. We can assume that in this case suppression of SA-induced reaction by JA wasn't present, but SA suppressed JA-induced reactions (Fig. 4).

Presumably, for observing the combined influence of SA and JA on plants the important factors are period, concentration and sequence of treatments, as well as a method of introduction of these compounds. JA application of *Brassica nigra* and *B. oleraceae* increased total shoot glucosinolate levels 1.5–3 times, but at root level it did not increase. JA-application only at root yielded a systemic response. Plants treated with JA to both organs had profiles similar to shoot-treated plants. SA-application did not disturb the organ-specific response to JA (Van Dam et al. 2004).

A combined application of SA and JA, peroxidase activity was localized in the leaf cells surrounding stomata, which indicates that these intermediates induce efficient local response preventing pathogen penetration into the leaf tissues through stomata. It is of importance that, in this case, enzyme activity in the zone of infection was much higher than after treatments with these compounds separately. Concentrating soluble peroxidases in the zone of infection is in agreement with the capability of cationic isoperoxidases with $pI \sim 9.3$, which are activated in this protein fraction by JA and inoculation with the late blight causal agent, to ion exchange interaction with *P. infestans* cell walls. Thus, in our experiments, JA and its mixture with SA improved plant resistance more efficiently than SA alone by enhancing H_2O_2 generation and activating soluble peroxidases binding with the pathogen mycelium.

6 Features of the Regulation of SAR and ISR by PGPR and Symbiotic Microorganisms

Data about the role of PGPR and other symbiotic microorganisms in SAR or ISR is of a great practical importance (Van Loon 2007). Investigations of the induction of plant resistance to pathogens under the influence of these symbionts provide a basis for the demonstration of their efficiency and possibility of their use in agriculture. But mechanisms of detection and development of the defence reaction with participation of endophytes are not clear. In 1991 three research groups (Alström 1991; Van Peer et al. 1991; Wei et al. 1999) discovered independently that induced by some bacteria of *Pseudomonas*, resistance of plants to a pathogens is specific but has some differences from SA-dependent SAR. Therefore, the SA-independent resistance was named "induced systemic resistance" (ISR). It's to be noted that in some cases in our experiments the SAR and ISR were induced simultaneously by different agents by suppressing the expected defence reaction. So, this caveat must be considered for effective use of agricultural preparations with PGPR (Pieterse et al. 2007; De Vleesschauwer et al. 2008).

Inoculation by endophytes increased the resistance of plants to a range of diseases (Berg 2009) and abiotic stress factors (Bultman et al. 2004; Campanile et al. 2007; Popay 2009; Saunders and Kohn 2009). The possibility of the establishment of ISR under the influence of rhizobacteria was evidenced for 15 plant species and the main features of this long-term defence against fungi, bacteria and viruses (and nematodes or insects in some cases) was described (Van Loon 2007).

PGPR differentially increased the sensibility of genes (sensitized) participating in SAR and ISR, so their expression during the following defence reaction against pathogens was more significant in comparison with non-treated, controls (Ryu et al. 2004; Verhagen et al. 2010; Conn et al. 2008; Yang et al. 2009; Valenzuela-Soto et al. 2010). *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, as well as detected in wheat tissues strain *Streptomyces* sp. EN28 induced ISR

against *E. car.* ssp. *carotovora* in Arabidopsis (Ryu et al. 2004; Conn et al. 2008; Choudhary and Johri 2009). Strain *B. subtilis* BEB-DN induced the expression of ISR-associated genes and promoted the resistance to insects in tomato (Valenzuela-Soto et al. 2010). The influence of *P. fluorescens* WCS417r led to the activation of ISR-associated genes to increase in tomato plants resistance to *Pseudomonas syringae* pv. *tomato* DC3000, *Xantomonas campestris* pv. *armoraciae, E. car.* subsp. *cartovora, F. oxysporium* f sp. *raphani, Alternaria brassicola, Botritis cinerea* и *Hyaloperonospora parasitica* (Pieterse et al. 2007) as well as the influence of *Penicillium simplicissimum* GP17-2 and its culture filtrate promoted the resistance of Arabidopsis to *P. syringae* pv. *tomato* DC3000 (Hossain et al. 2007). Strains *P. fluorescens* CHA0 and *P. aeruoginosa* 7NSK2 induced ISR to *B. cinerea* due to the activation of mechanisms of oxidative burst and phytoalexins synthesis (Verhagen et al. 2010). However the resistance of tomato to oomycete *Hyaloperonospora parasitica*, ascomycetes *Botrytis cinerea, Alternaria brassicicola and* bacteria *P. syringae* pv. *tomato* DC3000 established under the influence of *Penicillium chrysogenum,* obviously, was independent of both SA and JA/ET signaling pathway (Thuerig et al. 2006). It should be noted that one of the important transcription factors of ISR MYB72, triggered by *Pseudomonas* (Pozo et al. 2008) can be connected with ET-signalling. The efficiency of this factor during the activation of JA-induced reactions was independent of ET directly but required co-factor regulated by ET (Van der Ent et al. 2008).

Series of works devoted to the search of the most sensitive to influence of PGPR genes obtained the rapid response of about 200 genes, part of them were up-regulated and the others down-regulated (ratio 1:1) by PGPR (Pozo et al. 2008; Yang et al. 2009; Valenzuela-Soto et al. 2010). The expression of 70 % of these genes under the influence of PGPR was associated with ISR, 13 % with SAR and ISR, and 17 % genes were regulated differentially. So, in the establishment of resistance of *Capsicum annuum* to causal agent of root rot *Xantomonas axonopodis* pv. *vesicatoria* under the influence of *B. cereus* BS107 was contributed by a range of genes encoding PR-proteins among them PR1 (SA-induced), PR4 and PR10 (JA- and ethylene- induced) and some genes sensitive to H_2O_2 (Yang et al. 2009). Strain *Bacillus vallismortis* EXTN-1 capable to stimulate the immune reaction in broad spectrum of plants particularly in cucumber *Cucumis sativus* triggered an expression of PR1 genes (Park et al. 2009). The expression of SAR-associated genes was detected simultaneously with ISR-associated, in pathosystem *Capsicum annuum*— *X. axonopodis* pv. *vesicatoria* under the influence of *B. cereus* BS107 (Yang et al. 2009). The resistance of Arabidopsis to *F. oxysporum* was induced by actinomycete strains *Micromonospora* sp. EN43 and *Streptomyces* sp. EN27 by activation of SAR-associated genes (Conn et al. 2008). The *Streptomyces* sp. EN27 SAR induced resistance was dependent on NPR1, by contrast, *Micromonospora* sp. EN43 promoted NPR1-independent resistance. Interestingly, the activation of expression of defence-related genes in plants infected by disease caused by causal agents only was detected. It means that PGPR has an important role in the sensibilization of plant defence systems (Verhagen et al. 2010; Pieterse et al. 2007). So, there are

some facts evidencing the participation of SAR-associated genes in the development of plant resistance induced by endophytes and PGPR despite the conventional belief of ISR-associated nature of this interaction (Pieterse et al. 2007).

PGPR and other symbionts can influence the signaling systems responsible for the formation of both SAR and ISR. Consequently, the data on their influence on the activity of key player of these pathways - NPR1 is very interesting. According to Pieterse et al. (2007) inoculation of *npr1* mutants by *P. fluorescens* WCS417r did not promote ISR. Influence of *Bacillus* is similar to *Pseudomonas,* but, apparently, strains of Bacillus can activate NPR1-independent defence systems resulting in ISR formation (Kloepper et al. 2004).

According to Van Loon (2007) the influence of PGPR on plant defence systems is similar to reactions induced by actual pathogens in resistant plants. It is due to the ability of endophytes to secrete a broad spectrum of exo-metabolites, more importantly phytohormones (Forchetti et al. 2007), oligosacchharides (similar to NOD-factors of rhizobium), SA and JA (Visca et al. 1993; Shanmugam and Narayanasamy 2009). Some elicitors of PGPR are reported as lipopolysaccharides and flagellin of bacteria cell walls, siderophores pseudobactin and pioceoline, antibiotics (Bakker et al. 2007; Pieterse et al. 2007; Van Loon 2007; Verhagen et al. 2010).

Flagellin of endophyte *Pseudomonas* having high elicit properties established ISR not always and wasn't an effective trigger (Bakker et al. 2007) and also possibly, did not always take part in interactions of plants and PGPRs (Gomes–Gomes and Boller 2002). As well as bacterial LPS were rather good elicitors of local immune reaction at the infection site. So, LPS triggered local defence reactions to nematode *Globodera pallida* in potato (Reitz et al. 2000). LPS derived from *Pseudomonas* WCS417r was able to stimulate ISR effective against *Fusarium* ssp in radish and carnation plants (Bakker et al. 2007). But Dow et al. (2005) reported that LPS of *Pseudomonas,* despite of their important role in triggering of ISR, did not participate in the establishment of ISR. It may be possible that the mechanism of LPS or flagellin influence on plants was limited to detect invaders in tissues and elicitation of non-specific local defence reaction.

At last we can assume that PGPR have other class of molecules that induce ISR which can be specific for each strain, produced and secreted extra-cellular: antibiotic peptides and universal signaling molecules such as ET, SA and JA. Production of these chemicals was claimed more than 10 years ago (Maurhofer et al. 1998; De Meyer et al. 1999).

It is important to note that PGPR are able to establish resistance of plants to a range of pathogens and also sensitize plant genome to probable pathogenic attack. Unfortunately mechanisms of regulation of plant immunity by PGPR and the role of phytohormones (such as SA and JA) are not probably understood.

7 Interaction Between SA- and JA-Mediated Signaling Systems and Endophytic Strain *Bacillus subtilis* 26d During the Defence Reactions of Potato Against *Phytophthora infestans*

We reported earlier that the combination of SA and JA promoted potato resistance to haemibiotrophic oomycete *P. infestans.* The literature has a number of evidences of JA-mediated nature of *B. subtilis* influences on plant defence reactions. Therefore, in the next step, we conducted an experiment by using a combination of SA, JA and *B. subtilis* 26D suspension for the investigation of these signaling pathways interactions, during pathogenesis. Indeed, treatment of potato by *B. subtilis* 26D was an effective method of its protection from late blight symptoms (Fig. 1). The use of bacterium suspension with SA (0.05 M) resulted in poorer symptoms development in comparison with only *B. subtilis* treated plants. It can be assumed that in this case *B. subtilis* initiated JA signaling as a substitute of JA. A combined influence of exogenic SA and components of JA pathway induced by bacterial strain promoted maximal resistance of plants to the late blight pathogen as above. However, it is more difficult to explain the fact that combined treatment of potato by JA and *B. subtilis* 26D decreased plant resistance to *P. infestans* significantly in spite of high defence stimulating activity of JA alone (Fig. 5).

The influence of SA and JA in combination with *B. subtilis* 26D on transcriptional activity of gene M21334, encoding anionic peroxidase during late blight pathogenesis was investigated. It was found that infection led to progressive accumulation of M21334 transcripts. On 48 h post-infection the expression of this
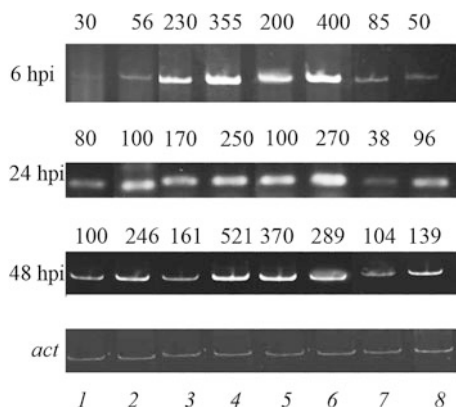
Fig. 5 The influence of SA, JA and *B. subtilis* 26D suspension on transcription of gene *M21334* encoding an anionic peroxidase of intact and infected by *P. infestans* potato plants (results normalized against transcription of gene encoding actine). (*1*) control; (*2*) *P. infestans*, (*3*) *B. subtilis*, (*4*) *B. subtilis* + *P. infestans*, (*5*) SA + *B. subtilis*, (*6*) SA + *B. subtilis* + *P. infestans*, (*7*) JA + *B. subtilis*, (*8*) JA + *B. subtilis* + *P. infestans*

gene, in infected plants was 2.5 times higher than in non-infected ones. In contrast the individual influence of *B. subtilis* 26D resulted in permanent maximal level of transcriptional activity of this gene in infected plants in comparison with non-treated control ones.

Simultaneous treatment of plants by a mix of SA and *B. subtilis* 26D led to rather high level of anionic peroxidase gene transcripts, i.e. the influence of Bacillus (like JA) is not being suppressed by SA. In infected plants treated by SA + *B. subtilis* 26D expression of *M21334* gene was highest among the all variants of the experiment. The simultaneous use of JA and *B. subtilis* 26D, activation of transcriptional activity of gene under study wasn't observed, but we found 50 % decrease of the expression of anionic peroxidase gene on the first day of post infection. It's to be noted that increasing of transcripts level was in inverse ratio to disease symptom development. Maximal expression of *M21334* led to minimal level of lesions.

According to our results, in potato plants during the development of the defence reactions against late blight pathogen JA-mediated signal system prevailed over SA-mediated system and was more efficient. Bacterial strain *B. subtilis* 26D displayed rather high defence stimulating activity similar to that of JA. Accordingly, on the basis of the data it can be possible to propose a preliminary hypothesis that ISR triggered by *B. subtilis* 26D is closely related to JA-mediated reactions. Another important conclusion based on our investigations is the possibility of using of composite preparation on the basis of SA and JA (or *B. subtilis* 26D) combines growth- and defence stimulating activities as well as SA-induced resistance to a rage of abiotic factors (Belkadhi et al. 2012).

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Chapter 13
Potential Benefits of Salicylic Acid in Food Production

R. Martín-Mex, A. Nexticapan-Garcez and A. Larqué-Saavedra

Abstract Plant species of Angiosperms and Gymnosperms applied with Salicylic acid respond in a positive manner when root system, flowering, stress or productivity is measured. Moreover, the published work indicates that Salicylic Acid application to plants of economic importance might be a good material to use it more widely to increase food production. The advantages to test this molecule is that (a) is a natural and eco-friendly product, (b) nanoquantities are required to produce positive effects, (c) is easy to be applied, and (d) is a cheap chemical available, almost anywhere.

1 Introduction

One of the objectives for this millennium, announced by the United Nations, is the global elimination of food poverty. This goal is undoubtedly far from being accomplished, as many developing countries have been unable to establish their policies for food security, which means that the strategies implemented by each country, along with the global strategies being carried out, must be carefully observed.

It is a well known fact that three permanent sources have been recognized as factors to improve food production. These are: biotechnology in its widest context, vegetable nutrition and biodiversity. It is in this context the purpose of this chapter is to present evidences that Salicylic Acid (SA) on its own could be consider as a chemical with the potential to enhance food production as is illustrated in the following diagram.

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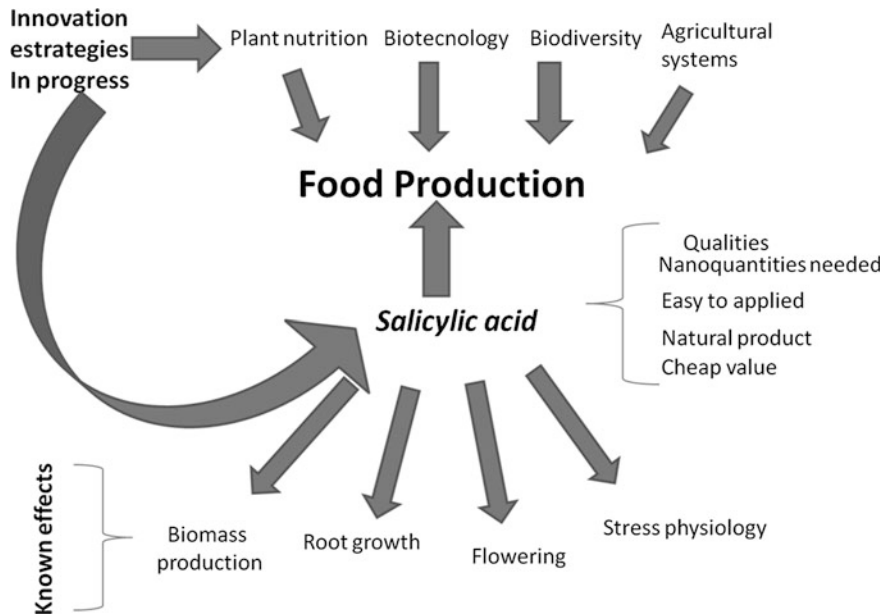
2 Physiological Studies of Exogenous Application of Salicylic Acid as a Basis to Increase Plant Productivity

(a) **Positive Effect of Salicylic Acid in Stress Physiology**

From the early 1970s, ongoing research and development has been carried out from which four basic contributions can be highlighted to support the premise that SA has potential in food production.

The first refers to 1978 when it was proposed that the application of aspirin to bean plantlets had an effect on the hydric status of the plant, a fact that was confirmed in specific bioassays carried out with stomata using the bioassay of *Commelina communis* epidermis strips (Larqué-Saavedra 1978, 1979). The news was given coverage by the international press, whose reports indicated that aspirin could be used to save crops in conditions of stress caused by drought (The Times 1978). This indication, to the effect that salicylates had the potential to participate in the physiology of plants subjected to stress, was later demonstrated by several authors thereafter (Shimakawa et al. 2012).

(b) **Positive Effect of Salicylic Acid in the Root System**

The second observation was when it was detected that SA favored growth in plant root systems (Gutiérrez-Coronado et al. 1998). This effect was estimated by the application of salicylates to intact soya beans plants, and was later validated in other plants. The discovery was confirmed using the bioassay of transformed

Catharantus roseus roots, in which concentrations at micromolar, nanomolar and femtomolar levels were sufficient to stimulate root growth and secondary root differentiation (Echevarria-Machado et al. 2007). This breakthrough was of great scientific importance as it demonstrated the sensitivity of plant tissue to the application of low concentrations of salicylates. Investigation carried out in bioassays with animal tissue has revealed no parallel reference to the high sensitivity registered in the case of plants.

Moreover, it has already been published that one micromole or less is sufficient to favor root growth, as in *Pinus patula* where concentrations of 1.0 and 0.01 μM increased root growth by 33 % and 30 %, respectively (San-Miguel et al. 2003), while in *Chrysanthemum* a concentration of 0.01 μM SA increased dry root weight significantly (Villanueva-Couoh et al. 2009). Dry root weight was also favored by the application of 1.0 μM or less in tobacco and cotton (Gutiérrez-Coronado et al. 1998).

This discovery is important because it was possible to demonstrate that SA affected the growth of one of the most important organs determining the productivity of plants, since it is generally accepted that one of the main functions of a root system is to extract water and nutrients from soil and transport them to the above-ground parts of the plant (Sperry et al. 2002). A great deal of work has been done towards developing the plant genetic material with larger roots for agricultural purposes, when production is the main objective. Larger and more vigorous root systems will contribute to better crops or horticultural plants (Bucher 2002).

(c) **Positive Effect of Salicylic Acid in Biomass Production**

The third contribution relevant to the effect of salicylates on plants resulted from the observation that plantlets sprayed with salicylic acid showed much greater vigor in comparison with control plants. In the case of papaya, for example, low concentrations of SA increased the height and diameter of the stem. Increases of 10 % in height and 3.5 % in diameter were obtained with picomolar applications of the product (Fig. 1)

This effect has been previously reported, indicating a greater accumulation of biomass in the case of Clitoria (Martin-Mex and Larqué-Saavedra 2001), and in

Fig. 1 Effect of foliar spraying with SA on stem height and diameter of Maradol papaya developed in field conditions. Values with the same letter are statistically equal (Tukey 0.05). Each value is the mean of 110 plants ± standard error
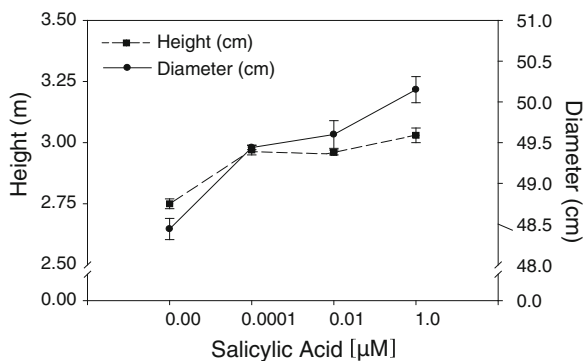
Fig. 2 Harvest index in wheat (relationship between grain yield and dry matter) grown in greenhouse and sprayed with different concentrations of SA (10, 1.0 and 0.01 uM)
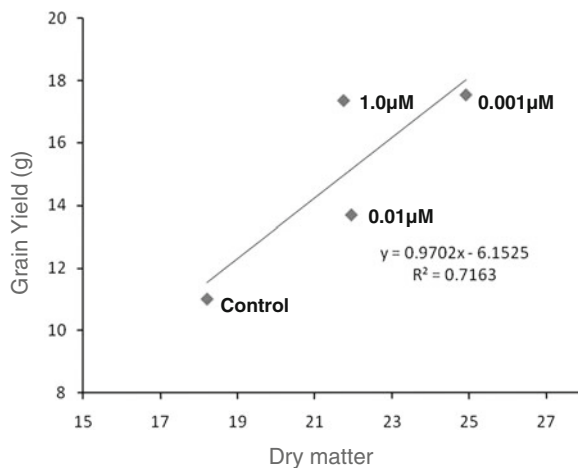
Gloxinia, Violet and *Tagetes erecta* (Larqué-Saavedra and Martín-Mex 2007; Sandoval-Yepiz 2004).

Ore recently, in other series of experiments with wheat, Hernández-Cervantes (unpublished data) demonstrated that the harvest index increased by 32 % in comparison with the control, as an effect of the application of 1.0 μM of salicylic acid (Fig. 2). The same effect of biomass increase by SA was reported for chrysanthemum and tomato, as shown in Table 1 and Fig. 3.

(d) **Positive Effect of Salicylic Acid on Flowering**

The fourth observation is in relation to the impact of salicylic acid on flowering in ornamental plants: In 1974, studies by Cleland and Ajami demonstrated that application of SA induced flowering in *Lemna gibba,* and indicated that it substituted photoperiodic requirements. Since then, numerous experiments have been conducted demonstrating the effect of SA on plants such as *Arabidopsi*s. Since the beginning of the year 2000, our group has demonstrated that, in ornamental plants developed in pots, such as Violet (*Saintpaulia Ionantha* Wendl.) (Martin-Mex et al. 2005) *Chrysanthemum morifolium* (Villanueva-Couoh et al. 2009) *Petunia* (Martín-Mex et al. 2010) and Gloxinia (*Sinningia speciosa* Benth.) (unpublished data). SA induced these species to produce significantly more flowers per plant. (Table 2, Figs. 4 and 5)

The economic impact of these results on commercial floriculture could be quite significant.

These four categories of effects reported for SA, resulting from continuous assays carried out at all levels; laboratory, greenhouse and field, motivated the proposal to test SA for increasing the productivity of food species that are of particular importance to humans, such as horticultural products and cereals.

Table 1 Effect of salicylic acid on height (cm) and diameter of *Chrysanthemum* plants var. Polaris White at 113 days after transplanting

SA treatment (μM)	Height (cm)	Stem diameter (mm)
0.0	75	6.3
0.00001	80	7.6
0.00000001	100	8.7
0.0000000001	93	8
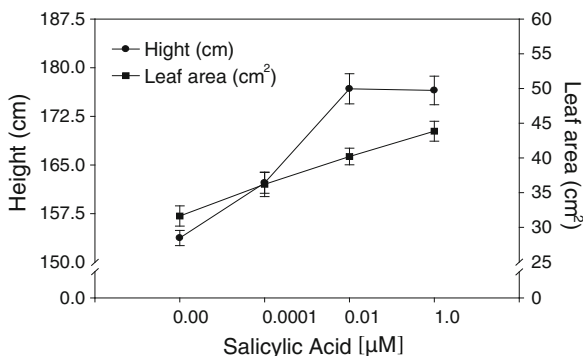
Fig. 3 Effect of different concentrations of Salicylic Acid on tomato plantlets (Lycopersicon esculentum cv. Maya), 20 days after emergence. Values with the same letter within columns are equal according to the Tukey test ($P \leq 0.05$). Each value is the mean of 24 replicates ± standard error

Table 2 The effect of salicylic acid on the number of flowers exposed in petunia, gloxinia and violet plants grown under greenhouse conditions. Same letter means no significant differences Tukey ($P < -0.05$)

SA Treatments	Number of flowers/plant		
	Petunia	Gloxinia	Violet
0.0	76	8	8
0.000001	99		
0.0001	104		
0.001		10	14
0.01	119	11	11
1.0	130	10	10

3 Impact of Salicylic Acid on Productivity of Plants of Economic Importance

To analyze the potential benefits of SA to enhance food production an extended series of experiments were conducted (1) with different plant species of interest for food production, (2) in different agro-ecosystems; taking into account climatic conditions of soil, temperature, humidity and the quality of water for irrigation,

Fig. 4 Effect of salicylic acid spray on the number (**a**), length (**b**), and width (**c**) of gloxinia flowers (*Sinningia speciosa*). $P \geq 0.05$, Tukey's. Same letter means no significant differences
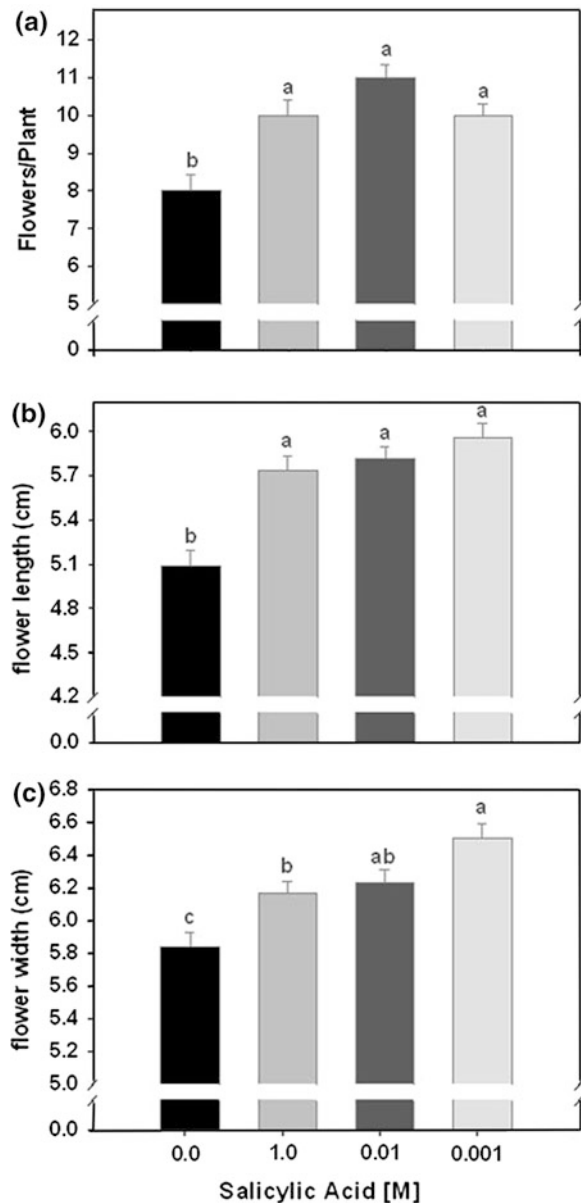
(3) employing the regular cultural practices used by the producers, with the only variant being the presence of SA, and (4) working directly with the producers.

The basic task was to demonstrate if there was in fact any effect, and if so, to specify "where, when, how, and the concentration of SA to apply", that are the fundamental part of the practices to be defined in a study of this type in the agricultural sector.

Fig. 5 Effect of salicylic acid spray on the number of flowers of African violet (*Sinningia speciosa*) plants. $P \geq 0.05$, Tukey's. Same letter means no significant differences
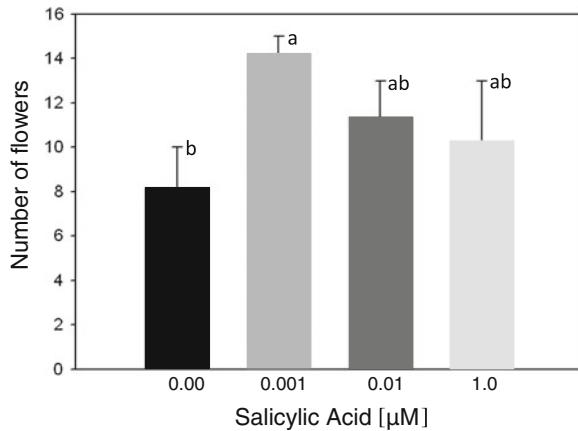
3.1 Effect of Salicylic Acid on the Productivity of Horticultural Plants

A series of experiments were planned with agribusinesses producing horticultural products in greenhouse conditions and outdoors; in following species with a high commercial value for export.

- Saladette tomato (*Lycopersicon esculentum*)
- Bunch tomato (*Lycopersicon esculentum*)
- Ball Tomato (*Lycopersicon esculentum*)
- Bell Pepper (*Capsicum annum*)
- European cucumber (*Cucumis sativus L.*)
- Habanero pepper *(Capsicum chínense)*
- Papaya *(Carica papaya)*

The experiments were conducted using cutting edge technology for intensive production in commercial greenhouses. Selected cultivars were of the highest commercial value, with highly rated seeds. Standard drip irrigation and the other cultural practices required in the intensive vegetable agribusiness were used throughout the development of the crop. Similarly, plant health control and fertilization of the substrate were carried out according to the recommendations for this type of agribusiness.

SA was sprayed on the plantlets in the first stage of development in seedbeds before transplanting. The recommended experimental designs were used to validate the effect of SA on the productivity of the vegetable species.

3.1.1 Effect of SA on the Productivity of Tomato (*Lycopersicon esculentum*)

The experiments were conducted at the facilities of the well known firm Pequeña Joya in La Paz, Baja California, Mexico, and a summary of the results from the application of different concentrations of salicylic spray can be seen in Fig. 6.

3.1.2 Effect of SA on the Productivity of Bell Pepper (*Capsicum annum*)

Similarly, experiments were established to estimate the effect of SA on other vegetables of commercial interest such as bell pepper (*Capsicum annum*).

The similarity in the pattern of the results obtained from two independent experiments conducted in greenhouse conditions can be appreciated, in spite of the fact that they were carried out two thousand kilometers from each other, one in the tropical area and the other in the dry arid zone of Mexico (Fig. 7).

3.1.3 Effect of SA on the Productivity of European Cucumber (*Cucumis sativum* L)

Cucumber is considered to be a horticultural plant which demands careful cultural practices and expert management. Experiments were established in two localities with extreme climates, one in the semi-desert area of northern Mexico and the other in the tropical area. The results are shown in Fig. 8.

Using the same contrasting approach of ecological conditions, the productivity and quality of the fruit was estimated. In every case, the application of SA increased the production of fruits by 33 %, while conserving the highest quality required for this vegetable.

3.1.4 Effect of SA on the Productivity of Habanero Pepper (*Capsicum chínense*)

The chili pepper is one of the most important horticultural crops in Mexico. Experiments were established in field conditions in the tropical area of the country, in three localities of Yucatan peninsula; separated from each other by approximately 200 km, Conkal, Tizimin, and Calkini.

The regular cultural practices traditionally used by the producers of Habanero pepper were employed, with the only variant being salicylic acid sprays at concentrations of 1 or less micromoles to estimate its effect on fruit production.

The results are shown in Fig. 9 where one can appreciate that, in every case, the application of SA increased fruit production by 30 % on an average.

Fig. 6 Effect of SA on the productivity of fruits from three varieties of Tomato (*Lycopersicon esculentum* Mill) **a** saladette, **b** bunch, **c** ball
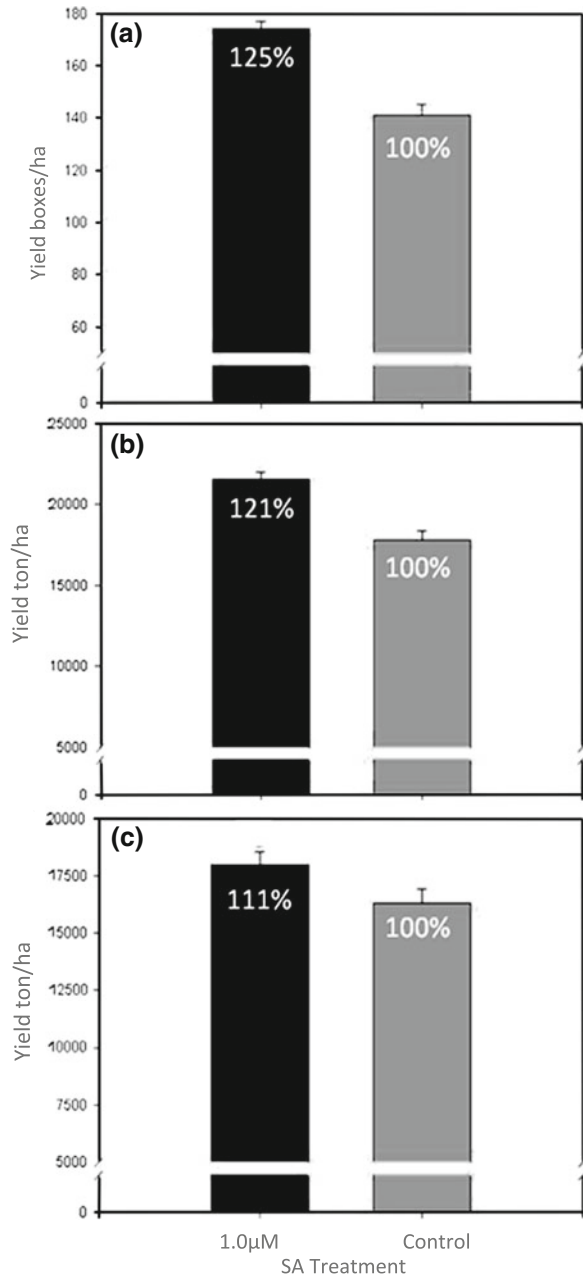
3.1.5 Effect of SA on the Productivity of Papaya

Over a period of two years, an experiment was conducted with papaya (*Carica papaya*) cv Maradol in the fields of producers in Yucatan. The effect of spray

Fig. 7 Effect of SA on the
productivity of Bell Pepper
(*Capsicum annum*) expressed
in kg for m² in **a** Timucuy,
Yucatán; number of boxes
with prime quality produce
for exportation in **b** La Paz,
Baja California Sur, México,
Tukey's $P \geq 0.05$. Same
letter means no significant
differences
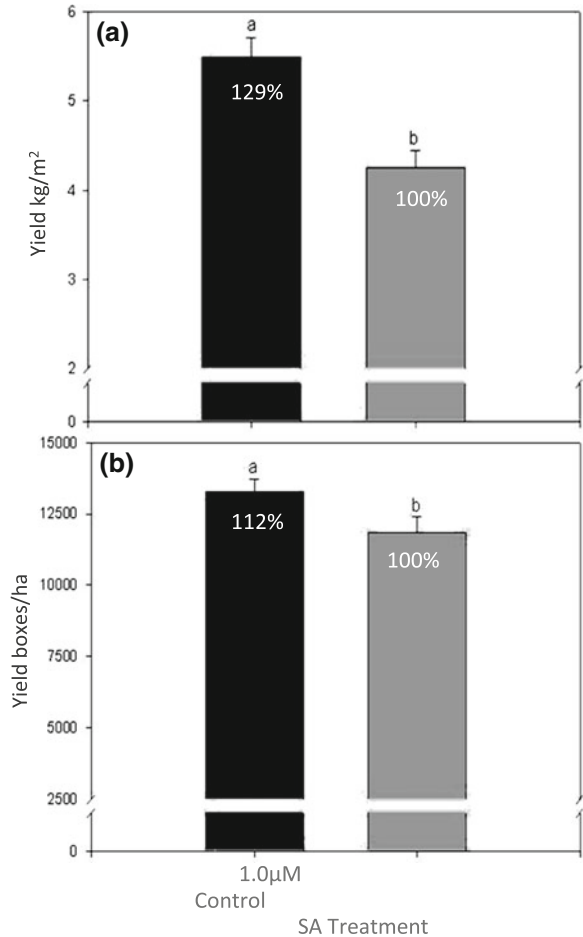
applications of SA at low concentrations on fruit productivity was studied. SA at
concentrations of 0.01 μM increased the number of fruits per plant by 19.7 % and
the yield per hectare by 21.9 % (Fig. 10).

No reports were found in the literature, consulted regarding the effect on
papaya, although the effect of SA on fruit production in tomato and cucumber has
been reported (Larqué-Saavedra and Martín-Mex 2007).

3.2 Effect of Salicylic Acid on the Productivity of Grammineae Plants

The importance of grains as a source of nourishment is undeniable; therefore,
based on the studies described above, experiments were conducted to determine if

Fig. 8 Effect of SA on the productivity of European cucumber (*Cucumis sativum* L) expressed in the number of boxes with prime quality fruit for exportation **a** Felipe Carrillo Puerto, Quintana Roo, **b** La Paz, BCS. Tukey's $P \geq 0.05$. Same letter means no significant differences
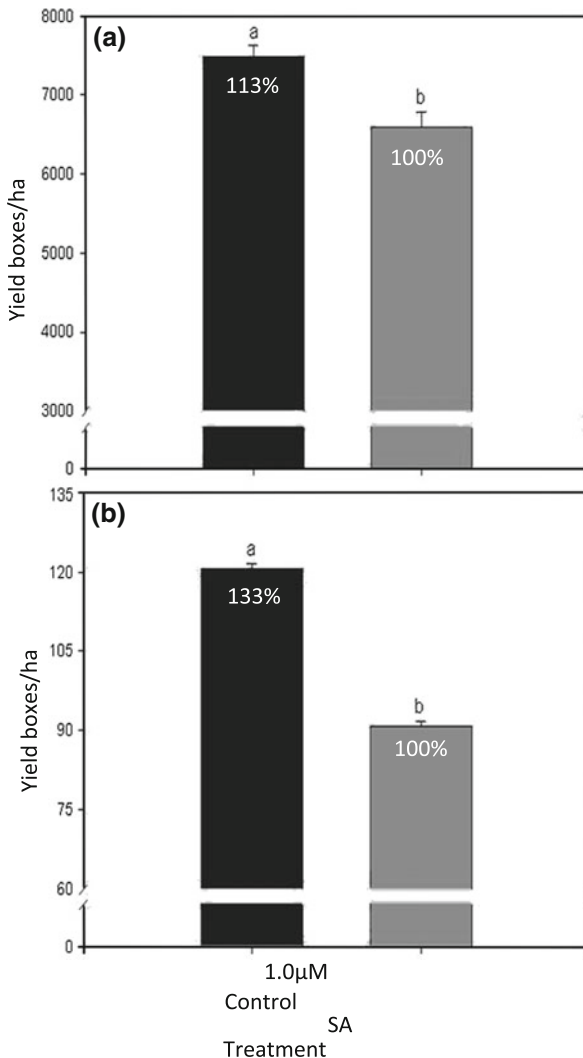
SA could have an effect on the productivity of these plants of great social significance.

Studies to estimate the effect of salicylic acid on grain productivity commenced at the beginning of 1980s. However, the data published showed a certain degree of inconsistency. In 2011, Hernández-Lopez, J.C. (unpublished data), investigated the effect of SA on grammineae, taking into consideration primarily to demonstrate the effect of SA on root growth and thereafter its effect on grain production.

The results reported by Hernandez-Lopez, J.C. are shown in Table 3, in which the response pattern of this species to this growth regulator can be seen. The application of SA always resulted in a higher biomass of the root system.

Fig. 9 Effect of Salicylic Acid on the productivity of Habanero pepper in three localities of the peninsula of Yucatan **a** Conkal, Yucatán, **b** Tizimín; Yucatán, and **c** Calkini, Campeche. Tukey's $P \geq 0.05$. Same letter means no significant differences
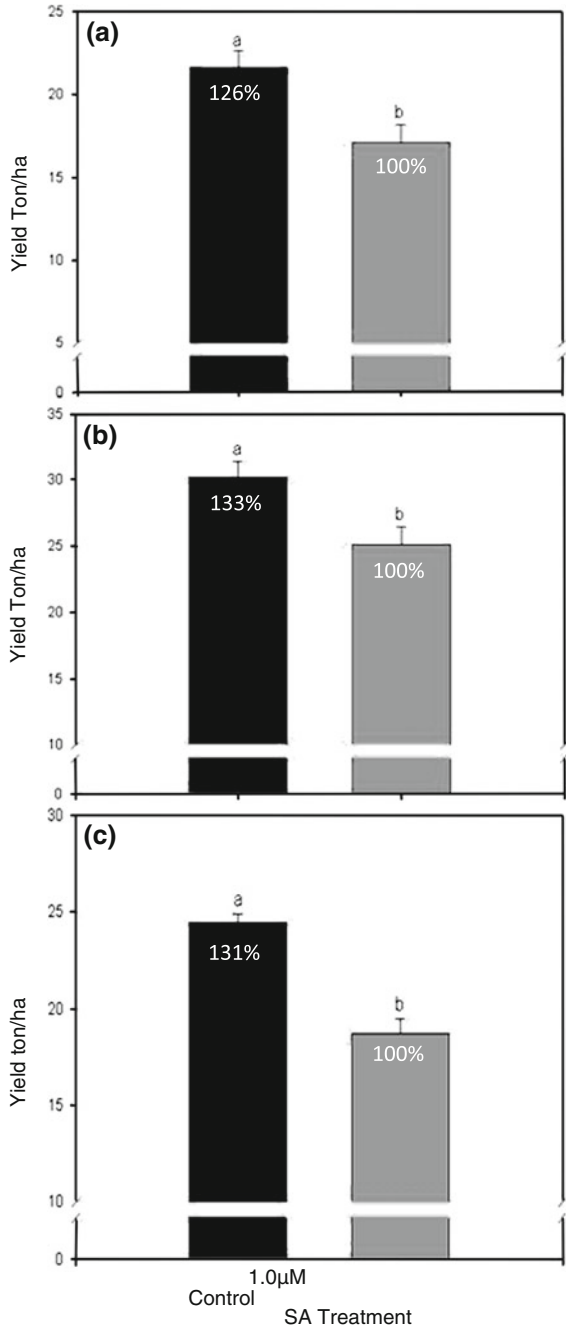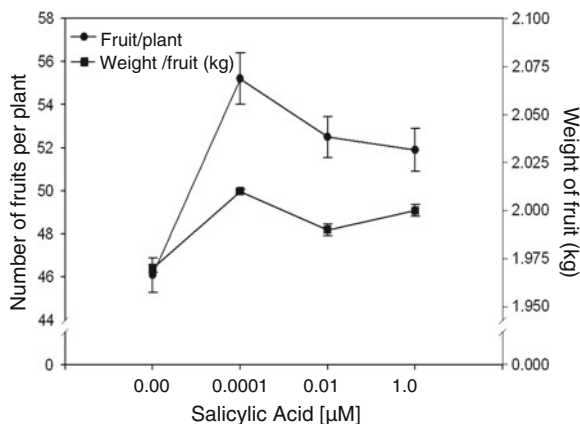
Fig. 10 Effect of foliar spraying of SA, on the yield of papaya Maradol under field conditions. Values with the same letter are statistically equal (Tukey 0.05). Each value is the mean of 110 plantlets ± standard error

Table 3 Effect of different treatments of SA, applied to seedling shoots, on the dry weight of the roots. Each data is the mean value of six readings ± standard error. Analysis of variance is shown (MSD $\alpha = 0.05$). Similar letter means no significant result

SA treatments	Root biomass (g)
0.0	10.22 ± 1.85a
0.01	12.32 ± 1.83a
1.0	12.27 ± 1.76a
10.0	11.15 ± 1.48a

Fig. 11 Effect of different treatments of SA, applied to seedling shoots, on the grain yield of wheat. Each data is the mean value of six readings ± standard error. Analysis of variance is shown (MSD $\alpha = 0.05$). Similar letter means no significant value
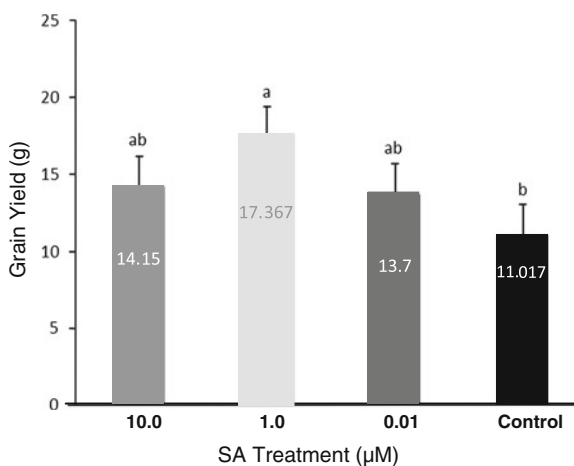
Such results are consistent with those reported by Echevarria-Machado et al. (2007) using transformed root bioassays of *Catharantus roseus*, and also with those reported by Larqué-Saavedra et al. (2010) for *Lycopersicon esculentum*, where concentrations as low as femtomolar favor root length.

The grain yield harvested in a parallel identical experiment with wheat plants treated with SA is shown in Fig. 11. From the pattern of this parameter, we can appreciate that all the concentrations of SA tested increased grain yield. The 1.0 μM SA treatment was significantly different and, therefore it was most effective with a 36.5 % increase in comparison with the control.

The result on grain yield obtained by Hernández Lopez, J.C. is consistent with those reported by Garcia (1982), who sprayed graded concentrations of acetyl-salicylic acid (ASA) ranging from 10^{-2} to 10^{-7} M on wheat plants, under greenhouse conditions and in the field. It was noted that concentrations of SA promoted grain production. According to Garcia, SA at a concentration of 10^{-2} M, had a toxic effect on the plants, but as the concentrations diminished, the yield showed a corresponding increase. It is also important to mention here that the results obtained by López et al. (1998) who reported that SA sprayed on wheat plants under field conditions, favors the number of grains per year by 2.19 % with a concentration of 10^{-6} M, and 7.7 % with 10^{-4} M, in comparison with the control.

However it must be emphasized that the publications mentioned above did not took into account the pH which is of great importance for this kind of study that might explain the toxic effect of SA reported by them.

4 Conclusions

1. The data referred to in the previous paragraphs gives support to the proposal that the application of salicylic acid spray to the foliage has a significant effect on flower production in ornamental plants, a fact which will undoubtedly have positive economic repercussions on floriculture.
2. The data obtained with plants of horticultural importance and papaya are consistent, in that productivity is favored, regardless of growth conditions, which can range from humid tropics to semi-desert areas; under greenhouse conditions or outdoor.
3. The data reported for grains are particularly encouraging, and the reproduction of these results in commercial plantations could represent a serious alternative to enhance food production.

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Chapter 14
Short and Long Term Effects of Salicylic Acid on Protection to Phytoplasma Associated Stress in Potato Plants

H. A. López-Delgado, M. E. Mora-Herrera, R. Martínez-Gutiérrez and S. Sánchez-Rojo

Abstract Salicylic acid (SA) activated the plant defense response in potato against phytoplasma attack, reduced infection symptoms, favored photosynthates translocation and improved the quality of tubers. SA induced effects at short and long terms and it was equally efficient when it was first applied on in vitro culture followed of transplanting or directly sprayed on greenhouse conditions. Low levels of exogenous SA (0.1 and 0.001 mM) showed higher biological activity. The reduction of damage was associated to high hydrogen peroxide and ascorbic acid contents, together with reduction of peroxidase activity suggesting an important role of SA on the regulation of these molecules and counteracting the pathogens effects.

Keywords Salicylic acid · Phytoplasma · Long term effects · Potato resistance · Biotic stress

1 Introduction

Phytoplasmas are bacteria in the class Mollicutes lacking cell walls that inhabit plant phloem sieve tubes (Liefting et al. 2004). They are responsible for several hundred plants diseases worldwide, affecting many economically important plants such as vineyard plants, tomatoes, and potatoes (Doi et al. 1967). Infected plants show a wide range of symptoms, such as 'witches' broom proliferation, flower

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alterations, stunted growth, and general decline (Christensen et al. 2005). Phytoplasmas do not possess genes for the *de novo* synthesis of amino acids, fatty acids, or nucleotides. They presumably cannot metabolize sugars and obtain their food supply from the high sugar concentrations in plant sieve tubes (Oshima et al. 2004).

In Mexico, at least two different types 16SrI and 16SrII of phytoplasma associated with potato purple top (PPT) symptoms have been reported using polymerase chain reaction (PCR) technology (Almeyda et al. 2001; Leyva et al. 2002).

Symptoms include foliar short internodes, stem thickening, apical rolling, purple leaflet coloration, chlorosis, axillary shoot proliferation, internode shortening, and aerial tuber formation. Tubers show different degrees of internal browning and produce abnormally thin, weak or absent sprouts, although in some varieties sprouting seems to be unaffected (Lee et al. 2000; Cadena-Hinojosa et al. 2003). Similar symptoms are produced when phloem transport of photosynthates is affected, such as diseases like PPT, psillid yellow, and Zebra Chip (Secor and Rivera 2004; Secor et al. 2009). *Candidatus Liberibacter psyllaurous*, a newly identified bacterial species, is associated with Zebra Chip symptoms (Hansen et al. 2008).

Under biotic stress conditions, plant defense responses include phytoalexin induction, reactive oxygen species (ROS) production, lignin and callose accumulation for structural strength, and antioxidant enhancement to counter pro-oxidants produced during the attack (Bolwell 1999; Baker et al. 2005). To minimize ROS damaging effects, aerobic organisms evolved both non-enzymatic and enzymatic antioxidants. Non-enzymatic defenses include compounds with intrinsic antioxidant properties such as ascorbic acid (AA), glutathione, and ß-carotene. Purely enzymatic defenses, such as superoxide dismutases (SOD), catalases (CAT), and peroxidases (POX) protect by directly scavenging superoxide radicals and hydrogen peroxide (H_2O_2), converting them to less reactive species (Scandalios 2005).

ROS can damage cellular elements but also are important as signals in transcriptional and pos-transcriptional regulation of gene expression, mediating plant response to biotic and abiotic stress (Foyer et al. 1994a, b; Dat et al. 2000; Foyer and Noctor 2005), leading to acclimation and cross tolerance in plants (Neill et al. 2002a, b; Bhattacharjee 2005).

H_2O_2 is one of the most important ROS in plants, it is produced continuously during the metabolism and the internal levels are enhanced as response to biotic or abiotic stress (Neill et al. 2002b; Desikan et al. 2004). The role of H_2O_2 in signaling responses to stress has been demonstrated during, hypersensitive response and systemic acquired resistance (Levine et al. 1994); production of proteins related to pathogenesis (León et al. 1995), cross protection and acclimation (Levine et al. 1994; Foyer et al. 1997; Gong et al. 2001; Neill et al. 2002a) and in different adaptive responses to stress (Desikan et al. 2001, 2004). Peroxidases have been associated with an ever-increasing number of physiological processes (Mehlhorn et al. 1996), including resistance to pathogens (Sgherri et al. 2001). Peroxidases act scavenging H_2O_2 to H_2O with a specific substrate

$(RH_2 + H_2O_2 \rightarrow R + 2H_2O)$. POX works in cell wall inhibiting growth (Djakovic and Jovanovic 2003).

AA is the major non-enzymatic antioxidant, protecting plants from oxidative damage resulting from aerobic metabolism and oxidative stress through the ascorbate–glutathione cycle, and participating in plant stress responses in association with other antioxidant system components (Smirnoff 1996, 2000). AA participates in many physiological process such as: photosynthesis, enzymatic cofactor, homeostasis of redox system (Smirnoff and Wheeler 2000), it is involved in growth, development and in modulation of cellular cycle and/or division (de Pinto and De Gara 2004) and cell elongation (Kato and Esaka 1999). It was proposed that AA from apoplast is the first defense mechanism against the damage of antioxidants such as ozone, SO_2 and NO_2 (Barnes et al. 2002).

Salicylic acid (SA) is involved in induction of tolerance response against stress, mainly through the oxidative and antioxidant system. High SA accumulation has been associated with many stress responses (Hayat et al. 2009).

Plants react to pathogen attack with an array of inducible defense mechanisms integrated by a complex signaling system, leading to protein phosphorylation cascades and transcription factor activation (Hayat et al. 2009). SA is important in the induction of resistance responses and is a key regulator of SAR and PR genes (Sticher et al. 1997; Dangl and Jones 2001). Mauch-Mani and Metraux (1998) reported that over-expression or suppression of putative candidate genes is involved in SAR, and SA has been identified as a signal molecule.

Exogenous SA application at non-toxic concentrations can regulate biotic and abiotic stress (Elwan and El-Hamahmy 2009). Exogenous application of SA or acetyl-salicylic acid (ASA) induce pathogenesis-related (PR) gene expression that promote resistance against several viral, fungal, and bacterial pathogens in a variety of dicots and monocots (Hayat et al. 2009). SA induction by H_2O_2 and SA inactivation of CAT both function in a cyclic mechanism to amplify the SA/H_2O_2 signal (León et al. 1995). SA and H_2O_2 are mediating the systemic acquired resistance (RSA; Levine et al. 1994). SA triggers a signaling cascade leading to blocking virus replication and transmission cell–cell and at a distance during virus infection (Singh et al. 2004). SA is known as the main SAR regulator and of the pathogenesis related gene induction. On the other hand, it contributes with HR and cell death producing high ROS production (Raffaele et al. 2006).

SA mediates H_2O_2 accumulation and protects against stress (López-Delgado et al. 1998; Scott et al. 1999; Dat et al. 2000; López-Delgado et al. 2004; Chao et al. 2009).

SA plays an important role in protection from biotic and abiotic stresses by regulating the antioxidant system (Horváth et al. 2007; He and Zhu 2008; Hayat et al. 2009).

In potato microplants, SA enhanced potato virus X elimination after thermotherapy and was associated with CAT activity inhibition and H_2O_2 accumulation (López-Delgado et al. 2004).

SA-induction of tolerance to extreme temperatures was mediated by the antioxidant system in cereals (Janda et al. 2003; Horváth et al. 2007; Yordanova and

Popova 2007), potato microplants (Mora-Herrera et al. 2005), and in orange (Huang et al. 2008).

Information about the SA effect on phytoplasma-induced disease is limited. Previously, phytoplasma-damage reduction by sprayed SA was associated with high hydrogen peroxide and ascorbic acid contents together with reduction of peroxidase activity, favored photosynthate translocation, and improved tuber quality, suggesting an important role of SA regulating these molecules counteracting pathogen effects (Sanchez-Rojo et al. 2011), however, the potential long term effects of SA is poorly understood, even less is known about phytoplasma-damage reduction in potato.

Hence, the present study evaluated the SA long-term effect on reducing phytoplasma-caused disease symptoms and physiological responses such as biomass accumulation, POX activity, and AA and H_2O_2 contents.

2 Materials and Methods

2.1 Plant Material

Virus-free *Solanum tuberosum* L. cv. Alpha microplants were obtained from the in vitro Germoplasm Bank of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias at Metepec, México. The plants were also negative to *C. Liberibacter* spp. Phytoplasma-infected potato plants were obtained from those with PPT symptoms under natural conditions. Single node cuttings were in vitro propagated in test tubes in Murashige and Skoog (1962) medium at 20 ± 1 C under 16 h photoperiod (fluorescent lights, 35 μmol m^{-2} s^{-1}, 400–700 nm; Espinoza et al. 1986).

2.2 Chemical Treatments

In vitro. Single node cuttings were obtained from in vitro phytoplasma infected plants and subcultured in test tubes containing Murashige and Skoog (1962) medium added with SA (0, 0.1, 0.01 and 0.001 mM) and incubated at 20 ± 1 C under 16 h of photoperiod (fluorescent lights, 35 μmol.m^{-2} s^{-1}, 400–700 nm). Negative and positive phytoplasma plants were micropropagated without SA as controls. Twenty to twenty five microplants 28–32-days-old from each treatment were transplanted to greenhouse in pots containing peat-moss. All plants were fertilized each 15 days and watered twice a week (Fig. 1).

Greenhouse. Twenty to twenty five in vitro phytoplasma infected plants 28–32-days-old were transplanted to greenhouse in pots containing peat-moss. Negative and positive phytoplasma plants were sprayed twice a week with SA 0, 0.1, 0.01
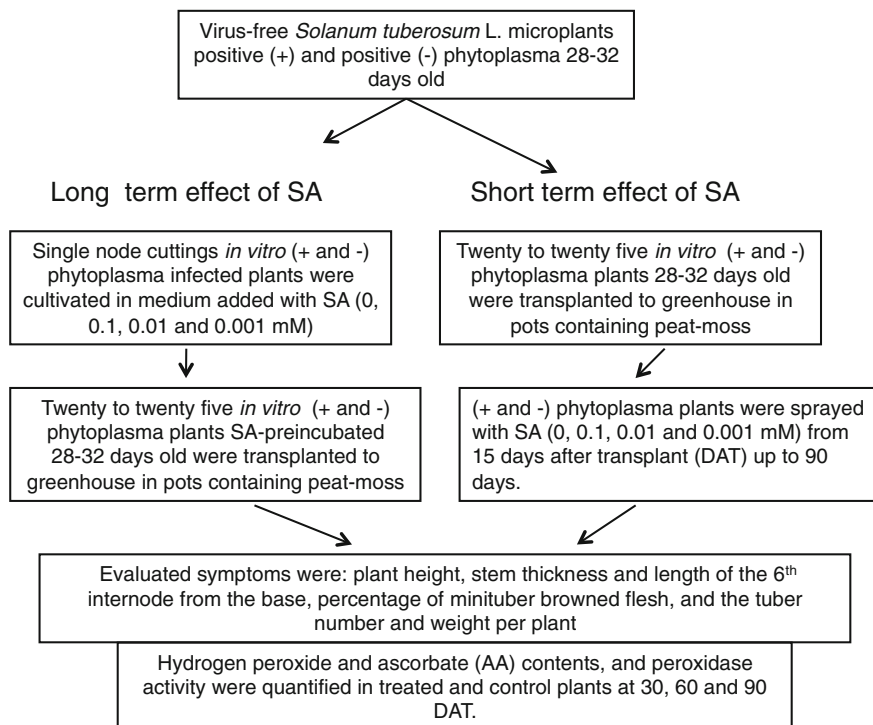
Fig. 1 Schematization of methodology for short and long term effects of SA, in phytoplasma-infected potato plants

and 0.001 mM pH 5.6–5.7, from 15 days after transplant (DAT) up to 90 days. Positive and negative controls were sprayed with distilled water (pH 5.6–5.7; Fig. 2).

Plants were maintained under greenhouse conditions for 95 days and each pot corresponded to an experimental unit. All plants were fertilized each 15 days and watered twice a week.

2.3 Symptom Analysis

Ninety DAT, stem and tuber symptoms were analyzed for 10 plants of each experimental unit. The evaluated symptoms were: plant height, stem thickness and length by the 6th internode from the base, percentage of browned mintuber flesh, and the tuber number and weight per plant.
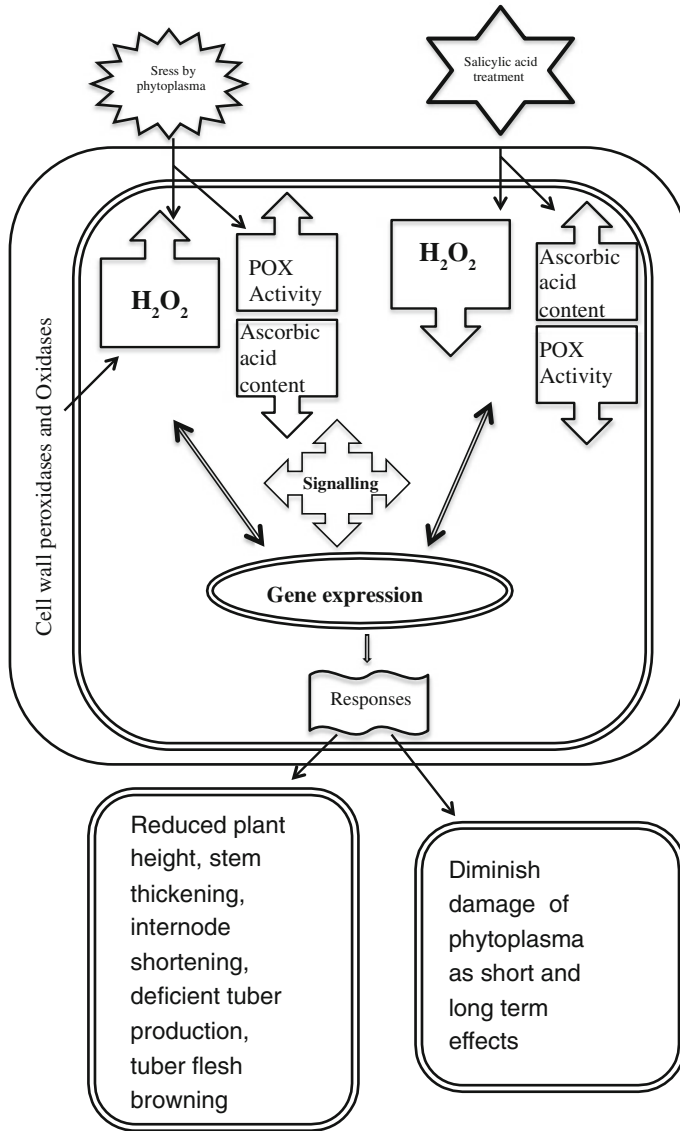
Fig. 2 Responses of phytoplasma-infected potato plants to SA treatment

2.4 H$_2$O$_2$ Content and Antioxidant Activity

Hydrogen peroxide content, peroxidase activity and ascorbate (AA) content were quantified in treated and control plants at 30, 60 and 90 DAT.

2.4.1 Determination of H_2O_2 Content

Leaf tissue (0.25 g) frozen in liquid nitrogen was powdered, extracted in 1.2 mL ice-cold 5 % (v/w) trichloroacetic acid (TCA) and clarified by centrifugation (15 min at 11,000 g) at 4 C. Samples (0.5 mL) of supernatants were passed through 0.5 g of Dowex-1 resin (Dow Chemical Company, Midland, MI) followed with 3.5 mL of 5 % TCA. H_2O_2 content was measured in the eluates using the luminol-dependent chemiluminescence method of Mora-Herrera et al. (2005): 0.5 mL of eluates was added to 0.5 mL luminol, the volume was made up to 4.5 mL with 0.2 M NH_4 OH (pH 9), and 450 mL of this mixture was analyzed using a Optocomp P luminometer (MGM Instruments, USA). Chemiluminescence was initiated by injecting 50 mL of 0.5 mM potassium ferricyanide in 0.2 M of NH_4 OH, and emitted photons were counted over 5 s. A parallel sample of each initial extract was processed after addition of a known concentration of H_2O_2 to provide a recovery correction factor b.

2.4.2 Peroxidase Activity

Leaf tissue (0.5 g) was crushed into fine powder in a mortar and pestle under liquid nitrogen. Soluble protein was extracted by homogenizing the powder in 2 mL of 50 mM potassium phosphate buffer (pH 7.2) containing 5 mM DTT, 1 mM EDTA and 1 % PVP (Anderson et al. 1995). Insoluble materials were removed by centrifugation at 11,000 g for 15 min at 4 C. Peroxidase activity was determined according to the Mora-Herrera and López-Delgado (2007) method. The total reaction mixture (3 mL) contained 50 mM sodium phosphate (pH 7.0), 3.33 mM guaiacol and 4 mM H_2O_2. The reaction was initiated by the addition of 20 mL of extract. Progress of the reaction was measured directly by the increment in absorbance at 470 nm (extinction coefficient 2.6 mM^{-1} cm^{-1}) at 30 s intervals for 3 min at 22 C. Protein content was determined using a NanoDrop 1,000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

2.4.3 Ascorbate Content

Leaf tissue (100 mg) was powdered using liquid nitrogen and extracted in 1 M perchloric acid ($HClO_4$). The samples were ground continuously until completely thawed. The homogenates were then clarified by centrifugation for 10 min at 17,000 g. The supernatant (0.5 mL) was transferred to fresh microtubes containing 120 mM sodium phosphate buffer (0.1 mL, pH 7.6). The pH of each sample was adjusted to 5.0 with 80 + μL of 2.5 M K_2CO_3. The insoluble KClO produced during neutralization was removed by centrifugation at 17,000 g. The pellet was discarded and the supernatant used for determining the oxidized and reduced ascorbate content of each tissue. The supernatant was then assayed for AA quantification as described by Foyer et al. (1983).

2.5 Experimental Design and Statistical Analysis

Treatments (n = 25) were distributed in complete randomized arrays for each chemical and control treatments and were replicated three times in independent experiments. ANOVA and Tukey post hoc test were carried out in Statgraphics Plus v 5.1 (StatPoint Technologies, USA) at 1–5 % probability level.

3 Results and Discussion

3.1 Effects of SA on Disease Symptom Reduction

SA induced reduction of symptoms associated with phytoplasma in both treatments, either by spraying (short term effect) or by pre-in vitro culture (long term effect). In this study, SA treatments reduced disease symptoms for internode length, plant height, tuber weight and internal browning.

In greenhouse conditions, phytoplasma-infected potato plants showed symptoms such as reduced plant height, stem thickening, internode shortening, deficient tuber production, and different degrees of tuber flesh browning comparing with no infected potato plants (Table 1).

Similar symptoms were reported previously under greenhouse conditions (Martínez-Gutiérrez et al. 2012; Sánchez-Rojo et al. 2011; Romero-Romero and López-Delgado 2009). Symptoms under field conditions generally are more drastic including: apical leaf rolling, purple coloration, chlorosis and aerial tuber formation (Cadena-Hinojosa et al. 2003; Secor et al. 2006; Martínez-Soriano et al. 2007). It was suggested that the impact of climatic differences can influence the phenotype expression of symptoms in grapes (Hren et al. 2009) and in potato (Mártinez-Gutiérrez et al. 2012) in phytoplasma infected plants.

Remarkably SA 0.01 mM significantly enhanced height and length in phytoplasma infected plants in both treatments (sprayed and in vitro pre-cultured), however, in short term effects, SA induced higher stems (11.8 %) in contrast to the positive control as the sprayed treatment showed (Table 1).

In short term, sprayed SA 0.01 mM significantly enhanced internode diameter comparing with the long term effect of SA in pre-cultured plants, which showed significant thinner diameter in contrast to the positive control. This response is counteracting the increase of stem diameter as one of the symptoms induced by phytoplasma infection, getting similar values to those of the negative controls (Table 1). Increase of biomass by SA has been reported under abiotic stress in banana (Bidabadi et al. 2012); SA increased radical and plumule length, fresh and dry weight in okra (Baghizadeh and Mahmood 2011), similarly SA treatment significantly increased the fresh and dry weights in both root and shoots of wheat plants under salt stress (Erdal et al. 2011). On abiotic stress, SA reduced symptoms

Table 1 Short and long term effects of SA on symptoms alleviation in potato plants infected by phytoplasma at 90 days after transplanting (*DAT*)

Sprayed plants with SA [mM]	Plant height (cm)	6th internode length (cm)	6th internode diameter (cm)	Tubers quantity per plant	Tubers weigh (g)	Weigh-number of tubers relation	Tubers browning on the inside (%)
0−	84.294 ± 0.97*	2.566 ± 0.04*	0.181 ± 0.006*	3.1 ± 0.08*	6.29 ± 0.06*	1.52 ± 0.02*	0.000 ± 0.00*
0+	63.187 ± 0.67	1.803 ± 0.05	0.231 ± 0.001	3.8 ± 0.15	2.97 ± 0.07	0.71 ± 0.05	16.90 ± 0.00
0.1	66.483 ± 0.49	1.940 ± 0.02*	0.236 ± 0.002	2.6 ± 0.10*	5.97 ± 0.06*	1.34 ± 0.03*	11.93 ± 0.10*
0.01	74.886 ± 0.84*	2.088 ± 0.02*	0.223 ± 0.003*	3.7 ± 0.09	3.48 ± 0.10*	0.96 ± 0.03	14.52 ± 0.27*
0.001	73.488 ± 0.66*	2.500 ± 0.05*	0.231 ± 0.003	3.2 ± 0.08*	3.74 ± 0.02*	1.17 ± 0.03*	10.32 ± 0.32*
In vitro treated with SA [mM]							
0−	80.19 ± 0.48*	3.20 ± 0.03*	0.25 ± 0.006*	5.5 ± 0.10	5.2 ± 0.11*	0.97 ± 0.01*	3.254 ± 0.27*
0+	74.41 ± 0.51	2.90 ± 0.02	0.295 ± 0.001	5.6 ± 0.06	3.3 ± 0.06	0.32 ± 0.00	17.533 ± 0.18
0.1	69.87 ± 0.34	2.86 ± 0.03*	0.219 ± 0.002*	7.2 ± 0.09*	3.7 ± 0.20	0.51 ± 0.02*	11.089 ± 0.49*
0.01	80.60 ± 0.53*	3.34 ± 0.02*	0.219 ± 0.003*	6.4 ± 0.05*	3.4 ± 0.04	0.70 ± 0.03	10.91 ± 0.04*
0.001	75.10 ± 0.61	3.56 ± 0.03*	0.23 ± 0.003*	5.1 ± 0.07*	4.5 ± 0.27*	0.93 ± 0.05*	10.88 ± 0.32*

Controls were phytoplasma-free (0−) and phytoplasma-infected plants (0+)

*Significantly different 0+

of *Fusarium* on tomato such as yellowing of stems and chlorosis (Mandal et al. 2009). In the present work, SA reduced symptoms of phytoplasma as result of short and long term effects (Table 1).

3.1.1 Minituber

The number of minitubers and internal browning were significantly reduced in plants as a short term effect of sprayed SA (0.1 and 0.001 mM) in contrast with infected plants no sprayed, presenting similar values to plants of the negative control. SA supplied in vitro as a long term effect (0.001 mM) significantly reduced the minitubers number and internal browning in contrast to positive control (Table 2). SA in both short and long terms significantly enhanced the dry matter and starch contents in the three concentrations tested in infected plants comparing with the positive control (Table 2). The relative growth rate was enhanced by SA in long term. Low SA concentrations (0.001 mM) are likely more biologically active in both short and long terms on the parameters evaluated in this research. Increase in dry matter and starch could be associated with augmentation of leaf area induced by SA in potato (Sánchez-Rojo et al. 2011) and more sugar translocation to the fruits involving increase of sucrose phosphate synthase and amylase (Elwan and El-Hamahmy 2009; Dong et al. 2011). It is likely that effects of SA reported in short term, could be extended in long term as observed in this research.

In short term effect, the tuber weight-number relationship, calculated to estimate tuber size, showed a significant increase with 0.1 and 0.001 mM SA sprayed on phytoplasma-infected potato plants. Whereas, in long term SA 0.001 mM

Table 2 Short and long term effects of SA on dry matter, starch tuber and growth in potato plants infected by phytoplasma at 90 days after transplanting (*DAT*)

SA sprayed plants [mM]	Tuber dry matter (%)	Tuber starch (%)	Relative growth rate (mg g^{-1} day^{-1})
0−	25.083 $\pm$ 0.26*	16.66 $\pm$ 0.59*	41.498 $\pm$ 1.08*
0+	22.962 $\pm$ 0.06	13.98 $\pm$ 0.41	33.931 $\pm$ 1.79
0.1	24.513 $\pm$ 0.03*	15.00 $\pm$ 0.29	39.760 $\pm$ 2.13*
0.01	24.177 $\pm$ 0.17*	15.76 $\pm$ 0.39	39.672 $\pm$ 0.96*
0.001	25.156 $\pm$ 0.20*	15.27 $\pm$ 0.51	41.016 $\pm$ 1.82*
SA in vitro treated [mM]			
0−	18.55 $\pm$ 0.33*	12.70 $\pm$ 0.31*	39.17 $\pm$ 3.56*
0+	14.29 $\pm$ 0.57	8.90 $\pm$ 0.54	20.64 $\pm$ 1.28
0.1	19.47 $\pm$ 0.23*	13.37 $\pm$ 0.22*	29.945 $\pm$ 0.87
0.01	18.76 $\pm$ 0.17*	12.96 $\pm$ 0.28*	26.99 $\pm$ 1.98
0.001	23.97 $\pm$ 0.47*	17.42 $\pm$ 0.38*	32.659 $\pm$ 3.29

Controls were phytoplasma-free (0−) and phytoplasma-infected plants (0+)

*Significantly different 0+

showed the same effect (Table 1). Tuber weight reflected efficient photosynthate translocation increasing tuber size and dry matter content.

SA 0.001 mM was the most physiologically active concentration for increasing and improving tuber production in phytoplasma-infected potato plants in both, long and short term effects. Similarly effects of SA in short term positively increased the average fruit weight, yield, and sugar translocation from leaves to fruits in *Capsicum annuum* L. (Elwan and El-Hamahmy 2009). There is no information about similar effects of SA in long term.

SA concentration effects depends on the physiological age of the tissue, kind of tissue and way of administration (López-Delgado et al. 2004, 2007; Mora-Herrera et al. 2005), probably the no-significant effect of 0.01 mM SA on weight-number relationship was linked to these factors.

On the other hand at physiological level, carbohydrate accumulation in mature leaves and decreased starch content in sink tissues are often associated with phytoplasma infection. Reduced translocation suggested a more indirect influence of the parasite on the host metabolism and phloem function (Christensen et al. 2005). In the present study, phytoplasma decreased biomass accumulation in potato plants (% tuber dry matter, % starch, plant dry matter) compared to non-infected control plants (Table 2). Leaf area index between negative and positive controls was not significantly different.

It was suggested that mechanisms involving SA/H_2O_2 signaling might mediate the biotic stress response to phytoplasma and provide protection leading to reduced infection symptoms. SA contributed to symptom suppression in TMV-infected tobacco leaves (Fodor et al. 1997). Accumulation of H_2O_2 by effect of SA it is well known (Huang et al. 2008).

SA treatments had an ameliorative effect on PPT symptoms. In particular, 0.001 mM SA significantly increased foliar area index, tuber dry matter content, and crop relative growth rate. SA also significantly affected tuber starch content (Table 2) in both short and long term effects of SA. Similar effects of SA in short term were observed when foliar applications enhanced leaf area and dry mass production in corn and soybean (Khan et al. 2003). Growth improvement by exogenous SA application has been associated with antioxidant activity (Eraslan et al. 2007; He and Zhu 2008). Low SA concentrations sprayed on *Brassica juncea* significantly enhanced dry matter accumulation, but higher concentrations had an opposite effect (Fariduddin et al. 2003). Conversely, SA exogenous applications had no inhibitory effects in this study.

Exogenous SA probably alleviated some symptoms related to phytoplasma in potato plants through changes in the antioxidative system, as demonstrated in *Cucurbit pepo* leaves infected with zucchini yellow mosaic virus (Radwan et al. 2006) and in sweet cherry infected with *Penicillium expansum* (Xu and Tian 2008).

Furthermore, crosstalk between plant responses to pathogens and abiotic stress suggest modulation by SA. SA alleviated some effects of abiotic stress in plants as in *Lycopersicum esculentum* exposed to NaCl (He and Zhu 2008), orange (Huang et al. 2008) and peach chilling injury during cold storage (Wang et al. 2006).

In potato, ameliorative effects of SA on abiotic stress have been previously reported (López-Delgado et al. 1998, 2004; Mora-Herrera et al. 2005). Most of the effects reported of SA are in short terms, in this work ameliorative long term effects of SA (90 than 90 days) against biotic stress are demonstrated.

3.2 H_2O_2 and Antioxidant Assessment

3.2.1 H_2O_2 Content

Phytoplasma-infected plants had significantly higher H_2O_2 levels than the free-phytoplasma plants during the 90 days of culture in greenhouse (Fig. 3). H_2O_2 overproduction occurred in phytoplasma-infected apple and apricot trees (Musetti et al. 2004, 2005). Similarly, in *Cucurbita pepo* H_2O_2 increased by effect of ZYMV virus (Radwan et al. 2010). In the present study, the absence of H_2O_2 overproduction in phytoplasma-free plants likely occurred because they lacked pathogen-induced oxidative stress. As short and long term effects of SA (0.001 mM), a significant increase of H_2O_2 content and tuber production was observed at 30 DAT compared to both controls. However, in long term effect both of them diminished at 0.1 mM respecting the positive controls.

H_2O_2 increase is a key requirement for plant protection against pathogens because the redox state regulates the nonexpresser of pathogenesis related genes 1 (NPR1), an essential component of the SA dependent plant defense response. This inactive oligomer accumulates in the cytosol and is reduced by ROS to release monomeric units, which in turn interact with the reduced TGA1 transcription factor to activate defense gene expression (Mou et al. 2003). However, SA reduction of H_2O_2 eventually protects against oxidative stress caused by ZYMV infection in *Cucurbita pepo* (Radwan et al. 2006). In *Lycopersicon esculentum* (He and Zhu 2008) and *Oryza sativa*, SA treatment also decreased H_2O_2 content, which alleviated oxidative damage (Choudhury and Panda 2004; Guo et al. 2009). In short and long term effects of SA we observed that plant age is important for SA response, since all the sprayed SA concentrations reduced H_2O_2 content at 60 days culture in contrast to the positive control. This low H_2O_2 concentration may have been linked to the SA-induced increased POX activity (Fig. 4) and AA content (Fig. 5) connected with the alleviation of phytoplasma-caused symptoms. However, some workers had found that SA increased H_2O_2 content (Agarwal et al. 2005; Chao et al. 2009; Erdal et al. 2011) or decreased it (Choudhury and Panda 2004; Huang et al. 2008; Khan et al. 2010), and sometimes no effect of SA on H_2O_2 content has been observed (Krantev et al. 2008), these effects could be associated with multiple factors, such as the species, SA concentration, physiological age, etc. (Sánchez-Rojo et al. 2011). At senescence (90 DAT) no significant differences occurred between treatments and the positive control in short term effects of SA. However, in long term effect SA significantly reduced H_2O_2 content respecting positive-phytoplasma plants (Fig. 2).

Fig. 3 Long and short terms effects of SA on hydrogen peroxide content in **a** 30, **b** 60, and **c** 90 days after transplanting (*DAT*) in greenhouse-grown potato plants infected with phytoplasma. Controls were phytoplasma-free (0 −), and phytoplasma-infected plants (0+). *Significantly different 0+
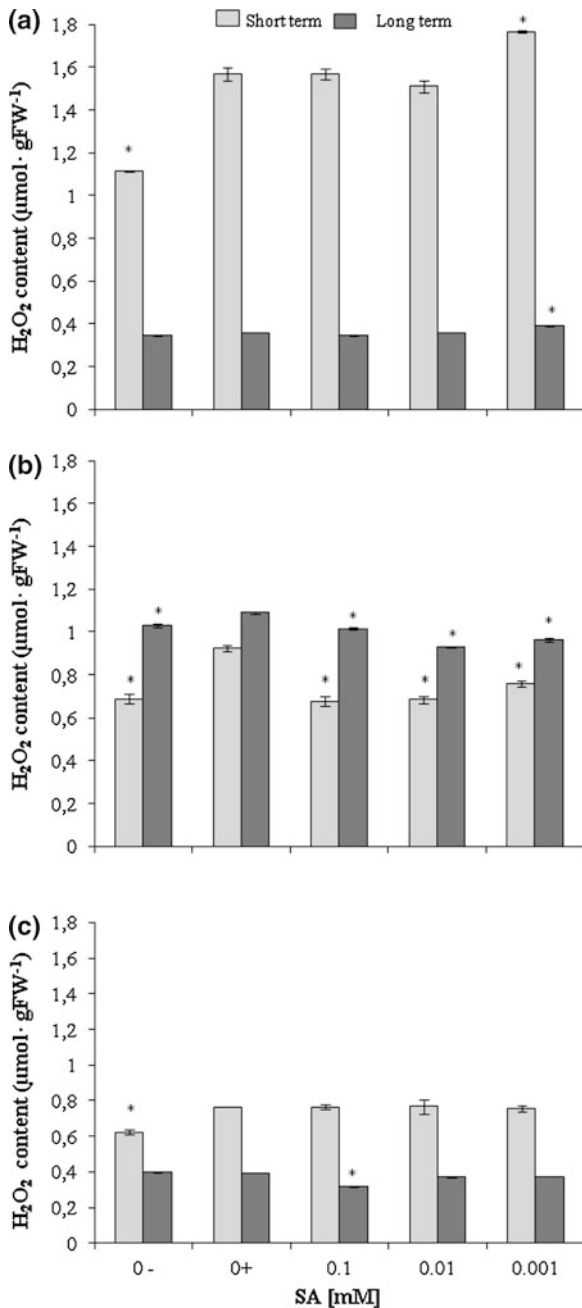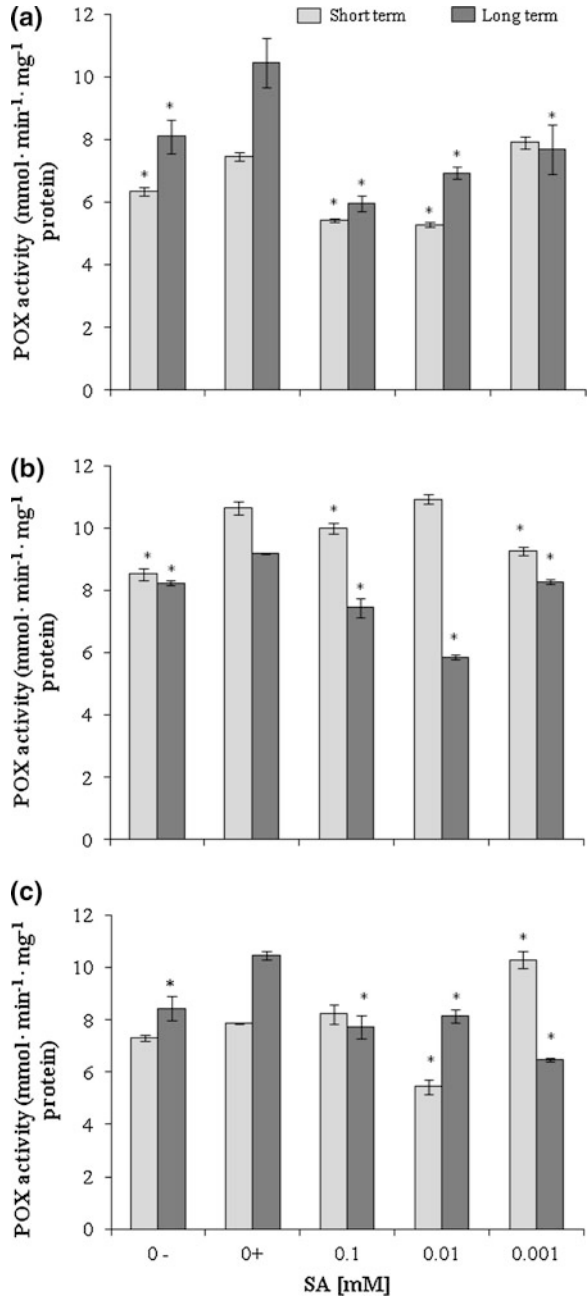
Fig. 4 Long and short terms effects of SA on POX activity in **a** 30, **b** 60, and **c** 90 days after transplanting (*DAT*) greenhouse-grown potato plants infected with phytoplasma. Controls were phytoplasma-free (0−), and phytoplasma-infected plants (0+). *Significantly different 0+

Fig. 5 Long and short terms effects of SA on ascorbate content in **a** 30, **b** 60, and **c** 90 days after transplanting (*DAT*) greenhouse-grown potato plants infected with phytoplasma. Controls were phytoplasma-free (0−), and phytoplasma-infected plants (0+). *Significantly different 0+
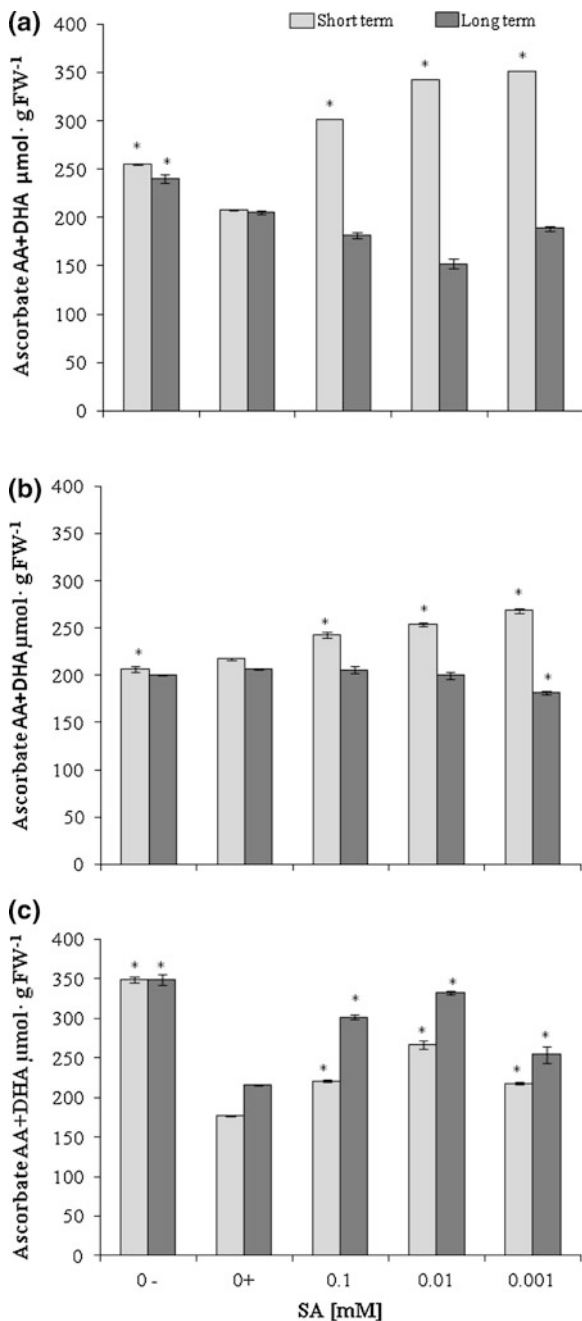
3.2.2 Peroxidase Activity

Phytoplasma-infected plants significantly enhanced POX activity from transplanting up to 60 DAT. This enhancement could be related to the phytoplasma attack, since Musetti et al. (2005), reported higher POX activity when apple trees were infected with the apple proliferation phytoplasma. Similarly, virus infections enhanced POX activity (Riedle-Bauer 2000; Clarke et al. 2002; Radwan et al. 2006, 2010). It is known that peroxidases are enzymes with numerous functions in plant cell, they participate in plant growth, differentiation and development processes, including auxin catabolism, ethylene biosynthesis, plasma membrane redox system and generation of H_2O_2 during cell wall edification, lignification and suberization, as well as response to pathogens (Mehlhorn et al. 1996). Thus, presumably peroxidase enhancement contributed to oxidative stress in systemic plant-virus interactions.

In this study, POX activity in phytoplasma-infected plants decreased in a short term effect of SA 0.1 mM at 30 and 60 DAT, whereas as long term effect decreased in all concentrations respecting the positive control (Fig. 4a, b). In short term effects of SA 0.01 mM POX decreased at 30 and 90 DAT. Whereas as a long term effect decreased in 30, 60 and 90 DAT respecting the positive control.

In a short term effect of SA 0.001 mM, POX diminished at 60 and increased at 90 DAT. In contrast, decrement of POX activity was observed at the three dates studied as a long term effect comparing the positive control (Fig. 4a, b and c).

POX activity likely depends on SA concentration and plant age, and other reports showed enhanced (Clarke et al. 2002; Choudhury and Panda 2004; He and Zhu 2008; Mandal et al. 2009) or unmodified (Krantev et al. 2008) POX activity by SA. In the present study, POX activity was inversely correlated with H_2O_2 content. Low POX activity also occurred during natural recovery from *Candidatus Phytoplasma prunorum* infection in apricot trees (Musetti et al. 2005). Remarkably in our study, a significantly high H_2O_2 content occurred in the early stage (30 DAT) only at the lowest SA concentration tested as a short and long term responses, affecting the increment of biomass assimilation and reducing symptoms, similar to Musetti et al. (2005). Low SA concentrations have induced augmentation of parameters involving productivity associated with decrease in POX (Elwan and El-Hamahmy 2009).

Alike the H_2O_2 content, peroxidase activity mediated by SA is depending on the concentration, species, application etc. (Sánchez-Rojo et al. 2011). In accordance, it was reported that SA increased the POX activity (Clarke et al. 2002; Horváth et al. 2007; Jing-Hua et al. 2008; Mahdavian et al. 2008; Mandal et al. 2009; Mutlu et al. 2009; Kumara et al. 2010; Erdal et al. 2011), on the contrary, POX activity was unmodified by SA (Noreen et al. 2009; Asthir et al. 2009).

Only 0.001 mM SA showed a different pattern of POX activity with a gradual increment from 30 to 90 DAT (Fig. 4). It is worth mentioning that this low SA concentration induced the greatest effects on alleviating symptoms, tuber production, and photosynthate translocation, probably because low levels of SA had optimal biological activity compared to high concentrations. Similarly, 0.001 mM

SA was the best treatment to increase sugar translocation from leaves to fruits, average fruit weight, and fruit yield, and decreased POX activity of greenhouse grown pepper (*Capsicum annuum* L.) under salinity stress (Elwan and El-Hamahmy 2009).

3.2.3 Ascorbate Content

In this study, decreased AA and high H_2O_2 levels occurred in infected plants as a response to phytoplasma stress in contrast to phytoplasma-free plants. Decreased AA in infected plants could be associated with phytoplasma infection, since an AA decline has been associated with TMV infection in tobacco (Fodor et al. 1997). In this work, all SA concentrations significantly increased AA levels, particularly at 30 DAT as a short term response, in opposition to the observed response as long term effect, where a reduction of AA concentration was noticed respecting the positive control (Fig. 5a). At 90 DAT not only in short but also in long term AA concentration increased respecting the positive control (Fig. 5c).

In agreement with previous studies showing that suitable concentrations of exogenous SA enhanced plant antioxidant system efficiency (Hayat et al. 2009). AA plays an important role in many cell processes, mainly as an antioxidant (Smirnorf 1996), and can increase resistance to abiotic (Shalata and Neumann 2001) and biotic (Noctor and Foyer 1998) stresses. Exogenous SA-induced augmentation of AA concentrations in wheat (Asthir et al. 2009), *Oryza sativa* (Choudhury and Panda 2004), carrot (Eraslan et al. 2007), tobacco (Fodor et al. 1997), *Lycopersicon esculentum* (He and Zhu 2008), orange (Huang et al. 2008), and maize (Krantev et al. 2008).

Greenhouse grown pepper treated with low SA concentrations under salinity stress had high fruit vitamin C (AA) and carotenoid levels (Elwan and El-Hamahmy 2009), and SA-induced AA participates in alleviating biotic (Fodor et al. 1997) and abiotic (Horváth et al. 2007; He and Zhu 2008; Huang et al. 2008) stresses. In our study, increased AA in SA-treated plants could be associated with reduced symptoms in potato plants infected by phytoplasma, possibly via PR gene expression because AA content can modulate PR gene expression (Foyer and Noctor 2005). Alterations in AA levels and/or redox state, as well as in their redox enzyme activities are important during plant-pathogen interactions (De Gara et al. 2003). The role of AA in plant growth is known (Pedreira et al. 2004), and perhaps SA-augmented AA contributed to increased tuber size and dry matter content. AA controls growth and it is mediated by H_2O_2 content (Pedreira et al. 2004). Hence, the significantly high H_2O_2 content in the early stage might be a key condition for the signaling response to reduce damage. SA can affect AA content either, increasing (Choudhury and Panda 2004; Eraslan et al. 2007; He and Zhu 2008) or reducing (Saruhan et al. 2012). Interestingly SA induced reduction of AA and gluthation contents in sensible cultivars but enhanced AA concentration in the tolerant (Saruhan et al. 2012).

4 Conclusions

Application of SA 0.1 and 0.001 mM in potato plants promoted biomass accumulation and reduced damage associated to phytoplasma infection.

Effects of SA were observed in short and long term and it is equally efficient applied first in vitro or sprayed under greenhouse.

The oxidative balance in plants is an elemental factor in defense responses to counteract the pathogen damage.

SA treatment is important during the early stages of development (in vitro treatment) in potato for stimulation of the response capacity against the pathogen.

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Chapter 15
Efficiency of Salicylic Acid Application on Postharvest Perishable Crops

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Abstract Salicylic acid is recognised as a plant growth regulator and is classified as a phenylpropanoid compound. Salicylic acid has been widely applied either at pre-harvest or post-harvest. It has been recently accepted that salicylic acid is a safe chemicals, used to control post-harvest quantity or quality losses of perishable crops. In this chapter, we have focused on salicylic acid application to post-harvest crops and its effects on physicochemical quality changes including that of biologically active compounds, physiological disorders and diseases of fruit, vegetables and ornamentals.

1 Introduction

Recently, the consumption of fresh fruit and vegetables has markedly increased as a health concern of consumers. Postharvest technology has been applied to reduce the losses and to maintain fresh-like quality of horticultural produce during storage. Moreover, safety and environmental friendly techniques are the challenge of postharvest technologists. The use of synthetic chemicals for fruits and vegetables storage is banned in many countries. Whereas, the application of natural compounds and/or physical treatments for postharvest produce have been replaced. Salicylic acid (SA) is one of natural and safe chemicals used for postharvest

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quality maintenance of horticultural and ornamental produces. In recent years, many postharvest technologies for perishable produces including fruit, vegetables and ornamentals have been adopted where SA is in use. SA, a simple plant phenolic compound, is known as an endogenous signal molecule regulating plant developmental processes and modulating both biotic and abiotic stresses (Ding and Wang 2003; Horváth et al. 2007; Asghari and Aghdam 2010). It is widely recognized that SA reduces respiratory rate (Raskin et al. 1989; Asghari and Aghdam 2010) inhibits ethylene biosynthesis (Leslie and Romani 1988), induces the expression of defense genes (Meena et al. 2001; Wen et al. 2005) and decreases lipid oxidation and membrane senescence (Kazemi et al. 2011a, b) in plants. In recent years, SA has been applied for postharvest purposes for delaying the processes related with ripening and senescence processes (Srivastava and Dwivedi 2000; Zhang et al. 2003a, b; Imran et al. 2007; Gerailoo and Ghasemnezhad, 2011), alleviating chilling injury (Sayyari et al. 2009; Luo et al. 2011; Yang et al. 2012; Luo et al. 2012), retarding browning reaction (Peng and Jiang 2006; Lu et al. 2011), enhancing bioactive compounds and antioxidant capacity (Elwan and El-Hamahmy 2009; Valero et al. 2011; Wei et al. 2011), maintaining firmness (Gholami et al. 2010; Tareen et al. 2012) and controlling postharvest decay (Babalar et al. 2007; Asghari and Aghdam 2010). Thus, the effects of SA application on postharvest physiology and quality of horticultural and ornamental crops are reviewed in this chapter.

2 The Application of Salicylic Acid

In the past, the purpose of application of exogenous SA and its derivatives, such as acetyl salicylate and methyl salicylate, was to control postharvest disease of fruits and vegetables by increasing the systemic acquired resistance and to increase stress resistance (Qin et al. 2003; Park et al. 2007; Babalar et al. 2007; Wang et al. 2011). Recently, many studies have reported that SA could be used as a commercial application for maintaining postharvest quality to prolong shelf-life of fruits, vegetables and ornamental produces. Both pre- and post-harvest SA applications in extending shelf-life and maintaining quality of postharvest produces have been investigated and developed for commercial use. Spraying and adding into growth medium are the approaches used for pre-harvest treatment. The foliar spray of SA (10^{-6} and 10^{-4} M) could improve the postharvest quality of pepper fruit by increasing fruit weight, the level of biologically active compounds and regulating sugar content (Elwan and El-Hamahmy 2009). Gholami et al. (2010) reported that SA pre-harvest treatment at three weeks before harvest improved the quality and reduced fungal infection of 'Mashhad' sweet cherry fruit. The common dipping technique is used to treat post-harvest fruits and vegetables, such as that of asparagus (Wei et al. 2011), banana fruit (Srivastava and Dwivedi 2000), kiwifruit (Zhang et al. 2003a, b), mandarin orange fruit (Zheng and Zhang 2004), peach fruit (Han et al. 2003; Tareen et al. 2012), pear fruit (Imran

et al. 2007), plum fruit (Luo et al. 2011), pomegranate fruit (Sayyari et al. 2009), sugar apple fruit (Mo et al. 2008), tomato fruit (Pila et al. 2010) and cut flowers (Gerailoo and Ghasemnezhad 2011; Hatamzadeh et al. 2012). Babalar et al. (2007) suggested that the use of pre-harvest treatment followed by postharvest treatment was the most effective strategy for preventing fungal decay and to maintain overall quality of Selva strawberry fruits. In a similar vein, Lu et al. (2011) reported that SA pre-harvest spray (2 mM) and/or postharvest dip (0.5 mM SA) could maintain the fruit quality and enhance the resistance to internal browning. Moreover, the combination treatment is used to enhance the effectiveness of SA. Yang et al. (2012) reported that the combined salicylic acid and ultrasound treatments induced better tolerance against chilling injury of cold-stored peach fruit better than salicylic alone. Kazemi et al. (2011a) reported that SA treatment incorporated with CaCl2 dip increased firmness and decreased the loss of fresh weight and decay percentage of kiwifruits during refrigerated storage for 60 days.

It is widely recognised that the concentration of SA is limited to non-toxic concentrations to plants (Xu and Tian 2008; Elwan and El-Hamahmy 2009). The optimum range of SA concentration used for harvested fresh produces is about 0.5–2 mM (Table 1). However, the application of SA at higher concentration is less effective in postharvest quality maintenance and may harm the produce. Babalar et al. (2007) reported that slight damage was found on the strawberry fruits, treated with 4 mM SA whereas the most effective concentration in retaining the fruit quality was 2 mM. Imran et al. (2007) reported that SA at lower concentrations (0.02–0.5 mM) could delay the senescence and weight loss of pear fruit cv. Huang Kum compared to higher concentrations (2.5–4 mM). In a similar vein, Kazemi et al. (2011b) addressed that SA was found to be positively correlated with lipid peroxidation of harvested carnation flower, as the SA concentration was increased, the lipid peroxidation increased. This indicated that at higher concentrations, SA could induce senescence of the cut flowers. However, the use of SA at proper concentration (1.5 mM) maintained the quality and the vase life of the flowers for a longer period.

3 Effects of Salicylic Acid on Postharvest Physiology of Perishable Produces

3.1 Respiratory Rate

Respiration has been known as a major factor contributing to the postharvest losses of perishable produces due to the conversion of stored sugars or organic acids to energy in the presence of oxygen and the loss of moisture from the produce (Luo et al. 2011). The increase in respiratory rate during storage advances the senescence process; therefore it has to be curtailed to a minimum level so as to maintain the quality and to extend the shelf-life of perishable produces. Storage at low

Table 1 Effects of SA on postharvest perishable crops

Commodity	Recommend concentration	Results	Author (s)
Tomato fruit	5.0 mM	Enhance defence response	Wang et al. (2011)
Fresh-cut bamboo shoot	1.0 mM	Chilling injury and browning prevention	Luo et al. (2011)
Banana fruit	1.0 mM	Delayed ripening	Srivastava and Dwivedi (2000)
Kiwifruit	1.0 mM	Delayed ripening	Yu et al. (2003); Zhang et al. (2003a, b)
Huang Kum pear fruit	0.2–0.5 mM	Delayed senescence	Imran et al. (2007)
Pomegranate fruit	2.0 mM	Chilling injury prevention	Sayyari et al. (2009)
Peach fruit	1.0 mM	Chilling injury prevention and quality maintenance	Yang et al. (2012); Tareen et al. (2012)
Strawberry fruit	1.0–2.0 mM	Decay control and quality maintenance	Babalar et al. (2007)
Sugar apple fruit	0.8–1.2 mM	Delayed ripening	Mo et al. (2008)
Cherry fruit	1.0 mM	Delayed ripening and quality maintenance	Velero et al. (2011); Gholami et al. (2010)
Navel orange fruit	1.0–2.0 mM	Enhanced defence response	Huang et al. (2008)
Pineapple fruit	0.5–2.0 mM	Quality maintenance and browning inhibition	Lu et al. (2011)
Asparagus	1.0 mM	Quality maintenance	Wei et al. (2011)
Rose	150 mg/L	Shelf-life extension	Gerailoo and Ghasemnezhad (2011)
Galdiolus grandiflora	150 mg/L	Shelf-life extension	Hatamzadeh et al. (2012)

temperature is widely adopted as the main technique that minimizes the respiratory and other metabolic rates of the produce. However, the exclusive use of low temperature storage is not enough to maintain the quality and to prolong shelf-life of the produce as the international trade of fresh produce including fruits, vegetables and ornamental crops has grown markedly. The application of additional techniques with low temperature storage is demanded to support the requirement of fresh produce in the market. SA is known as a potential natural chemical (plant hormone) inhibiting respiratory rate of fresh produce as it postpones the onset of respiratory burst in climacteric fruit and suppresses the rate of respiration, during storage (Leslie and Romani 1988; Han et al. 2003; Mo et al. 2008; Luo et al. 2011). The reduction of respiratory rate is associated with an increase in the expression of alternative oxidase, thus enhancing cyanide-resistance respiration (Rhoads and McIntosh 1992). It proved that SA treatment retards O_2- content which positively correlates with the increase in cyanide—resistant respiration

(Zhang et al. 2003a, b). Moreover, SA treatment also reduces the activities of respiratory pathways, such as glycolysis pathway, tricarboxylic acid pathway, pentose phosphate pathway, cytochrome pathway and related enzymes activities. However, Leslie and Romani (1988) addressed that only at concentrations exceeding 500 μM did inhibit respiration whilst at higher concentrations SA generally produced a respiratory rise in young fruit tissues of *Pyrus communis* cv Passe Crassane. This suggests that the effect of SA on respiratory inhibition is in a concentration dependent manner. Postharvest application of SA in reducing respiratory rate has been reported in banana fruit (Srivastava and Dwivedi 2000), peach fruit (Han et al. 2003), strawberry fruit (Babalar et al. 2007), sugar apple fruit (Mo et al. 2008) and plum fruit (Luo et al. 2011).

3.2 Ethylene Production

Ethylene is the most important gaseous plant hormone adversely affecting the postharvest quality and physiology of the produce. Ethylene triggers the ripening process and advances senescence. The elimination of ethylene during postharvest period is very important to extend the shelf-life and quality of the produce. SA is one of the potential ethylene inhibitors and is likely able to be effectively used at commercial scale for fresh produce. Many studies have proven that SA treatment decreases ethylene production by inhibiting ACC synthase and ACC oxidase production and their activities (Leslie and Romani 1988; Zhang et al. 2003a, b; Asghari and Aghdam 2010). Zhang et al. (2003a, b) reported that the application of acetylsalicylic acid elevated endogenous SA level in kiwifruit and slowed the early phase of ethylene climacteric rise. In cut flower, SA treatment at a concentration of 1.5 mM reduced ACC oxidase activity of cut carnation flower and prolonged its vase-life (Kazemi et al. 2011b). Moreover, the application of exogenous SA decreased lipoxygenase (LOX) and allene oxide synthase (AOS) which positively related to the increase in ethylene production (Marcelle 1991). Similarly, Zhang et al. (2003a, b) and Mo et al. (2008) addressed that SA treatment could inhibit wound-induced transcription and the activity of ACC synthase and LOX in fruits leading to the consequent reduction in free radical level and ethylene synthesis. Suppression of ethylene production in harvested fruits by SA treatment has been reported for peach (Han et al. 2003), 'Selva' strawberry (Babalar et al. 2007), sugar apple (Mo et al. 2008), 'Mashhad' sweet cherry (Gholami et al. 2010), 'Qingnai' plum (Luo et al. 2011) and tomato (Wang et al. 2011).

3.3 Ripening

Fruit ripening is triggered by ethylene production leading to physiomorphological changes such as tissue softening, pigments degradation and/or development, the

changes in sugars and organic acids content and release of aromatic compounds. In recent years, many researchers have investigated the effects of SA treatment on postharvest ripening of fruits. It is recognised that SA treatment delays postharvest ripening process in fruits (Srivastava and Dwivedi 2000; Zhang et al. 2003a, b; Valero et al. 2011). SA treatment could maintain texture, delay the rise in reducing sugars and enhance the level of biologically active compounds which provide the benefit to health and prevent certain diseases in human beings.

Modifications in cell wall components and cell membrane deterioration cause tissue softening during fruit ripening that are related to the climacteric rise in ethylene production (Supapvnaich and Tucker, 2011). The modification in cell wall components isinduced by the action of cell wall hydrolases such as polygalactosidase (PG), pectinmethylesterase (PME), β-galactosidase (β-Gal) and xylanase (Supapvanich and Tucker 2011; Srivastava and Dwivedi 2000). As an ethylene inhibitor, SA treatment delays the ripening process of fruit and prevents the fruit softening by reducing the activity of cell wall degrading enzymes. Srivastava and Dwivedi (2000) reported that SA treatment inhibited PG, xylanase and cellulase in banana fruit, in a concentration dependent manner. They also found that cellulase and PG seem to be most sensitive to SA treatment. Zhang et al. (2003a, b) also reported that firmness of kiwifruit, during storage, was maintained by SA treatment. The deterioration of cell membrane is known as a factor affecting fruit firmness which is related to membrane lipid peroxidation. The intermediates and terminal products of membrane lipid peroxidation, namely •OH2, H2O2, -O2 play an important role in fruit ripening, including softening mechanism. LOX activity and superoxide free radical production are widely known as the important factors stimulating membrane dysfunction and deterioration during fruit ripening. It has been demonstrated that SA treatment helps the maintenance of cell membrane structure and integrity by reducing LOX activity and superoxide free radical production in fruits (Zhang et al. 2003a, b; Mo et al. 2008). Moreover, it is proved that exogenous application of SA enhances defense mechanism and the production of antioxidants in fruits leading to the decrease in lipid peroxidation of cell membrane (Imran et al. 2007; Huang et al. 2008; Mo et al. 2008; Wei et al. 2011). It proves that exogenous SA treatment potentially maintains fruit firmness during postharvest period by reducing the activity of cell wall hydrolases and cell membrane degradation.

The increase in sucrose contents of fruits during development is because of its biosynthesis by the action of sucrose-phosphate synthase and sucrose phosphatase (Hubbard et al. 1991; Asghari and Aghdam 2010). Sucrose is the most common form of carbohydrate produced during photosynthesis and transferred from source to sink, leading to an increase in total soluble solids (TSS) and soluble sugar content in the fruits. During ripening, the decrease in non-reducing sugar content, mainly sucrose, is associated with the increase in reducing sugars (fructose and glucose). This process is closely related to the increase in invertase activity. Both sucrose-phosphate synthase and invertase are activated by the action of ethylene burst during ripening process (Langenkamper et al. 1998; Srivastava and Dwivedi 2000; Asghari and Aghdam 2010). As a potential ethylene inhibitor, SA treatment

could delay the increase in reducing sugar content and maintains a lower TSS but in fruits during storage (Asghari and Aghdam 2010). Srivastava and Dwivedi (2000) found that SA treatment leads to a decrease in invertase activity and that of reducing sugars and also delayed breakdown of starch in banana fruit during ripening. Aghdam et al. (2009) suggested that a lower TSS of kiwifruit treated with methylsalicylic acid was concomitant with reduced ethylene production and decreased sucrose-phosphate synthase activity. Moreover, pre-harvest treatment of SA (10^{-6} M) regulated sugar contents (reducing and nonreducing) in both pepper leaf and fruit (translocation from source to sink) and stimulated invertase activity (Elwan and El-Hamahmy 2009). This indicates that SA treatment can delay the fruit ripening during storage and also enhance fruit quality by increasing total sugar content when pre-harvest treatment of SA is applied.

Titratable acidity (TA) is directly related to the organic acids content in fruits. Normally, TA and total organic acid content decline throughout ripening process. However, treatment of kiwifruit with SA maintained higher TA than control fruit during storage (Kazemi et al. 2011a, b). In the similar vein, both pre- and post-harvest SA treatments maintained higher TA of winter pineapple fruit than the control (Lu et al. 2011). In fresh-cut product, SA treatment also delayed the loss of TA in Chinese water chestnut during storage for 4 days (Peng and Jiang 2006).

Colour changes during storage such as the loss of greenness, the increase in yellowness and the development of certain pigments relating to ripening and senescence processes, are the important factors affecting the quality of horticultural produce during postharvest period. Chlorophyll content is a key factor for green vegetables and florist green. It is widely recognized that the loss of chlorophyll content results to the loss of greenness affecting visual quality and marketability. Ethylene is widely accepted as a key factor playing major role in the loss of chlorophyll during senescence. The regulation of ethylene production or its action could maintain surface colour of the produce during postharvest period. The application of SA, an ethylene inhibitor, can delay the loss of greenness of vegetables and florist green. Wei et al. (2011) found that the chlorophyll content in asparagus was maintained by SA treatment, however, the high concentrations of SA cause deterioration of the pigment. SA treatment can prolong the shelf-life and delay chlorophyll degradation of detached leaves of *Hippearstrum x chmielii* (Lukaszewska and Kobyliński 2009). In the similar vein, total chlorophyll content of carnation cut flowers was also delayed by SA treatment (Kazemi et al. 2011a, b). Moreover, SA treatment could also maintain the level of other pigments in the produce. Huang et al. (2008) reported that pre-harvest treatment of SA could enhance lycopene content in tomato fruits during development stages and slowed down the degradation of lycopene and β—carotene during storage. This indicates that SA can activate the lycopene biosynthesis pathway, including up-regulation of the gene encoding the enzymes relating to lycopene level, during fruit development. Moreover, Elwan and El-Hamahmy (2009) suggested that SA applied to the intact foliage induced the accumulation of carotenoids in pepper fruits. In contrast, postharvest treatment of SA delayed the accumulation of carotenoids and lycopene in tomato fruits whilst the accumulation of total chlorophyll was detected (Pila

et al. 2010; Wang et al. 2011). In sweet cherry fruit, SA treatment (1 mM) let to a continuous increase in anthocyanin content until the end of storage whilst the loss of anthocyanin content of the control fruit was detected (Gholami et al. 2010; Valero et al. 2011).

3.4 Bioactive Compounds

In recent years, the demand of fresh fruits and vegetables has increased markedly, as the concern of human health has got promoted. It is universally recognized that fresh fruits and vegetables are the excellent source of biologically active components that impart health benefits, beyond basic nutrients (Erkan et al. 2008). Varied postharvest treatments are applied to maintain not only quality as visual appeal but also to improve the bioactive compounds, which are known to provide additional health benefits. The researchers have been trying to locate an alternative simple eco-friendly treatment that can be applied as a commercial practice. SA is widely recognized as a natural plant hormone with the ability to improve bioactive compounds expressing antioxidant properties of postharvest commodities. According to the results of Sayyari et al. (2009) SA treatments at the concentration of 2 mM was highly effective in reducing ascorbic acid loss in the husk of pomegranate. Similarly, postharvest treatment of asparagus shoots with 1.0 mmol/L SA effectively induced the maximum concentration of ascorbic acid and that of phenolic compounds. The treatment also promoted total flavonoids that affected the antioxidant activity as indicated by the resultant increase in ferric reducing antioxidant power (FRAP) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Wei et al. 2011). Asghari (2006) reported that SA at the concentration of 2 mM could enhance total antioxidant potential and ascorbic acid content in strawberry fruits. Shafiee et al. (2010) also reported that hot calcium chloride at 45 °C incorporated with SA treatment and enhanced ascorbic acid content in strawberry fruits, during storage. Similarly SA treatment (32 μL/L) to kiwifruit increased its ascorbic acid content (Aghdam et al. 2011). Huang et al. (2007) suggested that high ascorbic acid contents in the pulp of navel orange (Citrus sinensis L. Osbeck.) pretreated with SA may result from an acceleration of biosynthetic pathways or a decrease in catabolism, through an accumulation of dehydroascorbate (DHAA). In addition, Pila et al. (2010) demonstrated that application of SA at 0.1 mM to tomato fruits was beneficial in retarding degradation of ascorbic acid and caused accumulation of chlorophyll which is in agreement with Turkyylmaz et al. (2005) who reported that foliar spray with SA increased Chl.a, Chl.b and other photosynthetic pigments in bean (Phaseolus vulgaris). Moreover, the treatment of SA also delayed the accumulation of carotenoid content in tomato fruits. A similar kind of phenomenon was observed in navel orange fruits by Renhua et al. (2008). Both pre- and post-harvest SA treatments can stimulate the accumulation of anthocyanin content of the fruits, during development and storage. Gholami et al. (2010) applied 2 and 3 mmol/L SA in 'Mashhad' sweet cherry (Prunus avium L.) fruit at postharvest

stage that resulted in anthocyanin accumulation in the fruits. Moreover, pre-harvest SA treatment can slow down the degradation of carotenoids (lycopene, α-carotene) in 'Cara cara' navel orange fruit pulp during storage. Huang et al. (2008) carried out an experiment where SA activated some of the fruits defense mechanisms and biosynthesis of nutrition components, including the increase in antioxidant activity, and the accumulation of biologically active compounds such as ascorbic acid, glutathione, antioxidant activity, total phenolics and flavonoid content. Additionally, Sarikhani et al. (2010) observed that SA treatment induced much higher total phenolic contents in treated grape fruits. The accumulation of phenolic compounds in grapes by SA treatment could be induced through an increase in phenylalanine ammonia-lyase (PAL) activity (Chen et al. 2006). It, therefore shows a close relationship between SA concentration and berry phenol contents as phenolic compounds are known to be important group of grapes secondary metabolites that strongly influence the berry quality such as color, flavor, bitterness, astringency (Chamkha et al. 2003) and antioxidant content (Frankel et al. 1995).

3.5 Senescence

Plant senescence is generally recognized as the association of a loss of membrane integrity, climacteric rise of respiration and ethylene biosynthesis. Morphological, biochemical and biophysical deterioration of plant consists of declining protein content and lipid fluidity in membranes. The senescence in plants is also concomitant with increased reactive oxygen species leading to the degradation of proteins, lipids and nucleic acid (Arora et al. 2007). The increase in reactive oxygen species such as superoxide anions closely relates to membrane phospholipids breakdown affecting membrane permeability dysfunction (Paulin et al. 1986). Kumar et al. (2008) and Gerailoo and Ghasemnezhad (2011) suggested that a decline in the level of antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and peroxidases (PODs), and increased endogenous H_2O_2 levels may partly be responsible for initiating the senescence in plants. SA treatment can delay the post-harvest senescence of horticultural produces by stimulating the accumulation of biologically active compounds and antioxidant enzymes leading to a decrease in free radical levels and lipid peroxidation (Zhang et al. 2003a, b; Imran et al. 2007; Huang et al. 2008; Kazemi et al. 2011a, b; Gerailoo and Ghasemnezhad 2011; Hatamzadeh et al. 2012). The application of SA (150 mg/L) delayed petal senescence and extended the quality of gladiolus flowers during storage by maintaining higher spike fresh weight, antioxidant enzyme activity and stability of membrane (Hatamzadeh et al. 2012). Similar results have been also reported for cut carnation flowers and cut rose flowers (Kazemi et al. 2011a, b; Gerailoo and Ghasemnezhad 2011). SA treatment also delays the senescence in many fruits such as kiwi (Zhang et al. 2003a, b), Huang Kum pear (Imran et al. 2007), 'Cara car' navel orange (Huang et al. 2008) and sweet cherry (Valero et al.

2011). However, the application of SA at high concentrations causes oxidative damage that is unable to be overcome resulting in the death of plants in contrast to the use of SA at lower concentrations (Horváth et al. 2007).

4 Alliviating Chilling Injury (CI) of Harvested Produce by Salicylic Acid

Even thought storage at low temperature is compulsory to preserve the quality of post-harvest produce, but it could harm the chilling sensitive produce and may limit the long term storage. Chilling injury is widely accepted as a major problem tropical and sub-tropical fruits during low temperature storage. The symptoms of CI are a consequence of oxidative burst from the excess of reactive oxygen species (ROS) caused by low temperature above freezing point of fruit tissues (Asghari and Aghdam 2010; Yang et al. 2012). In the recent years, many studies have proven that SA treatment, at non-toxic concentrations, can be used commercially to alleviate chilling injury (CI) in many fruits (Lu et al. 2011; Luo et al. 2011, 2012; Yang et al. 2012). SA induces the chilling tolerance by modulating anti-oxidant systems such as increased glutathione reductase, glutathione transferase, SOD and guiacol-POD and decreased CAT (Horváth et al. 2007; Yang et al. 2012) that would prevent the accumulation of ROS. SA treatment also induced polyamines biosynthesis, namely putrescine, spermidine and spermine, leading to an increase in CI tolerance in plum fruits (Luo et al. 2012). Asghari and Aghdam (2010) suggested that the accumulation of heat shock proteins in tropical and sub-tropical produces with SA or its derivatives, such as methylsalicylic acid or acetylsalicylic acid, would prevent chilling injury development during refrigerated storage. On other hand SA can delay membrane deterioration, due to lipid peroxidation, which is known as one of the adverse effects of CI, leading to malonaldehyde (MDA) accumulation. SA treatment at the concentration of 1 mM could maintain membrane integrity in pomegranates and reduced electrolyte leakage, the accumulation of MDA and the percentage of chilling injury incidence (Sayyari et al. 2009). Similar results were also reported for bamboo shoot (Luo et al. 2012). Luo et al. (2011) suggested that SA treatment at the concentration of 1.5 mM is optimal for alleviating postharvest chilling injury of 'Qingnai' plum fruits. They reported that SA application delayed the increase in electrolyte leakage, the MDA accumulation and both PPO and PODs activities which lead to lower chilling injury incidence in plum fruits during refrigerated storage. Moreover, Lu et al. (2011) also reported that both pre- and post-harvest SA treatment alleviated internal browning, a chilling injury symptom, in winter pineapple fruits which was concomitant with the inhibition of browning of enzymes, namely PPO and PAL. In cut Anthurium flowers, SA treatment (2 mM) could inhibit CI symptoms, the desiccation of sapadix and browning of spathe, which was concomitant with the reduction of ROS accumulation and lipid peroxidation, during chilling storage (Promyou et al. 2012).

5   Control of Post-Harvest Diseases by Salicylic Acid

Salicylic acid (SA) is an endogenous signal molecule, playing a significant role in the control of post-harvest diseases and also an inducer of disease resistance in plants (Joyce and Johnson 1999). Some of the results reported by the researchers regarding the effect of SA on the control of post-harvest diseases in different horticultural commodities are showed in Table 2. Beasley et al. (1999) reported that SA postharvest applications can reduce storage diseases caused by *Alternaria* and *Epicoccum* sp. in Geraldton waxflower cv. CWA Pink (*Chamelaucium uncinatum*). The SA application is also found to induce systemic resistance in tomato by incompatible race of *Cladosporium fulvum* (Cai and Zheng 1999). Foliar application of SA has led to protection of postharvest Rock melon and Hami melon fruits from diseases (Huang et al. 2000). Treatment with 2.0 mg/L of SA could suppress postharvest anthracnose disease severity caused by *Collectotrichum gloeosporioides* in mango fruit cv. Kensington Pride and also improved resistance against in vitro antifungal activity of *Cladosporium cladosporioides* (Zainuri et al. 2001). These induced defense responses involve an increase in PAL, chitinase, β-1,3-glucanase and POD activities (Meena et al. 2001). According to Qin et al. (2003), a study on sweet cherry fruit, SA treatment at the concentration of 0.5 mmol/L was effective to inhibit blue mould (*Penicillum expansum*) and alternaria (*Alternaria alternate*) rots without any surface injury. Yao and Tian (2005) demonstrated that SA treatment at the concentration of 2.0 mmol/L could exhibit antifungal effects against pathogens and fungal toxicity of *Monilinia fructicola* in sweet cherry fruits during stored at 25°C and alsosignificantly inhibited the mycelia growth and spore germination of the pathogen in in vitro. Exogenous application of SA also increased resistance to *Botrytis* rot in certain produces, such as lily leaves (Lu and Chen 2005) and table grape (Asghari et al. 2009). Harvested cluster of *vitis vinifery* L. 'Bidaneh Sefid' and 'Bidaneh Ghermez' treated with SA at the concentration of 1, 2 and 4 mmol/L effectively reduced water loss and fungal decay and increased berry firmness (Sarikhani et al. 2010). Such an increase in resistance was correlated with enhanced expression or/and activities of glucanase and chitinase (Yao and Tian 2005; Derckel et al. 1998). Aghdam et al. (2011) reported that the vapour treatment of SA at a nontoxic concentration (32 µL/L) was effective in reducing fungal decay in Hayward kiwifruit by reducing CAT and ascorbate-POD activities, which subsequently increase intra-cellular H_2O_2 concentration. The increase in H_2O_2 concentration may be involved in the enhancement of disease resistance (Zeng et al. 2006). Similarly, Janda et al. (2003) reported that SA treatment resulted in temporary reduction of CAT and increased H_2O_2 level where H_2O_2 acts as a second messenger in the activation and expression of defense related genes. Additionally, postharvest applications of SA in a concentration dependent manner from 1 to 2 mmol/L effectively reduced fungal decay in Selva strawberry fruits (Babalar et al. 2007). Gholami et al. (2010) found that the immersion in 2–3 mmol/L SA for 5 min before cold storage was effective to the fungal decay resistance of sweet cherry fruits, during storage. These findings reveal that

Table 2 Effect of SA on control postharvest diseases in different postharvest horticultural commodities

Commodity	Recommend concentration	Pathogen resistance	Author (s)
Chamelaucium uncinatum (Geraldton waxflower)	2.0 mmol/L	*Alternaria* sp., *Epicoccum* sp.	Beasley et al. (1999)
Mangifera indica (mango)	2.0 mmol/	*Collectotrichum gloeosporioides*	Zainuri et al. (2001)
Prunus avium (sweet cherry)	0.5 mmol/L	*Penicillum expansum*	Qin et al. (2003)
	2.0–3.0 mmol/L	*A. alternate*	Gholami et al. (2010)
	2.0 mmol/L	*Monilinia fructicola*	Yao and Tian (2005)
Fragaria ananassa cv. Selva (strawberry)	1.0–2.0 mmol/L	*Botrytis cinerea*	Babalar et al. (2007)
Pyrus pyriflora L. (pear)	1.0 mmol/L	*Botrytis cinerea*	Asghari et al. (2009)
Vitis vinifery L.(grape)	1.0–4.0 mmol/L	*Botrytis cinerea*	Sarikhani et al. (2010)
Lycopersicon esculentum L cv. Fenhong (tomato)	5.0 mmol/L	*Botrytis cinerea*	Wang et al. (2011)
Actinidia deliciosa (kiwifruit)	32 μL/L	*Botrytis cinerea*	Aghdam et al. (2011)

exogenous SA may have an anti-pathogenic function in fruits where endogenous SA level is correlated with induced resistance to the invading pathogens (Malamy et al. 1990). SA is shown to regulate the expression of pathogenesis related protein genes, which suggests its role as a signal molecule in providing resistance against pathogen attack. Such signaling molecules, when exogenously applied move systemically through plants, resulting in the expression of a set of defense genes that are activated by pathogen infection, thus inducing resistance against the pathogens (Epple et al. 1997). SA mediates hypersensitive and systemic acquired resistance against pathogen attack by inhibiting catalase activity, which subsequently raises intracellular H_2O_2 concentration resulting in activated expression of defense related genes. Several reports indicated that exogenous application of SA could induce the expression of many defense genes (Loake and Grant 2007; Wang et al. 2007). Moreover, SA treatment is subsequently found to induce pathogenesis-related (PR) proteins gene expression and/or resistance to viral, bacterial, and fungal pathogens in many plant species. Moreover, Wang et al. (2011) confirmed that SA significantly suppressed decay and disease incidence from *Botrytis cinerea* showing a typical gray mold symptom in tomato fruits at both mature green and breaker stages, along with higher expression level of *PR* gene after 2 days of treatment. Furthermore, an accumulation of *PRs* gene with the acquisition of resistance in fruit at different maturity stages are defined as proteins which are induced in plant tissues in response to pathogenic attack or related stimuli.

6   Conclusion

Salicylic acid, a phenylpropanoid compound, regulates plant growth and development, and also influences post-harvest physiological and metabolism of perishable crops. The observations reported above indicate that salicylic acid treatment plays an effective role in controlling the quality of horticultural crops, slows down pace of respiration, decreases the rate of ethylene production and senescence, delays ripening process, maintains or enhances the amount of bioactive compounds including antioxidants, alleviates chilling injury and induces post-harvest disease resistance, during storage.

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Chapter 16
Recent Advances and Future Prospects on Practical Use of Salicylic Acid

L. P. Popova

Abstract Plants are exposed to various pathogens, insects and different environmental constrains. To counteract against these stresses, plants have evolved defensive strategies. One very sophisticated strategy is to emit a variety of volatile substances from flowers, fruits, and vegetative tissues. Volatile compounds act as a language that plants use for communication and interaction with the surrounding environment. The volatile blends emitted by plants can be manipulated by interfering with the signal transduction pathways leading to volatile emissions. The manipulation of the volatile emission of a plant using a chemical elicitor allows for the investigation of the possible effects of plant volatiles on community ecology. Many chemicals are critical for plant growth and development and play an important role in integrating various stress signals and controlling downstream stress responses by modulating gene expression machinery and regulating various transporters/pumps and biochemical reactions. Signal molecules such as salicylic acid, jasmonates and NO play key roles in the plants' defense responses. Their defense pathways have been shown to cross-communicate, providing the plant with a regulatory potential to fine-tune the defense reaction depending on the type of attacker encountered. However, detailed understanding of the effects of these chemicals on key physiological processes that determine plant productivity in relation to stress tolerance is warranted prior to practical application. Furthermore such studies may provide an insight into the molecular mechanisms governing stress tolerance in plants and may also facilitate genetic engineering of plants to tolerate stresses. The effects of SA on plant resistance to abiotic and biotic stresses were found contradictionary, and the actual role of SA remains unresolved. The dual role of salicylic acid on different physiological processes will be discussed in this chapter. Another important objective of this study is to apply the potential of SA as an effective tool in increasing plant production and quality. The agricultural

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and ecological use of SA for improving various physiological parameters and crop yield will also be studied.

Keywords Salicylic acid · Jasmonic acid · Nitric oxide · Plant volatiles · Systemic acquired resistance

1 Plant Volatiles

In nature, plants are part of complex communities that include herbivores, carnivores, pollinators, microbes and neighboring plants. Terrestrial plants encounter numerous environmental conditions that have the potential to injure or destroy above- and/or below-ground tissues. In addition to coping with abiotic factors such as environmental changes and oxidative stress, plants are under selection to maximize fitness within a complex setting comprising biotic interactions with positive and negative outcomes.

Although plants might seem to passively undergo the attack, this is far from true. Upon perception of the attack, they quickly initiate a suite of responses that result in defenses against the attackers (Dicke et al. 2009). The defense strategies are generally organized as a network of responses including initially the infected/damaged cell, surrounding cells, as well as the entire plant referred to as the systemic response. Induced defense involves a wide range of different chemical and physical defense barriers, ranging from toxic metabolites that target the attacker's physiology, to cell wall appositions that prevent invasions by pathogenic fungi. Despite this diversity in mechanisms, the induced defense is not always sufficient to protect the plant against damage by pathogens and insects. This is why plants have evolved an additional regulatory system that allows them to fine-tune their inducible defense system. The idea that plants actively respond to tissue injury dates back to the work by Wiesner in the 19th century (Wiesner 1892). Wound signaling research in plants was revitalized in 1970s following the discovery that leaf damage induces systemic accumulation of proteinase inhibitors (PIs) that impair insect digestive enzymes (Green and Ryan 1972). This pioneer paper introduced the idea that mobile signals generated at the site of injury trigger systemic protection against insect herbivores. During the past 35 years, systemic wound responses have been demonstrated in species throughout the plant kingdom, and intense research efforts have been focused on the identification of systemic wound signals and the mechanisms by which they are generated, transported, and perceived (Karban and Baldwin 1997; Ryan 2000; Leon et al. 2001). The concept that biotic agents activate local and systemic host defense responses is a cornerstone of current theories of plant immunity (Jones and Dangl 2006; Browse and Howe 2008; Howe and Jander 2008; Boller and He 2009; Wu and Baldwin 2009). Baldwin is one of the first in the field to investigate chemical signaling among plants. He was studying trees with biologist Jack Schultz, and in

1983 they published a controversial hypothesis: airborne chemical cues from damaged maple and poplar trees seemed to boost the chemical defenses of undamaged trees nearby (Baldwin and Schultz 1983). In 1983 Rhoades first proposed the Talking Trees Hypothesis whereby damaged trees might warn their neighbours from an imminent herbivore attack, thus resulting in increased resistance. He was the first to find lower performance of herbivore insects on trees exposed to volatile compounds from conspecific infested trees. He proposed that plants are able to capture warning signals from damaged neighbours and thus produce defensive metabolites against herbivores. This author was strongly criticized for his methodological approach and several ecologists rejected the hypothesis. In spite of public appeal and of this being the 25th year of the Talking Trees Hypothesis, only recently the most sceptical scientists got convinced. The advent of modern molecular biology and molecular genetics has revolutionized our ability to put in an upper level the complexities of plant signal transduction pathways. A variety of techniques are now available to identify the genes that control the plants development and ability to adapt to its environment. Each technique has its own strengths and weaknesses and must be carefully selected by the researchers according to the question that they would like to ask. Carefully controlled experiments over the last 10 years have shown that interplant communication is possible, even though the phenomenon is apparently not widely distributed over the plant kingdom. One recurring point of the controversy on the role of volatile organic compounds (VOCs) signaling between plants has been the distance over which the herbivore-induced volatiles can be received (Baldwin et al. 2002; Karban et al. 2003; Kessler et al. 2006). Direction and dynamics of the transport are dependent on temperature, convective transport, and wind for aboveground signalling or water for below-ground signalling. Small highly volatile compounds such as ethylene, methanol, isoprene, acrolein, methacrolein and some monoterpenes diffuse rapidly into the headspace and are diluted in the atmosphere. For such compounds, signalling function is likely limited to the foliage of the emitter and of neighbours with intertwined canopies. On the other hand, compounds with less volatility such as terpene alcohols, methyl jasmonate (MeJA), and methyl salicylate (MeSA) are more likely to work as long-distance signals. Because the slower dispersal and development of plumes of higher concentration, such signals would be carried farther in functional levels by turbulent atmospheric flows (Baldwin et al. 2006). However, the greater the distance over which a signal is to function, the greater the released amounts must be (Firn and Jones 1995). Once these criteria are met, a compound may be considered as a potential airborne signal (reviewed by Campos et al. 2008).

The quantities of plant volatiles released into the air are not equal. Almost one-fifth of the atmospheric CO_2 fixed by land plants is released back into the air each day as volatiles. Plant volatiles constitute about 1 % of plant secondary metabolites; they act as a means for communication between plants, insects, and herbivores, moving freely through the atmosphere and soil. Volatiles emitted into the atmosphere can serve to defend plants against herbivores and pathogens by their ability to either directly repel microbes and animals, or attract natural predators of

attacking insects/herbivores. These warning volatiles also induce the emission of similar defensive volatiles by neighboring plants. Volatiles can also enhance a plants reproductive by attracting pollinators and/or seed dispersers. Volatiles emitted from roots can contribute to below ground defense in similar ways: acting as antimicrobial and antiherbivore substances, or attracting enemies of root feeding herbivores. The presence of volatiles, and abilities for plants to produce them, greatly increases plants resiliency to a variety of threats (reviewed by Dudareva et al. 2006).

Plant volatiles provide herbivorous arthropods with information that allow them to discriminate between host and non-host plants. Volatiles may also indicate plant stress status, and natural enemies can use herbivore-induced plant volatiles as cues for prey location. Neighbouring plants may also make use of volatiles cues to prepare for herbivore attack. Since both constitutive and inducible plant volatile emissions can be modified by plant breeding or metabolic engineering, the possibility exists to improve plant resistance against important pests both directly and indirectly via improved biological control.

Plants are always producing volatiles, the most common of which are the typical green-leaf volatiles (GLVs), which include several saturated and unsaturated six-carbon alcohols, aldehydes, and esters. These GLVs are typically released in higher amounts after mechanical damage (Pare and Tumlinson 1999).

Plants also have the ability to produce volatiles after being induced by insect feeding. The most obvious result of induction is a marked increase in the amount of terpenes produced by the plant, especially 1,3,6-octatriene, 3,7-dimethyl (β-ocimene), and 1,6-octadien-3-ol, 3,7-dimethyl (linalool) (Pare and Tumlinson 1999). Induced volatile production can lead to attraction of natural insect enemies of the herbivorous insect (Turlings et al. 1993). There is typically a delay between the onset of insect feeding and emission of induced volatile release, therefore, a series of biochemical reactions must be involved (Pare and Tumlinson 1999).

In recent years, major advances have been made in identifying metabolites that are candidate for systemic signals in plant defense against different attacks. Many chemicals are critical for plant growth and development and play an important role in integrating various stress signals and controlling downstream stress responses by modulating gene expression machinery and regulating various transporters/ pumps and biochemical reactions. These chemicals include calcium (Ca^{2+}), cyclic nucleotides, polyphosphoinositides, nitric oxide (NO), sugars. Plant hormones: abscisic acid (ABA), jasmonates (JAs), salicylic acid (SA) and polyamines, which occupy a central role in regulating these highly dynamic and adaptive responses (Satner and Estelle 2009).

MeSA, JAs, azelaic acid and a diterpenoid have been implicated as mobile signals associated with the activation of systemic acquired resistance (SAR), which confers enhanced resistance against a broad spectrum of pathogens. By contrast, auxins probably contribute to negative regulation of systemic defenses.

The first systematic studies into the priming phenomenon were carried out by Kauss and Conrath laboratories in 1990s. They discovered that exogenous application of SA parsley cells can enhanced cellular defenses upon secondary

treatment with a pathogen elicitor (Kauss et al. 1992, 1993). Latter, Park et al. (2007) suggested that MeSA is a critical, phloem-mobile SAR long-distance signal in tobacco. In addition, MeSA has been suggested to act as a volatile intraplant signal that is capable of activating SAR in distant leaves of the same plant (Shulaev et al. 1997). The authors conclude that MeSA may function as an airborne signal which activates disease resistance and the expression of defense-related genes in neighbouring plants and in the healthy tissues of the infected plant. Another recent study extended this putative signaling function of MeSA to SAR in *Arabidopsis* (Vlot et al. 2008).

Among the compounds that are thought to be involved in interplant communication are two jasmonates (cis-jasmone and MeJA), MeSA, terpenes, and some C6-C10 alkenals and alkanals (Preston et al. 2001).

Since the pioneer work reported by Farmer and Ryan (1990), JAs have been the most studied compounds as well as the stronger candidates for aerial signals in interplant communication (Shonle and Bergelson 1995; Farmer 2001; Preston et al. 2001, 2004). Different approaches have been used for understanding the mechanisms of interplant communications. Most of them are based on the integration of molecular biology, biochemistry, physiology, and ecology. Manipulation of the volatile emission of a plant using a chemical elicitor allows for the investigation of the possible effects of plant volatiles on community ecology. The use of an elicitor has the advantage of being able to induce (part of) the volatile blend without removal of plant tissue, and offers the possibility to apply a controlled dose, whereas it is difficult to control the amount of damage inflicted by herbivore feeding (Bruinsma et al. 2009).

Plant defenses against pathogens and insects are regulated differentially by cross-communicating signal transduction pathways in which SA and JA play key roles. SA and JA accumulate in response to pathogen infection or herbivore damage, resulting in the activation of distinct sets of defense-related genes. Mutant and transgenic plants that accumulate SA are often more susceptible to pathogen infection than wild-type plants (Delaney et al. 1994; Wildermuth et al. 2001). Blocking the response to JA generally renders plants more susceptible to herbivore damage (Howe et al. 1996; McConn et al. 1997), although enhanced susceptibility toward necrotrophic pathogens has been reported as well (Thomma et al. 2001). SA- and JA-dependent defense pathways have been shown to cross-communicate (Felton and Korth 2000; Feys and Parker 2000), providing the plant with a regulatory potential to fine-tune the defense reactions depending on the type of attacker encountered.

Recently nitric oxide (NO) has emerged as a key signalling molecule in plants. It is a small, water and lipid soluble gas that in recent years has been pointed as a major signalling molecule of ancient origin and ubiquitous importance However, research on NO and plant signalling was mainly restricted to a few 'pioneers' such as Leshem (Leshem and Haramaty 1996) and Laxalt (Laxalt et al. 1997) until the landmark publication in 1998 describing NO as a plant defense signal (Delledonne et al. 1998). Since then, studies on NO and its role on plant biology have increased dramatically and intensive reviewed (Durner and Klessig 1999; Beligni and

Lamattina 2001; Wendehenne et al. 2001; Popova and Tuan 2010). NO is able to move freely through the lipid phase of membranes. Once produced, it can move from one cell to another or within a cell. However, being a reactive free radical, it has a relatively short half-life, in the order of a few seconds. Typically, NO rapidly reacts with O_2 to form nitrogen dioxide (NO_2), and rapidly degrades to nitrite and nitrate in aqueous solution. The range of its effects is limited to the cell in which it is generated, or to cells in the near neighbourhood (Neill et al. 2003).

2 Signaling Cross Talk

2.1 Signaling

Like humans, plants also get diseases and use their immune systems to respond. Sometimes, the immune system overreacts and exhibits responses with deleterious effects. Plant neurobiology is a newly focused field of plant biology research that aims to understand how plants process the information they obtain from their environment to develop, prosper and reproduce optimally. The behavior plants exhibit is coordinated across the whole organism by some form of integrated signaling, communication and response system. Plant neurobiology claims to study the role of signalling, communication and behaviour to integrate the data obtained at the genetic, molecular, biochemical and cellular levels, with the physiology, development and behaviour of individual organisms, plant ecosystems and evolution.

Plant disease resistance can be triggered by specific recognition of microbial effectors by plant nucleotide binding-leucine rich repeat (NB-LRR) receptors, which are believed to work as key receptors of plant immune systems and are used for fighting disease. Over the last few years, many efforts have greatly improved the understanding of effector and NB-LRR function, but have left a lot of questions as to how effector perception activates NB-LRR induction of defense-signaling. Later, scientists identified related genes in humans. These human genes also code for proteins involved in immunity and inflammatory responses, and their study has provided clues about the mechanisms behind inflammatory diseases (Bernoux et al. 2011).

Certain protein kinases [receptor-like kinase (RLK) gene-family] have been shown to be crucial for plant cell signaling pathways associated with plant immune responses. More recently, it has been shown that the LRR (cytoplasmic protein kinase) gene domains of RLK interact with a diverse group of proteins leading to combinatorial variations in signal response specificity. Therefore, RLK appears to play a central role in signaling during pathogen recognition, the subsequent activation of plant defense mechanisms, and developmental control (Afzal et al. 2008).

The role of phytohormones in the regulation of these induced defenses is well established. SA, JA, NO, and ethylene (ET) are recognized as key players in the

regulation of the signaling pathways involved. Other plant hormones, including ABA (Mauch-Mani and Mauch 2005), brassinosteroids (Nakashita et al. 2003), and auxin (Navarro et al. 2006), have also been implicated in plant defense, but their significance is poorly studied. The major signal transduction pathway underlying insect-induced plant defenses is the octadecanoid pathway (Kessler and Baldwin 2002). The phytohormone JA is the main octadecanoid of this pathway, while 12-oxophyto-dienoic acid (OPDA) is an intermediate that acts as a weaker elicitor of plant responses (van Poecke and Dicke 2002; Kessler and Baldwin 2002). JA is often used to manipulate the volatile emission. Treatment of plants with JA or MeJA has been reported to induce volatile emission, similar to herbivore induction, extrafloral nectar production, increased levels of endogenous secondary metabolites, reduced development and oviposition of herbivores, increased attraction of predators and parasitoids, and enhanced parasitism rates of herbivores for a wide variety of plant species (Kessler and Baldwin 2001; Mumm et al. 2003; Heil 2004).

2.2 Crosstalk

It may be defined as occurring when a common cellular component is used in more than one signal transduction chains and leads to an exchange of information between different signalling pathways. It is used in many cases to explain how two or more signalling pathways might interact and lead to different cellular outcomes. In cellular terms, 'crosstalk' is studied to increase understanding of the control of signalling pathways and networks, and how they are regulated (reviewed by Taylor et al. 2004). Ecologically or agriculturally, 'crosstalk' provides possibilities to address series of studies about how a plant interacts with its environment.

Evidence for hormone cross talk comes largely from the analysis of mutant phenotypes. Frequently mutants that are affected in one hormone pathway also display changes in other hormone responses. Almost all plant hormones interact with one or more additional hormones by affecting synthesis, transport or response. The type of interaction very often depends on the tissues, developmental stage or environmental conditions (Satner and Estelle 2009).

3 Crosstalk Between Salicylates and Jasmonates

One of the best-characterized examples of defense-related signal cross talk is the interaction between the SA and JA response pathways. MeSA, a volatile liquid, also known as oil of wintergreen, is made by a number of plants. It is synthesized from SA, a nonvolatile chemical signal required for the establishment of acquired resistance and local and systemic induction of antimicrobial pathogenesis-related proteins. MeSA acts by being converted back to SA. Whitham et al. (1994) and

Shulaev et al. (1997) showed that MeSA is a major volatile compound produced by tobacco plants inoculated with tobacco mosaic virus. They concluded that MeSA may function as an airborne signal which activates disease resistance and the expression of defense-related genes in neighbouring plants and in the healthy tissues of the infected plant.

The discovery of JA as a potent elicitor of proteinase inhibitor expression in tomato (Farmer and Ryan 1990) and secondary metabolism in plant cell cultures (Gundlach et al. 1992) provided initial insight into the importance of JA as a defense hormone. Subsequent genetic studies established the importance of JA in promoting resistance to herbivores and pathogens that lead to decrease in their presence by activating JA synthesis in damaged or infected host tissue (Howe et al. 1996; Vijayan et al. 1998; Kessler et al. 2004; Howe and Jander 2008). JA exerts its protective effects by regulating a wide range of defense-related processes, including the synthesis of toxic secondary metabolites (Pauwels et al. 2009), production of morphological barriers (Yoshida et al. 2009), and changes in the rate of vegetative growth (Popova et al. 1988; Yan et al. 2007; Balbi and Devoto 2008; Zhang and Turner 2008). These broad effects of JA imply a general role for the hormone in controlling tradeoffs between growth and defense (Herms and Mattson 1992; Baldwin 1998; Popova 2012). The interactions between SA and JA signaling appear to be complex, and there is evidence for both positive and negative interactions between these pathways.

Although most reports indicate a mutually antagonistic interaction between SA- and JA-dependent signaling, synergistic interactions have been described as well. A few studies provide evidence for synergistic interactions between SA and JA signaling mechanisms. For example, the co-application of SA and JA conferred higher levels of expression of the basic PR-1 gene (PR-1B) in tobacco than each molecule applied individually (Xu et al. 1994).

Many examples of trade-offs between SA-dependent resistance against biotrophic pathogens and JA-dependent defense against insect herbivory and necrotrophic pathogens have been reported (Pieterse et al. 2001; Bostock 2005).

The inhibitory effects of salicylates on JA biosynthesis and signaling have been extensively investigated. Several studies have demonstrated that exogenous application of aspirin, SA, and MeSA, suppresses the expression of JA biosynthesis genes, suggesting that salicylates may target the JA biosynthesis pathway to suppress downstream JA signaling (Peña-Cortes et al. 1993; Niki et al. 1998; Stout et al. 1999. As exogenously applied jasmonates reverse the inhibitory effects of SA application, this effect has been linked to the suppression of JA biosynthesis (Sivasankar et al. 2000).

One of the most studied induced defense responses in plants is systemic acquired resistance (SAR). SAR is triggered after local infection with pathogens, causing hypersensitive necrosis, and is effective against a broad spectrum of plant pathogens. Two main metabolic pathways are involved in SAR, the salicylic acid pathway, generally induced by pathogens, and the jasmonic acid pathway, often induced by insect attack. Considerable "crosstalk" occurs between these two pathways—sometimes they are additive and synergistic, and other times salicylate

activity suppresses the jasmonic acid pathway. The activation of SAR has been shown to suppress JA signaling in plants, thereby prioritizing SA-dependent resistance to microbial pathogens over JA-dependent defense against insect herbivory (Pieterse et al. 2001). Pharmacological and genetic experiments have shown that SA is a potent suppressor of JA-inducible gene expression (Peña-Cortes et al. 1993).

It appears that different branches of these two pathways combine in different situations, making for a complex interplay. Complicating this is the induction of jasmonic-SAR, or really a priming or strengthening of SAR, by rhizosphere bacteria, composts, and certain bacterial isolates. Additionally, some insects induce SAR channels that are normally induced only by microbes. Common to both the SA and JA pathways is the shikimic acid pathway, a branch of Krebs cycle phospoenolpyruvate, and source of the majority of phenolics in the plant. One of the three aromatic (ring-structured) amino acids, phenylalanine, is an important intermediate here. In SAR, phenylalanine is diverted from protein synthesis by an enzyme, phenylalanine ammonialyase (PAL), which de-ammoniates the molecule and converts it to phenol precursor cinnamic acid.

JA and MeJA mediate plants defense against insects, and appears to mobilize antimicrobial defense response, while SA-mediated defense responses are effective against biothropic fungi, bacteria and viruses (Thomma et al. 2001).

Besides SA/JA cross talk, interactions between SA and ET, JA and ABA, and JA and ET have been shown to function in the adaptive response of plants to herbivores and pathogens with different lifestyles. Cross talk between defense-signaling pathways is thought to help the plant decide which defensive strategy to follow, depending on the type of attacker it is encountering. It seems that attackers have also evolved to manipulate plants for their own benefit by suppressing induced defenses or modulating the defense-signaling network (Pieterse and Dicke 2007). SA-, JA-, and ET-dependent pathways regulate defense responses that are differentially effective against specific types of attackers. In general it can be stated that pathogens with a biotrophic lifestyle are more sensitive to SA-dependent responses, whereas necrotrophic pathogens and herbivorous insects are resisted by JA/ET-dependent defenses (Thomma et al. 2001). However, there are many exceptions to this basic framework, and recent work suggests that interactions between the JA and SA pathways may play important roles in fine-tuning the defense responses (Thaler et al. 2004). The few studies that have addressed plant responses to parasitism by other plants suggest that both salicylates and jasmonates can mediate effective defenses. A question remains as to the relevance of synergistic signal interactions to resistance responses. Often simultaneous activation of signaling pathways has no additive effects with resistance patterns to discrete pathogens and pests being maintained (van Wees et al. 2000), although there are instances where both SA-and JA-signaling pathways are required (Ellis et al. 2002). As discussed by Mur et al. (2006), it seems likely that variably employed synergistic/antagonistic mechanisms, which need not involve only SA and JA but also, for example, ET may represent more flexible plant response to a particular stress.

4 Signaling Role of Nitric Oxide and Crosstalk with Salicylic Acid and Jasmonates

Although less is known about the role of NO in plants several lines of evidence indicate an interrelationship between SA and NO signaling pathways, and also between JA and NO signaling pathways (Grun et al. 2006).

NO-related signaling can be attributed to various NO derivates, collectively referred to as reactive nitrogen species (RNS). RNS comprise not only NO radical (NO*) and its nitroxyl (NO^-) and nitrosonium ions (NO^+) but also peroxinitrite ($ONOO^-$) and dinitrosil ion complexes. The term NO-related signaling is used to summarize effects accused by all their RNS (Leitner et al. 2009).

NO acts as a key signal in plant resistance to incompatible pathogens by triggering resistance-associated hypersensitive cell death. In addition, NO activates the expression of several defense genes (e.g., pathogenesis-related genes, phenylalanine ammonialyase, chalcone synthase) and could play a role in pathways leading to systemic acquired resistance (Romero Puertas and Delledonne 2003). NO has been associated with plant defense responses during microbial attack, and with induction and/or regulation of programmed cell death (Huang et al. 2004). The expression of genes in response to NO has also been shown to mediate via SA and JA signaling pathway (Grun et al. 2006).

Graziano and Lamattina (2007) reported that NO accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. They suggested that NO action is closely linked to SA and thus it could mediate many processes via SA signaling pathway. Using different biochemical, molecular and genetic approaces Huang et al. (2004) and Laxalt et al. (2007) floated an idea for an interaction between NO, ROS, Ca^{2+}, SA, JA and ET in many biotic stress responses and also in developing cross tolerance in plants.

One of the fastest reactions of NO within biological systems is the combination with reactive oxygen species (ROS). This function is very often described as either toxic or protective. Under severe stress conditions the production of ROS is predominant and under such conditions NO may act as a chain breaker and thus limit damage. NO itself is a very reactive nitrogen species and therefore can start chain reactions that cause cell injury. It is interesting to note that ROS and NO exert reciprocal control on each other in several ways. NO can regulate ROS levels by inhibiting the activities of antioxidant enzymes (Clark et al. 2000). However, *Arabidopsis* plants unable to express AtNOS1, the only NO synthase so far identified in plants, showed higher levels of ROS, suggesting that in certain conditions NO can also act as an antioxidant, reducing cell damage and senescence (Guo and Crawford 2005). Several other lines of study have shown that the protective effect of NO against abiotic stresses is closely related to the NO-mediated reduction of ROS in plants (Lamattina et al. 2003). On the other hand, the relative rates of production of O_2^- and H_2O_2 are critical in modulating the reactivity of NO. A model has been proposed in which if the NO-O_2^- balance is in favor of O_2^-, NO is scavenged before it can react with H_2O_2. If the balance is in favor of

NO, O_2^- is scavenged before it is dismutated to H_2O_2. Whereas the cooperation of NO and H_2O_2 leads to the death of the cell, reciprocal scavenging of NO and O_2^- leads to the formation of $ONOO^-$, which appears to be non-toxic in plants (Delledone et al. 2001; Zaninotto et al. 2006).

Both ROS and SA have been shown to synergize with NO to enhance host cell death in soybean suspension cells (Delledonne et al. 1998). In addition, SA may mediate and/or potentiate NO's effects by altering the activity of various NO-regulated enzymes. SA also induces the synthesis of a pathogen-inducible oxygenase in plants that has strong homology to a mammalian cyclooxygenase (Sanz et al. 1998). This enzyme is posttranslationally activated by NO in mammals (Nogawa et al. 1998). Thus, SA and NO may work synergistically to transduce the defense signal by targeting the same effector proteins and/or their genes.

In other cases SA may antagonize the NO signaling pathway. It has been reported that in plants SA may antagonize NO's ability to inhibit respiration (and thereby cause oxidative stress) by activating the NO-insensitive alternative oxidase (Millar and Day 1997). Considering the many interactions that are currently emerging between the pleiotropic effectors SA, NO, and other ROS, it is apparent that researchers are at a very early stage in understanding the complexity of their action in disease resistance.

NO also functions independently of ROS in the induction of various defense genes, including those found in pathogenesis-related proteins and enzymes of phenylpropanoid metabolism involved in the production of lignin, antibiotics and the secondary signal salicylic acid. The mobile nature of NO and its chemical reactivity with various cellular targets means that downstream effects of NO may be directly induced by interaction with various cellular components, like ion channels or proteins that modulates gene expression, or indirectly following interaction with signaling proteins such as protein kinases. NO signaling functions depend on its reactivity and ROS are key modulators of NO in triggering cell death, although through mechanisms different from those commonly observed in animals (Delledonne et al. 2001). As ROS initiate various signaling pathways, the preservation of suitable ROS levels might correspond to survival response. NO interacts with ROS in different ways and serves as an antioxidant during various stresses (Wellburn 1990). In the pathogen-activated hypersensitive response, both NO and ROS act as signal molecules (Delledonne 2005). ROS and NO are also involved in the regulation of SA biosynthesis (Durner et al. 1998).

Several models suggest that redox signalling through NO and ROS is enhanced by SA in a self-amplifying process (Klessig et al. 2000). Nonetheless, the relationship between NO, SA, and ROS in the activation of defense genes and/or induction of host cell death is not clearly defined. Several lines of evidence point to an inter-relationship between NO and SA in plant defense. It is well documented that both SA and NO play important role in activation of plant defense responses after pathogen attacks, but the interrelationship between their respective signaling pathways is still unclear.

Treatment of tobacco and *A. thaliana* leaves with NO induces a substantial increase in endogenous SA (Durner et al. 1998). Song and Goodman (2001)

suggested that NO is involved in both SA biosynthesis and action. It has been also shown that SA induced the production of ROS, such as H_2O_2 and NO (van Camp et al. 1998). In addition, SA may mediate and/or potentiate NO' effects by altering the activity of various NO-regulated enzymes.

The global picture of ROS-NO-SA interactions is far from being complete, but it already has been revealed as a fascinating cross talk of mechanisms able to fine-tune resistance responses and other plant reactions to environmental stimuli, as well as important developmental aspects in the life of the plant. This dual role of NO can be accomplished by a signal transduction pathway through a signaling cascade. Involvement of another signaling molecules, such as salicylic acid, jasmonic acid, abscisic acid, ethylene and Ca^{2+} activate a very complex network (Beligni and Lamatina 2001).

It should also be considered that the SA-induced NO production is probably controlled by multiple mechanisms, as suggested by the experiments on calcium signalling. There is increasing evidence of the existence of cross talk between NO and calcium signalling systems in plants (Lamotte et al. 2004). It has been proved that both ROS and calcium signals are intimately interconnected. How this cross talk can finally modulate the translocation and/or the activity of nuclear proteins leading to the control of specific genes is still unsolved problem (Mazars et al. 2010).

Wang and Wu (2005) reported that exogenously supplied MeJA at 100 μM induced rapid production of NO in *Taxus* cell cultures, reaching a maximum within 6 h of MeJA supply. Several other responses were documented, like the production of H_2O_2, and the increases in intracellular malondialdehyde (MDA) content, lipoxygenase (LOX) and phenylalanine ammonium-lyase (PAL) activities. The MeJA-induced H_2O_2 production was suppressed by an NO donor, sodium nitro-prusside (SNP), but enhanced by NO inhibitors, N'.OMEGA.'-nitro-L-arginine (L-NNA) and 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO). In contrast, the MeJA-induced MDA, LOX and PAL were all enhanced by the NO donor but suppressed by the NO inhibitors. According to the authors, the results are suggestive of a role for NO as a signal element for activating the MeJA-induced defense responses and secondary metabolism activities of plant cells.

Supplying young excised tomato plants with the NO generators SNP and SNAP before wounding caused a nearly complete inhibition of the induction of synthesis of proteinase Inh I, one of several wound-inducible proteinase inhibitor proteins in tomato leaves. NO also blocked the H_2O_2 production and proteinase inhibitor synthesis that was induced by systemin, oligouronides, and JA. Although the expression of proteinase inhibitor genes in response to JA was inhibited by NO but not that of wound signaling-associated genes was not. The inhibition of wound-inducible H_2O_2 generation and proteinase inhibitor gene expression by NO was not due to an increase in SA, which is known to inhibit the octadecanoid pathway. Instead, NO appears to be interacting directly with the signaling pathway downstream from JA synthesis, upstream of H_2O_2 synthesis. The authors suggested that NO may have a role in down-regulating the expression of wound-inducible defense genes during pathogenesis (Orozco-Gardenas and Rayan 2002).

5 The Dual Role of Salicylic Acid

The first indication for a physiological effect of SA was the discovery of flower-inducing action and bud formation in tobacco cell cultures (Eberhard et al. 1989). The most important effects of SA are connected with regulation of plant flowering, heat production and plant desease resistance (Raskin 1992; Popova et al. 1997 for reviews). Some observations that SA exerted inhibitory effect on stomata function, chlorophyll content, photosynthetic gas-exchange and the activity of carboxylating enzymes (Pancheva et al. 1996; Pancheva and Popova 1998) point to a possible regulatory function of SA connected with photosynthetic reactions.

It has been shown that SA provides protection in maize plants against low-temperature stress (Janda et al. 1999; Tasgin et al. 2003), induces thermotolerence in mustard seedlings (Chen et al. 1997; Dat et al. 1998a, 1998b) or modulates plant responses to salt and osmotic stresses (Borsani et al. 2001), ozone or UV light (Sharma et al. 1996), drought (Senaratna et al. 2000; Singh and Usha 2003), herbicides (Ananieva et al. 2004) or pathogens (Malamy et al. 1990; Durner et al. 1997). Furthermore, SA is also known to be involved in plant protection to heavy metals. SA pretreatment alleviates Pb- and Hg-induced membrane damage in rice (Mishra and Choudhuri 1999) and Cd toxicity in barley (Metwally et al. 2003), pea and maize plants (Pal et al. 2002; Krantev et al. 2008; Popova et al. 2009). SA has been shown to accumulate in plants in response to various oxidizing stresses, such as H_2O_2 (Leon et al. 1995), ozone (Sharma et al. 1996), heat (Dat et al. 1998a) and it has been suggested that it is directly involved in signaling different antioxidant responses (Larkindale and Knight 2002).

The effects of SA on plant resistance to abiotic and biotic stresses were found contradictionary, and the actual role of SA remains unresolved. Generally, deficiency of SA or a very high level of SA increases the plant susceptibility to abiotic stress. The optimal levels for the highest stress tolerance range from 0.1 to 0.5 mM for most plants. But the role of SA at a certain level in moderate and severe abiotic stress may be different. This can be attributed to redox regulations in plant cells (Yuan and Lin 2008). Although SA has a beneficial effect against different abiotic and biotic stresses, the compound itself stressed the plants. This was confirmed by observations of many authors. Pancheva et al. (1996) showed that long-term treatment (7 days) of barley seedlings with 500 μM or 1 mM SA reduced root and seedlings growth, chlorophyll and protein content and the rate of CO_2 assimilation. Treatment with SA exerted inhibitory effect on stomata function, and the activity of carboxylating enzymes (Pancheva and Popova 1998). Different types of photosynthetic responses (both positive and negative) have been described. For instance, Sahu et al. (2002) showed that low concentrations of SA (20–100 μmol/L) favoured photosynthetic activity while the high concentrations (>500 μmol/L) induced drastic attenuation of photosynthetic activity. In contrast, Zhou et al. (1999) showed that stem injection of corn with 10^{-2} mol/L SA increased the photosynthetic rate. In addition, Khan et al. (2003) demonstrated that 48 h after foliar application of 10^{-5} mol/L SA to corn and soybean plants the rate of

photosynthesis increased. Furthermore, the observations showed that the effect of SA on the capacity of the plants for CO_2 fixation is time-dependent. Long-term (7-d) treatment with SA decreased the rate of photosynthesis and the level and activity of RuBPCase in barley. In short-term (2 h) treated plants no changes in photosynthetic reactions were observed, while 24 h after treatment of barley seedlings with SA the rate of photosynthesis declined (Pancheva et al. 1996). Long-term treatment (7 d) of barley seedlings with SA caused alterations in chloroplast ultrastructure (Uzunova and Popova 2000) and changed the polypeptide composition of thylakoid membranes (Maslenkova and Toncheva 1998). Very scantly information is available for the effect of SA on photosynthetic electron-transport activity. Under long-term treatment (7 d) Maslenkova and Toncheva (1998) observed the inhibitory effect of SA on PS II oxygen-evolving reactions. The effect of SA depended on the treatment durations, no changes in these parameters were observed when barley seedlings were treated with SA for 2 h, the inhibition appeared 6 h after the start of treatment. Authors suggested that SA plays different roles based on its endogenous levels in particular plant species under specific developmental and environmental conditions. Similar observations were reported by Sahu et al. (2002) studying changes in electron-transport activity in wheat plants treated with SA for 7 days. They showed that the effect of SA is a concentration-dependent. The low concentration of SA (50 µmol/L) favoured PS II- and PS I-catalyzed electron flow, whereas electron transport activity of chloroplasts was drastically disturbed by high SA concentrations (500 and 1,000 µmol/L). Janda et al. (1999) showed that treatment of maize plants with 500 µmol/L SA for 24 h caused only a slight decrease in the ratio of variable to maximal fluorescence (Fv/Fm) and the observed decrease in photosynthetic activity was not due to a depression in PS II.

SA reduced the negative affects of salinity stress on shoot and root dry weights in *Rosemary* seedlings (Sharareh et al. 2009). The authors demonstrated that concentration of 150 ppm SA had positive effect on plant photosynthesis and stomatal conductance when plants were under stress conditions. Transpiration rates may be decreased by the application of salicylates and are likely to be concentration and plant species dependent. Stomatal closure has been observed within 13 min when *Commelina communis* leaves were treated with a 10 mM acetyl-salicylic acid (ASA) solution (Larque-Saavedra 1979). SA application is shown to decrease stomatal apertures at much lower concentrations (Rai et al. 1986). In *Arabidopsis*, SA has been proposed to have a dual role, which may explain differences in plant responses to SA (Borsani et al. 2001). Excessive SA accumulation can induce a programmed cell death pathway (Rao and Davis 1999), with adverse effects of SA above 1 mM being observed in tomato and bean plants (Senaratna et al. 2000).

Canakci (2003) reported that treatment of bean seedlings with 100 ppm ASA solutions had no significant influence on weight alteration, pigment and protein amounts but 250 and 500 ppm ASA caused an increase on weight loss. ASA at higher concentrations (250 and 500 ppm), generally, caused a decrease on pigment amounts but 100 ppm ASA had no considerably significant influence on them,

none of the ASA treatments caused a statistically significant influence on carotenoid amount. 100 and 250 ppm ASA treatments did not cause a significant influence on protein amount, however 500 ppm ASA treatment caused an increase on protein injury.

Although SA participates in the development of stress symptoms, it is also needed for the adaptation process and the induction of stress tolerance. Most abiotic and biotic stresses increase the plant concentration of SA, which point to its involvement in stress signaling.

There is strong evidence that SA mediates the oxidative burst. SA specifically binds to catalase and inhibits the activity of the enzyme (Chen et al. 1993) thus increasing the H_2O_2 level. SA is a direct scavenger of hydroxyl radical and an iron-chelating compound, thereby inhibiting the direct impact of hydroxyl radical. While the moderate doses enhance the antioxidant status and induce stress resistance, higher concentrations activate a hypersensitive cell death pathway and increase stress sensitivity. When applied at suitable concentrations, exogenous SA may cause transient oxidative stress, which acts as a hardening process, increasing the antioxidative capacity of the plants. In some cases SA potentates the activation of antioxidant defense responses to minimize the oxidative stress induced by different environmental factors, while in the other cases high levels of SA led to the activation of oxidative burst and cell death. These examples demonstrate that SA is an important component in modifying stress responses and may have a pro- or antioxidative role depending on its endogenous level (Yang et al. 2004).

6 Agricultural and Ecological Use of Salicylic Acid

Phyhormones play vital role in plant tolerance against environmental stresses. Plant resistance can be induced by adopting various strategies. One of these, exogenous use of various growth regulators and other chemicals has been proven worthwhile in producing resistance to many stresses in a number of plants. Another important function of plant growth regulators is their agricultural use for improving various physiological parameters and crop yield. Plant growth substances help to bring rapid changes in the phenotypes of the plants and also improve the growth, translocation of nutrients to economic parts and ultimately increase the productivity of the crops. Seed priming is a short-term and a very pragmatic approach for agricultural use. Another strategy is foliar application of plant growth regulators, both natural and synthetic. Among other regulators SA has been proven to be effective in increasing plant production and quality of the produce. People aren't the only ones to benefit from SA. Research has shown that spraying this naturally occurring compound onto some plants triggers natural defenses that keep harmful fungi, bacteria, and viruses far from the plants.

Jeyakumar et al. (2008) reported that application of SA (125 ppm) increased the dry matter production and seed yield in black gram. Nagasubramaniam et al. (2007) showed that foliar application of SA (100 ppm) on baby corn increased the

plant height, leaf area, crop growth rate and total dry matter production. Moreover, and the cob yield of baby corn was the highest by the foliar application of SA. Sujatha (2001) reported that foliar application of SA (100 ppm) on green gram at 75 days after sowing increased plant height, root length, number of leaves and leaf area index. Authors also demonstrated that foliar application of SA increased number of pods plant^{-1}, number of seeds pod^{-1}, seed weight plant^{-1}, 100 seed weight and grain yield. In green gram, foliar application of SA at branching, flower bud initiation stages increased the number of flowers, pods and seeds plant^{-1} and seed yield (Singh et al. 1980). Treatment of mungbean with SA significantly increased the pod number plant^{-1} and yield (Singh and Kaur 1981).

The quality parameters were also improved by SA treatment. Jeyakumar et al. (2008) reported that the highest seed protein content in black gram was recorded by foliar application of SA (125 ppm). Hussein et al. (2007) indicated that in maize hybrid, all determinated amino acids concentration such as tyrosine, lysine, arginine, alanine, leucine, except methionine increased with the application of SA (200 ppm). Proline concentration increased when using SA acid as foliar application under salt stress condition. Sujatha (2001) revealed that foliar application of SA (100 ppm) on green gram at 75 DAS increased seed protein and soluble protein. Kalpana (1997) found that foliar spray of SA increased the soluble protein in rice. Results indicated that seed imbibition with SA affected physiological processes related to growth and development in cucumber plants. At lower concentrations, SA significantly increase rate of seed germination and plant dry mass even if added NO_3 was 20 μM. Plants treated with 10 and 50 μM SA had higher chlorophyll levels and NO_3-assimilation through the induction of nitrate reductase (NR) activity. However, 100 and 500 μM were detrimental to plant health. Exogenous application of SA to cucumber plants improved their growth and 50 μM SA is the optimum physiological concentration that increased nitrogen use efficiency during germination and seedling growth (Kumar et al. 2010).

Plant species vary widely in their tolerance to applications of SA and aspirin at varying concentrations. Often at high concentrations, plant damage occurs. However, relatively large concentrations are needed to induce resistance because much of the SA become immobilized in the plant tissues that initially get contact during application.

Plants make SA acid to trigger natural defenses against bacteria, fungi, and viruses. However, plants often do not produce the acid quickly enough to prevent injury when attacked by a microbe. Plants have always had some means to defend themselves; it is just that some do not recognize their microbial attackers in time.

In 2009, Science News published a very interesting paper concerning how to use SA and what are the benefits for farmers and for plants of increased level of SA. The dilemma is that spraying SA puts plants defenses on high-alert against future attacks. But a rise in SA levels also causes the plant to slow its growth, perhaps saving its strength for the battle against the pathogen. That sets up a challenging situation for both the plant-grow faster or protect myself better? and farmers, who might view SA as a tool to protect their plants from disease. A plant that makes high levels of SA all the time will be safe from infection but will grow

slowly. A plant that makes little or no SA will grow faster but be very susceptible to infection (Science News, 2009). For effective application and commercial use of exogenous SA as inducer of plant tolerance, its mechanism of action, the most optimal concentration, and appropriate plant development stages, must be carefully determine. Hadi and Baladi (2010) showed that treatment with 0.2 mM SA resulted in 72 % reduction in the infection symptoms of the potato tuber (sclerocia) caused by *Rizoctonia solani*. The authors believe that artificial application of SA as spray on potato plants leaves can be used to control of SAR against fungal pathogen.

When a plant is attacked, levels of SA in tissues can rise to 180 times normal amounts. These high levels are correlated with high levels of resistance proteins, substances that actually enable the plant to defend itself. These proteins cause changes in the plant that make it difficult for disease organisms to penetrate plant tissues or survive, if they succeed in penetrating. Gardeners in Vermont (USA) sprayed selected eggplants, tomatoes, basil and bean plants with aspirin water (1.5 aspirins to 2 gallons of water) every three weeks. Yields of all sprayed plants were much greater, but in tomato the effect was the best. The sprayed plants produced twice as many tomatoes as the unsprayed plants (Szilard, Tropicalplantsociety, USA).

Greenhouse-cultivated tomato faces economic damage due to grey mould caused by the necrotrophic pathogen *Botrytis cinerea* (Eden et al. 1996). The fungus has a wide host range and infects several plant tissues including stems, leaves, flowers, fruits and seeds. Chemical control by use of fungicides often leads to unsatisfactory results. The phenomenon of induced resistance has reinforced the potential for pesticide-free disease control strategies. The modern strategies leading to increase in plant defense mechanisms involve phytohormones such as SA, JA and ET (for review—van Loon 2000). The SA-dependent defense pathway can be activated by treatment of plants with chemical inducers such as benzo (1,2,3)-thiadiazole-7-carbothioic acid S methyl ester (BTH), (Tally et al. 1999). BTH is a chemical analogue of SA and has been used successfully to induce resistance to a wide range of diseases on field crops. According to Achuo et al. (2004) the plant defense responses activated by the SA-dependent pathway depend on the specific host–pathogen system. Therefore studies on one system may not be extrapolated even to another closely related system.

The ability of four plant growth-promoting *rhizobacteria*, to induce systemic resistance on *Arabidopsis thaliana* Col 0 against biotic and abiotic stress was evaluated (Barriuso et al. 2008). All the bacteria enhanced protection against the foliar pathogen *Pseudomonas syringae* DC 3000 and increased plant tolerance to salt stress (NaCl 60 mM). In addition, it was shown that the SA-dependent pathway is involved in the response triggered by the *Arthrobacter oxidant* strain BB1. The authors concluded that the assayed strains are able to trigger *A. thaliana* systemic resistance which is effective against biotic (pathogen DC 3000) and abiotic (salt) stress. The investigated strains could be used in intensive agricultural production processes to decrease chemical inputs, and increase crop yield in saline soils.

Evidence has been found that shows SA can stimulate flowering. Normally, plants flower at a particular season, reflecting day length and/or temperature cues.

However, they can surpass this seasonal regulation and show precocious flowering under environmental stresses. Martinez et al. (2004) showed that SA is involved in *A. thaliana* flowering regulation which make it a good candidate to be a link between stress-activated responses and developmental programs in *Arabidopsis.*

The effect of SA treatment at different concentrations during different growth stages of strawberry (*Fragaria × ananassa* 'Selva') on fruit Total Antioxidant Activity (TAA) was studied. All tested SA concentrations (1, 2 and 4 mmol L^{-1}) effectively enhanced fruit TAA when applied constantly at least at 2 stages and it was most effective when applied at vegetative, fruit development and postharvest stages. A single treatment strategy was not effective in increasing fruit TAA. Although SA at concentrations of 1, 2 and 4 mmol L^{-1} effectively caused an increase in fruit TAA but a concentration of 2 mmol L^{-1} was more effective than 1 mmol L^{-1} and the 4 mmol L^{-1} SA was less effective in increasing fruit TAA. Postharvest treatment with 4 mmol L^{-1} salicylic acid caused damage to the fruit and decreased the fruit TAA compared to the control (Asghari and Babalar 2010).

One of the problems in oregano tissue culture is hyperhydricity and modifications of media are being used to control this physiological malformation. Andarwulan and Shetty (1999) reported for a reduction of hyperhydricity, stimulation of rosmarinic acid biosynthesis and lignification in oregano clonal line O-1 and O-5 in response to acetyl salicylic acid (ASA), fish protein hydrolysate (FPH) and combination of FPH/ASA. This research provides strategies to prevent hyperhydricity in tissue culture by ASA and combination of FPH/ASA and these were linked to lignification, high levels of total phenolics and rosmarinic acid. This improvement is important for efficiency and quality of in vitro plant tissue propagation and outdoor transplanting of elite phenolic antioxidant-producing oregano cultures.

Bi et al. (2007) tested the hypothesis that allelopathy is an inducible defense mechanism, and that the JA and SA signaling pathways may activate allelochemicals release. Exogenous application of MeJA and MeSA to rice (*Oryza sativa* L.) enhanced rice allelopathic potential and led to accumulation of phenolics, an increase in enzymatic activities, and gene transcription of phenylalanine ammonialyase and cinnamate 4-hydroxylase. The authors suggested that allelopathy may be an active defense mechanism, and that plant signaling compounds are potentially valuable in its regulation.

Scientists at the National Center for Atmospheric Research (NCAR) in Boulder, Colorado (USA) have discovered that plants in a forest respond to stress by producing significant amounts of aspirin, The finding opens up new prospects of research into the behavior of plants and their impacts on air quality, and it also has the potential to give farmers an early warning signal about crops that are failing (Karl et al. 2008). For years, scientists have known that plants in a laboratory may produce MeSA, which is a chemical form of acetylsalicylic acid, or aspirin. But researchers had never before detected MeSA in an ecosystem or verified that plants emit the chemical in significant quantities into the atmosphere (Karl et al. 2008). The authors speculated that the MeSA has two functions. One of these is to stimulate plants to begin a process known as systemic acquired resistance, which is analogous to an immune response in an animal. This helps a plant to both resist

and recover from disease. The MeSA also may be a mechanism whereby a stressed plant communicates to neighboring plants, warning them of the threat. Researchers have demonstrated that a plant may build up its defenses if it is linked in some way to another plant that is emitting the chemical. The NCAR team has demonstrated that MeSA can build up in the atmosphere above a stressed forest. Scientists are, therefore, speculating that plants may use the chemical to activate an ecosystem-wide immune response. The discovery raises the possibility that farmers, forest managers, and others may eventually be able to start monitoring plants for early signs of a disease, an insect infestation, or other types of stress. The discovery also can help scientists resolve a central mystery about VOCs. For years, atmospheric chemists have speculated that there are more VOCs in the atmosphere than they have been able to find. Now it appears that some fraction of the missing VOCs may be MeSA and other plant hormones. This finding can help scientists better track the impact of VOCs on the behavior of clouds and the development of ground-level ozone, an important pollutant (Karl et al. 2008).

Hence, it may be resolved from the survey of literature cited above that SA plays diverse physiological roles in plants and potentially alleviates the devastating effects generated by biotic and abiotic stresses. In future, the exogenous application of this phytohormone might act as a powerful tool in enhancing the growth, productivity and also in combating the ill effects generated by various abiotic stresses in plants. The future applications of this plant hormone holds a great promise as a management tool for providing tolerance to our agricultural crops against the aforesaid constrains consequently aiding to accelerate potential crop yield in near future.

7 Conclusion and Perspectives

During the last years, our understanding of the molecular mechanisms of hormone biosynthesis, perception and response has improved dramatically. Knowledge of the hormone metabolic and transport pathways will lead to new opportunities to manipulate hormone levels and thus regulate plant growth. Receptors for many of the hormone classes have been identified, thus leading to exiting new models for hormone perception. Detailed knowledge of the receptor function may stimulate the development of new plant growth regulators. A new information on the nature of chemicals and the signaling pathways that they are involved has been generated. Earlier, it was thought that calcium, phosphoinositides and ABA were the key molecules that participated in abitoic stress signaling. Plant hormones like SA, JA, NO, which were thought to respond to biotic stresses, have now been implicated in abiotic stresses also. Other molecules like simple sugars and polyamines are also involved in these signaling pathways. In future more work should be done to find out their exact role. Cellular perception of NO may occur through its reaction with biologically active molecules that could function as 'NO-sensors'. But exactly how NO evolution relates to its bioactivity in plants remains to be established. The

overall progress of research on chemical regulated stress responsive genes and their products reflect their central role in plant growth and development under stress conditions. The mechanisms, by which the plants maintain their physiology and development under abiotic stress using different chemical signals at different times and in response to different stress conditions, and cross talk between each of them, still remained to be fully understood (Toteja and Sopory 2008).

Some research on horticultural uses of MeJA has focused on pre-harvest or post-harvest treatments to protect against microbial development on harvest tissues. As biological control becomes more important and useful in horticultural crop production the use of jasmonates-induced defenses may provide valuable augmentation of integrated pest management strategies. Jasmonates have a crucial role in protecting ornamental and food crops from post-harvest disorders and diseases. Jasmonates may also enhance plant quality or to be used in propagation. Jasmonates can alter physiological processes in plants, to make plants more valuable for humans (Popova 2012).

NO as a signaling molecule in all living organisms from mammals dawn to bacteria and plant suggests that it could be one of the most widespread biological messengers in all the living species (Torreilles 2001). The most important challenge is to gain donors which increase stability and longer half-life, with controlled NO release rates and donors exhibiting high tissues and cells specificity. The relations between NO, SA and ET must be better clarified in the future. In order to supply evidence for long distance transmission between different cells and neighbor tissues, further experiments should be done in whole plants (Palavan-Ursal and Arisan 2009; Popova and Tuan 2010).

Based upon the work reviewed by Yang et al. (2009) we can be reasonably sure that global climate changes will have an impact on VOCs emission and their ecological function, but our knowledge about the details is still limited. Although only a few studies address these effects, the current ecological role of VOCs remains a significant challenge.

A major challenge for current biology is to integrate research approaches that address different levels of biological organization, from molecular to ecological systems (Zheng and Dicke 2008). Interplant communication is a research topic that properly suits the practice of interdisciplinary cooperation.

Many of the future questions concerning the very attractive field of interplant communication were summarized by Campos et al. (2008). According to the authors statement the future scope of interplant communication will open new perspectives applied to pest and disease control in plants. Volatile mixtures directly sprayed on plants against herbivores and pathogens, and the development of plants that are more sensitive to defence inducers are some of the possibilities. Several studies have shown that it is indeed possible both to reduce herbivory and to enhance natural enemy attraction simultaneously. If such effects can be translated into increased and more stable yields in important crops, this strategy might be explored by the plant breeding industry and eventually become available to plant growers in the form of resistant cultivars. There are, however, ecological challenges associated with this approach, and the modified plant volatile

composition should preferably be inducible specifically by the target pests, or by field application of specific elicitors based on forecasts of pest attack (Campos et al. 2008).

Salicylic acid is perhaps the only compound on the surface of the earth to mediate so diverse functions as ranging from curing various human ailments to protect the plants from various biotic and abiotic stresses and affecting various physiological and biochemical processes of plants (Popova et al. 2012). However, this recently recognized phytohormone still demands a lot of work to be carried out to elucidate the exact pathways of its biosynthesis. The work is also needed on how this plant hormone interacts and being regulated by the cross talk in harmony with other established phytohormones and plant growth regulators working at long range (auxins, cytokinins, gibberellins, ethylene etc.), short range (NO, jasmonates, brassinosteroids etc.) and very short range (ROS, H_2O_2).

In future, biochemical and physiological changes in plants by SA may be explored to use than as biochemical markers, which may further be transformed into genetic markers and be utilized in genetic engineering of plants for making them tolerant to various stresses.

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