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Biosynthesis and Regulation of Salicylic Acid and N-Hydroxypipecolic Acid in Plant Immunity

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ABSTRACT

Salicylic acid (SA) has long been known to be essential for basal defense and systemic acquired resistance (SAR). N-Hydroxypipecolic acid (NHP), a recently discovered plant metabolite, also plays a key role in SAR and to a lesser extent in basal resistance. Following pathogen infection, levels of both compounds are dramatically increased. Analysis of SA- or SAR-deficient mutants has uncovered how SA and NHP are biosynthesized. The completion of the SA and NHP biosynthetic pathways in *Arabidopsis* allowed better understanding of how they are regulated. In this review, we discuss recent progress on SA and NHP biosynthesis and their regulation in plant immunity.

Key words: plant immunity, SAR, SA, NHP, pipecolic acid

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INTRODUCTION

Systemic acquired resistance (SAR) is a phenomenon whereby activation of local defense leads to enhanced disease resistance in distal part of the plants. Due to its induced nature remotely resembling adaptive immunity in mammals and broad effects against different pathogens, extensive studies have been carried out to understand the molecular mechanism of SAR (Fu and Dong, 2013). Salicylic acid (SA) emerged as a signaling molecule in SAR during early 1990s because pathogen infection leads to drastic increase of SA levels (Malamy et al., 1990; Metraux et al., 1990), and accumulation of SA was shown to be essential for SAR in studies using transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase from bacteria (Gaffney et al., 1993). Later studies on *Arabidopsis* mutants defective in SA biosynthesis or perception further confirmed the importance of SA in SAR (Cao et al., 1994; Delaney et al., 1995; Nawrath and Metraux, 1999). In *Arabidopsis*, SA is perceived by two classes of receptors, NPR1 and NPR3/NPR4, which have opposite roles in regulating SA-responsive genes (Zhang and Li, 2019). SA inhibits the transcriptional repression activities of NPR3/NPR4, while promoting the transcriptional activation activity of NPR1 (Ding et al., 2018).

Despite its central role in SAR, SA is unlikely a long-distance signal based on early data from grafting experiments in tobacco (Vernooij et al., 1994). Methyl salicylate (MeSA) was proposed as a mobile signal for SAR in tobacco (Park et al., 2007), but it was shown not to be required for SAR in *Arabidopsis* (Attaran et al., 2009). Recent analysis of a group of SAR-deficient mutants that do not affect SA levels or signaling identified L-pipecolic acid (Pip) and its derivative N-hydroxypipecolic acid (NHP) as two critical metabolites involved in establishment of SAR (Navarova et al., 2012; Chen et al., 2018; Hartmann et al., 2018; Hartmann and Zeier, 2019). Blocking Pip or NHP biosynthesis results in total loss of SAR, but with only minor effects on local disease resistance (Song et al., 2004b; Mishina and Zeier, 2006; Jing et al., 2011), suggesting that their main role is in long-distance signaling. In this review, we focus on recent developments in deciphering the biosynthetic pathways of SA and NHP and their regulation in plant immunity.

SA Biosynthesis in Higher Plants

Plants use two independent routes to produce SA, the isochorismate synthase (ICS) and phenylalanine (Phe) ammonia-lyase (PAL) pathways (Figure 1) (Dempsey et al., 2011; Hartmann and Zeier, 2019; Zhang and Li, 2019), both initiating in plastids from chorismate, a product of the shikimate pathway.

The ICS pathway of SA biosynthesis was mostly deciphered using *Arabidopsis* mutants defective in pathogen-induced SA biosynthesis. It was originally proposed with the identification of SA Induction Deficient 2 (SID2) as an ICS (Wildermuth et al.,

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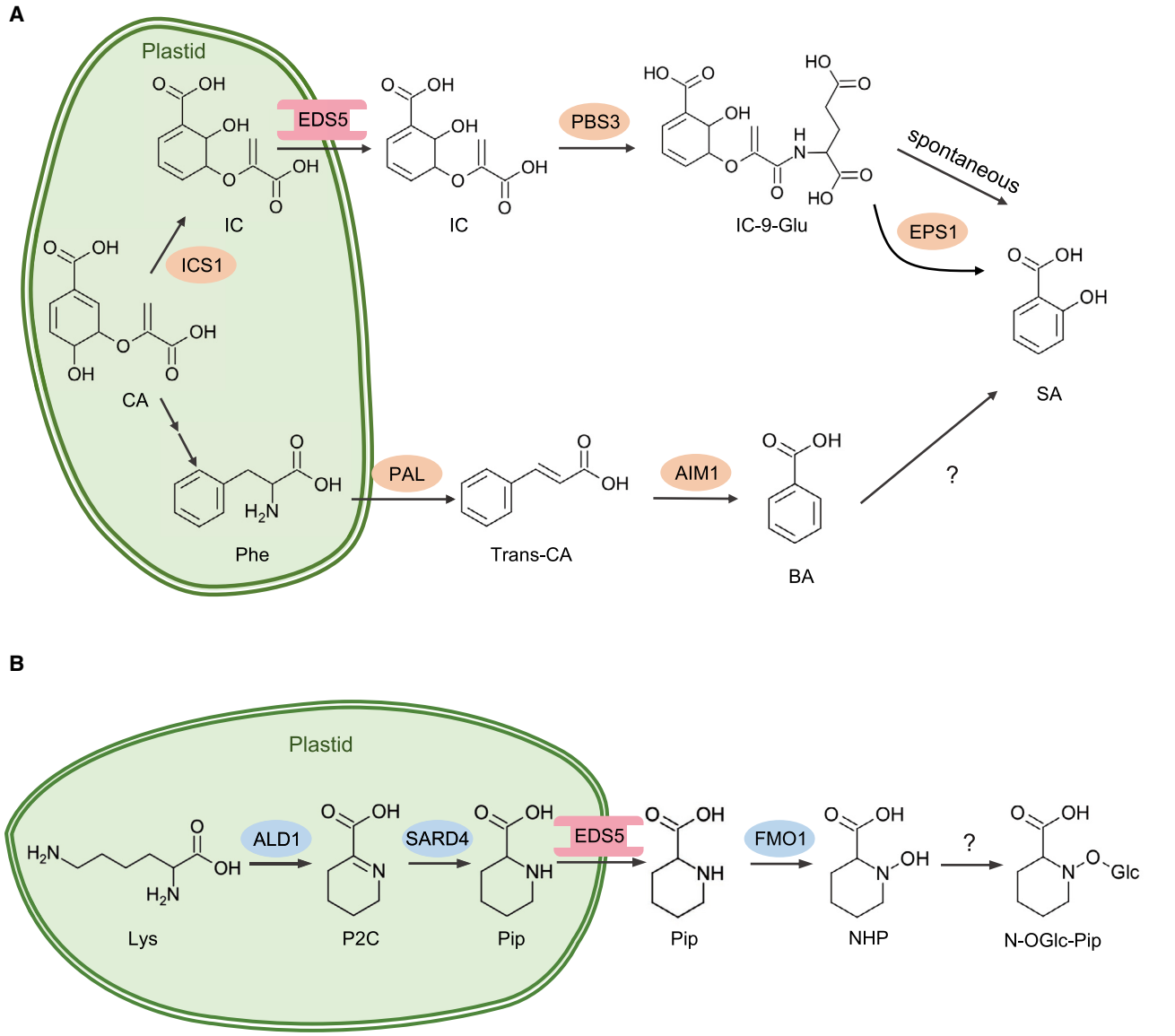

Figure 1. The Biosynthetic Pathways for Salicylic Acid (SA) and N-Hydroxypipecolic Acid (NHP).

(A) In the isochorismate synthase (ICS) pathway, ICS converts chorismite (CA) to isochorismate (IC) in the plastids. IC is transported by the MATE transporter EDS5 to the cytosol, where it is converted to IC-9-Glu by PBS3. IC-9-Glu is further broken down into SA. EPS1 enhances the breakdown of IC-9-Glu in Brassicaceae. In the phenylalanine ammonia-lyase (PAL) pathway, PALs convert Phe to trans-CA, which is further converted to benzoic acid (BA) via β-oxidation by AIM1. SA is subsequently produced from BA by a yet-to-be-identified BA 2-hydroxylase.

(B) In plastids, Pip is made from Lys in a two-step reaction catalyzed by the aminotransferase ALD1 and reductase SARD4. EDS5 transports Pip from the plastids to the cytosol, where it is oxidized into NHP by FMO1. NHP can be glycosylated to form N-OGlc-Pip by an unknown glucosyltransferase.

2001). *Arabidopsis* has two *ICS* genes, *ICS1* and *ICS2*. In the *sid2/ics1* single mutant, pathogen-induced SA accumulation is largely blocked and SA levels are further reduced in *ics1 ics2* double-mutant plants, suggesting that ICS1 plays a major role, whereas ICS2 plays a minor role, in pathogen-induced SA synthesis (Nawrath and Metraux, 1999; Garcion et al., 2008).

Both ICS1 and ICS2 are localized to the plastids, suggesting that the conversion of chorismate to isochorismate occurs in plastids (Wildermuth et al., 2001; Garcion et al., 2008). In some bacteria, isochorismate is directly converted to SA by an isochorismate pyruvate lyase (IPL) (Serino et al., 1995). However, no *IPL* homologs have been found in plant genomes, suggesting that plants likely use a different mechanism to produce SA from isochorismate. Studies of *sid1/eds5* mutants revealed that mutations in the MATE transporter Enhanced Disease Susceptibility 5 (EDS5) lead to greatly decreased SA levels (Nawrath and Metraux, 1999; Nawrath et al., 2002). EDS5 localizes to the chloroplast envelop, implying that it transports either SA or its precursor to the cytosol (Serrano et al., 2013). Recently, it was shown that the conversion of isochorismate to SA occurs in the cytosol, and EDS5 most likely facilitates the transport of isochorismate from plastids to the cytosol (Rekhter et al., 2019a).

Mutations in *avrPphB Susceptible 3 (PBS3)*, which encodes an aminotransferase, also result in reduced pathogen-induced SA accumulation (Nobuta et al., 2007). However, how PBS3 contributes to SA accumulation was unknown for many years. Recently, it was uncovered that PBS3 catalyzes the conjugation of glutamate to isochorismate in the cytosol (Figure 1) (Torrens-Spence et al., 2019). The resulted product isochorismate-9-glutamate spontaneously decays to yield SA. Analysis of another SA-deficient mutant, *enhanced pseudomonas susceptibility 1* (*eps1*) (Zheng et al., 2009), revealed that the newly evolved EPS1 in Brassicaceae can further boost SA production by stimulating the decay of isochorismate-9-glutamate (Torrens-Spence et al., 2019).

The PAL pathway (Figure 1) has long been known to contribute to SA biosynthesis in tobacco. Isotope labeling experiments showed that SA can be synthesized from Phe via trans-cinnamic acid (CA) and benzoic acid (BA) in tobacco (Yalpani et al., 1993; Ribnicky et al., 1998). PALs convert Phe to CA in higher plants, which is the first step for phenylpropanoid production (Vogt, 2010). Upon infection, genes encoding PALs, but not ICSs, are induced in tobacco, suggesting that pathogen-induced SA in tobacco might be produced primarily through the PAL pathway (Ogawa et al., 2006). Even in *Arabidopsis*, loss of all four *PAL* genes leads to ~75% reduction of basal SA and ~50% decrease in pathogen-induced SA production, suggesting that *PALs* are not only involved in basal SA production but also contribute to pathogen-induced SA biosynthesis (Huang et al., 2010).

Abnormal Inflorescence Meristem 1 (AIM1) mediates the conversion of CA to BA. *AIM1* encodes a hydroxyacyl-CoA hydrolyase involved in beta-oxidation (Richmond and Bleecker, 1999), which is required for the production of benzoylated metabolites in *Arabidopsis* seeds (Bussell et al., 2014). In the rice *aim1* mutant, CA level is about sixfold higher, whereas BA level is only half and SA level is ~25%, as in wild-type plants, further supporting that BA may be a beta-oxidation product of CA produced by AIM1 in the second step of the PAL pathway (Xu et al., 2017). A putative BA 2-hydroxylase was proposed to hydroxylate BA to SA (Leon et al., 1993, 1995), but the gene encoding this enzyme has not been identified yet.

Exogenous leaf application of SA precursors, such as Phe, BA, or CA, induces *PR* gene expression and enhances resistance against tobacco mosaic virus (Yalpani et al., 1993). On the other hand, pretreatment with a PAL inhibitor, 2-aminoindan-2-phosphonic acid (AIP), leads to increased growth of the oomycete pathogen *Hyaloperonospora arabidopsidis* Emwa1 on *Arabidopsis* Col-0 ecotype (Mauch-Mani and Slusarenko, 1996). These data support that the PAL pathway indeed contributes to local defense, most likely through production of SA. How the PAL pathway and ICS pathway are used preferentially or work together in different plants will be an interesting question to address.

Evolution of the SA Biosynthetic Pathways

SA is biosynthesized in plants as well as some bacterial species. In both plants and bacteria, isochorismate (IC) is used as an intermediate for making SA. The conversion of chorismate to IC in plants is catalyzed by ICSs with high similarity to bacterial ICS and it takes place in plastids (Wildermuth et al., 2001; Garcion et al., 2008), suggesting that the IC-derived SA biosynthesis pathway may have a prokaryotic origin. However, the SA biosynthesis pathways in bacteria and plants diverge after IC. Unlike bacteria, in which IPL is used to break down IC, *Arabidopsis* uses PBS3 to conjugate glutamate to IC to make IC-9-Glu, which subsequently breaks down into SA (Rekhter et al., 2019a; Torrens-Spence et al., 2019).

PBS3 is a member of the Glycoside Hydrolase 3 (GH3) family (Nobuta et al., 2007), which is widespread in plants. Phylogenetic analysis revealed that they form three separate clades, with PBS3 in group III (Zhang et al., 2018). Although genes encoding group I and group II GH3 proteins can be found in all sequenced plant genomes, group III proteins are found mostly in Brassicaceae (Zhang et al., 2018). Interestingly, in plants such as soybean and *Nicotiana benthamiana*, which do not have group III GH3 proteins, the ICS pathway still contributes to SA biosynthesis (Catinot et al., 2008; Shine et al., 2016). It is possible that some GH3 proteins in group I or group II may have functions similar to PBS3 in conjugating glutamate to IC.

The PAL pathway was shown to contribute to SA biosynthesis in a number of plant species, such as *Arabidopsis*, rice, soybean, and tobacco (Yalpani et al., 1993; Huang et al., 2010; Shine et al., 2016; Xu et al., 2017). In *Physcomitrella patens*, one of the *PAL* genes is strongly induced by *Botrytis cinerea*, suggesting that the PAL pathway may also be used to produce SA in the mosses (Ponce De Leon et al., 2012). As the committing step for SA production in the PAL pathway is the conversion of BA to SA, it is critical to identify the genes encoding the BA 2-hydrogenase in order to better understand how the pathway has evolved.

SA Metabolism

SA levels change dynamically during plant immune responses. SA can be further metabolized into inactive or storage forms to modulate its activity, increase membrane permeability, and fine-tune its levels (Dempsey et al., 2011). Following its biosynthesis, SA can undergo glucosylation or methylation to form SA 2-O-β-D-glucoside (SAG) and MeSA, respectively. SAG is believed to be a detoxified and vacuole storage form of SA. At least three *Arabidopsis* UDP-glucosyltransferases were reported to be involved in the conversion of SA to SAG (Lim et al., 2002; Song, 2006; Dean and Delaney, 2008). Loss-of-function mutants of UGT74F1 or UGT76b1 exhibit reduced SAG levels and increased SA accumulation, leading to enhanced disease resistance in *Arabidopsis* (Noutoshi et al., 2012). The conversion of SA to MeSA is facilitated by a BA/SA carboxyl methyltransferase (Chen et al., 2003; Attaran et al., 2009). Methylation confers SA better membrane permeability and volatility, facilitating its release from plants. Interestingly, MeSA can be converted back to SA by SA-binding protein 2 in tobacco (Park et al., 2007).

In addition to glucosylation and methylation, SA can also be hydroxylated in plants, likely serving a storage function. *Arabidopsis* SA 3-hydroxylase (S3H) and SA 5-hydroxylase (S5H) catalyze the

Regulator	Role	Reference
SARD1	+	Zhang et al., 2010; Wang et al., 2011
CBP60g	+	Wang et al., 2009; Zhang et al., 2010
WRKY28/46	+	van Verk et al., 2011
WRKY8/48	+	Gao et al., 2013
WRKY75	+	Guo et al., 2017
TCP8/9	+	Wang et al., 2015
NTL9	+	Zheng et al., 2015
CHE	+	Zheng et al., 2015
ANAC019/055/072	–	Zheng et al., 2012
EIN3/EIL1	–	Chen et al., 2009
CBP60a	–	Truman et al., 2013
WRKY18/40	–	Birkenbihl et al., 2017
WRKY70/54	–	Wang et al., 2006
DEL1	–	Chandran et al., 2014

Table 1. Transcriptional Factors Regulating Genes Involved in SA Biosynthesis.
+, positive regulator; –, negative regulator.

conversion of SA to 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA), respectively (Zhang et al., 2013, 2017). S3H primarily functions during senescence, as its expression levels are very low in young plants (Zhang et al., 2017). In the *s5h/dmr6* single mutant, SA level is elevated, which is further increased in the *s5h s3h* double mutant (Zeilmaker et al., 2015). Consistent with the increased SA accumulation, the *s5h* single mutant and the *s5h s3h* double mutant exhibit enhanced resistance to pathogens (Zeilmaker et al., 2015; Zhang et al., 2017).

Transcriptional Regulation of SA Biosynthetic Genes

In *Arabidopsis*, a large number of transcription factors are involved in regulating genes involved in SA biosynthesis (Table 1). Pathogen-induced *ICS1* expression and SA biosynthesis are mainly controlled by two plant-specific transcription factors, SAR-Deficient 1 (SARD1) and CaM-Binding Protein 60 g (CBP60g) (Wang et al., 2009, 2011; Zhang et al., 2010). Loss of both SARD1 and CBP60g leads to almost complete block of the induction of *ICS1* and SA accumulation by bacterial pathogens (Zhang et al., 2010; Wang et al., 2011). In addition, constitutive expression of *ICS1* and elevated SA accumulation in the autoimmune mutants *snc1* and *snc2-1D* are largely dependent on SARD1 and CBP60g (Sun et al., 2015, 2018b). Chromatin immunoprecipitation (ChIP) analysis revealed that SARD1 and CBP60g target not only *ICS1*, but also *EDS5* and *PBS3* (Sun et al., 2015). Similar to *ICS1*, the induction of *EDS5* and *PBS3* by the bacterial pathogen *Psm* ES4326 is greatly reduced in the *sard1 cbp60g* double mutant, suggesting that genes involved in the pathogen-induced SA biosynthesis pathway are coordinately regulated by SARD1 and CBP60g (Figure 2).

A number of WRKY transcription factors have been implicated in positive regulation of *ICS1* expression and SA biosynthesis. Overexpression of WRKY28 and WRKY46 in *Arabidopsis* protoplasts resulted in increased *ICS1* expression (van Verk et al., 2011). EMSA (electrophoretic mobility shift assay) and ChIP assays showed that WRKY28 binds to the *ICS1* promoter both *in vitro* and *in vivo* (van Verk et al., 2011). The induction of *ICS1* expression by *Pst* DC3000 *avrRpm1*, *avrRpt2* and *avrB*, but not *Pst* DC3000 is greatly reduced in *wrky8* and *wrky48* mutant plants, suggesting that the effector-induced *ICS1* expression is largely dependent on WRKY8 and WRKY48 (Gao et al., 2013). Whether WRKY8 and WRKY48 directly regulate *ICS1* is still unclear. Another transcription factor WRKY75 directly binds to the promoter of *ICS1* and promotes *ICS1* expression and SA production during senescence (Guo et al., 2017). As a consequence, age-dependent leaf senescence is delayed in WRKY75 knockdown or knockout mutants and accelerated in WRKY75 overexpression lines.

On the promoter of *ICS1*, there is also a typical TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP)-binding site (Wang et al., 2015). Yeast one-hybrid analysis showed that transcription factors TCP8 and TCP9 bind to this site. The binding of TCP8 to the *ICS1* promoter was further confirmed by EMSA and ChIP assays. The induction of *ICS1* expression and SA accumulation was modestly reduced in the *tcp8 tcp9* double mutant at late stage of infection by *Psm* ES4326, suggesting that TCP8 and TCP9 function redundantly in promoting *ICS1* expression and SA biosynthesis (Wang et al., 2015). Yeast one-hybrid analysis also identified the transcription factors NTM1-LIKE 9 (NTL9) and CCA1 HIKING EXPEDITION (CHE) that bind directly to the *ICS1* promoter (Zheng et al., 2015). Further analysis revealed that they regulate *ICS1* expression and SA biosynthesis in specific immune responses (Zheng et al., 2015). NTL9 promotes *ICS1* expression and SA biosynthesis in stomatal immunity. On the other hand, CHE is involved in the regulation of circadian oscillations of *ICS1* and SA levels as well as induction of *ICS1* expression and SA accumulation in distal tissue during SAR.

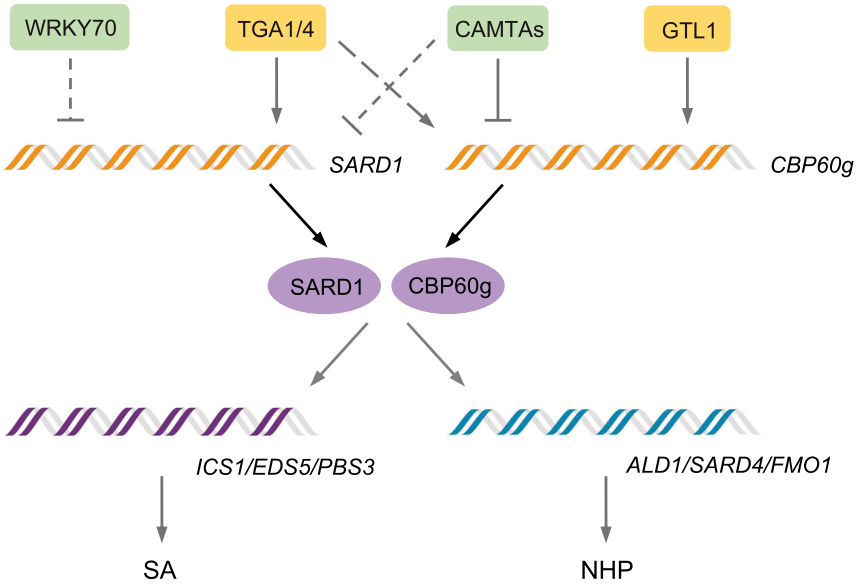

Figure 2. A Model Illustrating Transcriptional Regulation of *SARD1*/*CBP60g* and SA/NHP Biosynthesis.

Biosynthetic genes for SA (*ICS1*/*EDS5*/*PBS3*) and NHP (*ALD1*/*SARD4*/*FMO1*) are coordinately regulated by the master immune transcription factors SARD1 and CBP60g. Their activation leads to increased SA and NHP biosynthesis. Upstream of *SARD1* and *CBP60g*, TGA1/TGA4 and GTL1 serve as positive regulators, whereas WRKY70 and CAMTA1/2/3 serve as negative regulators of their transcription.

Several transcription factors are involved in the negative regulation of SA biosynthetic genes. ANAC019, ANAC055, and ANAC072 were shown to function redundantly in direct repression of *ICS1* expression during coronatine-induced suppression of SA biosynthesis and in activation of *BENZOIC ACID/SA CARBOXYL METHYLTRANSFERASE 1* (*BSMT1*) (Zheng et al., 2012). Following infection by *Psm* ES4326, the induction of *ICS1* is modestly increased, whereas the induction of *BSMT1* is greatly reduced in the *nac* triple mutant. Consistent with the gene expression data, there is an increase in SA accumulation and dramatic reduction in MeSA level in the *nac* triple mutant. Two ethylene signaling components ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE1 (EIL1) are also involved in negative regulation of SA biosynthesis by repressing *ICS1*. EIN3 binds to the promoter of *ICS1* and the *ein3-1 eil1-1* double mutant displayed elevated *ICS1* and SA levels in the absence of pathogen attack (Chen et al., 2009). CBP60a is another transcription factor implicated in negative regulation of SA biosynthesis (Truman et al., 2013). In *cbp60a* mutant plants, there is an increase in basal *ICS1* and SA levels, suggesting that CBP60a either directly or indirectly affects the expression of *ICS1* and SA biosynthesis. Two WRKY transcription factors, WRKY18 and WRKY40, were shown to directly target *ICS1*, *EDS5,* and *PBS3,* and negatively regulate their expression (Birkenbihl et al., 2017). Two other WRKY transcription factors, WRKY70 and WRKY54, also contribute to negative regulation of SA biosynthesis (Wang et al., 2006). In the *wrky54 wrky70* double mutant, basal expression level of *ICS1* is elevated. The SA level in *wrky54 wrky70* plants treated with *Psm* ES4326 *avrRpt2* is also much higher than in wild-type plants. Furthermore, the atypical E2F transcriptional repressor DEL1 was shown to negatively regulate SA biosynthesis (Chandran et al., 2014). *EDS5* is a direct target of DEL1. Loss of function of DEL1 results in elevated *EDS5* expression and SA accumulation.

NHP Biosynthesis

NHP was lately identified as a critical and likely mobile plant defense signal for SAR (Hartmann and Zeier, 2019; Tian and Zhang, 2019).

Studies on *Arabidopsis SAR-deficient* (*sard*) mutants largely contributed to the elucidation of the NHP biosynthesis pathway (Figure 1). *ALD1* encodes an aminotransferase essential for the onset of SAR and was found to be required for the production of the immediate precursor of NHP, Pip (Song et al., 2004a, 2004b; Navarova et al., 2012). It uses lysine as a substrate and catalyzes its conversion to ϵ-amino-α-keto caproic acid, which cyclizes spontaneously to form Δ1-piperideine-2-carboxylic acid (P2C) (Ding et al., 2016; Hartmann et al., 2017).

Studies on *sard4* mutants showed that SARD4 is required for Pip accumulation in distal leaves and to a lesser extent in local tissue after bacterial pathogen infection (Ding et al., 2016). SARD4 catalyzes the reduction of P2C to Pip (Ding et al., 2016; Hartmann et al., 2017). The full Pip pathway can be reconstituted by introducing just *ALD1* and *SARD4* into *Escherichia coli* (Ding et al., 2016). Studies on another SAR-deficient mutant, *flavin-dependent monooxygenase 1* (*fmo1*) revealed that FMO1 is required for the production of NHP (Chen et al., 2018; Hartmann et al., 2018). In pathogen-treated *fmo1* mutants, Pip accumulates to higher levels and no NHP can be detected. Biochemical analysis revealed that FMO1 catalyzes the N-hydroxylation of Pip, yielding NHP (Chen et al., 2018; Hartmann et al., 2018).

In another recent study, it was found that UV-induced NHP accumulation was greatly reduced in *eds5* mutant plants (Rekhter et al., 2019b). In contrast, *sid2* mutant plants showed normal NHP accumulation. The reduced NHP accumulation in *eds5* mutant plants cannot be rescued by external SA application, suggesting that EDS5 is required for NHP biosynthesis. Considering that Pip is made in plastids and FMO1 is predicted to be localized in the cytosol, EDS5 most likely facilitates the transport of Pip from plastid to cytosol, where Pip is further converted to NHP by FMO1 (Rekhter et al., 2019b).

NHP exists as both free and glucosylated forms. Pathogen infection dramatically induces the accumulation of NHP as well as N-OGlc-Pip in *Arabidopsis* (Chen et al., 2018; Hartmann et al., 2018). Treatment of lower leaves of *fmo1* mutant plants with NHP results in detectable N-OGlc-Pip accumulation in the distal leaves, suggesting that either NHP or N-OGlc-Pip move systemically (Chen et al., 2018). It remains to be determined whether NHP or N-OGlc-Pip serves as a mobile signal for SAR.

Evolution of the NHP Biosynthetic Pathway

Pip has been detected in many plant species long before it was shown to play a critical role in plant defense (Zacharius et al., 1952). Similar to *Arabidopsis*, the toothed clubmoss *Huperzia serrata* also makes Pip via a two-step reaction catalyzed by *Hs*ALD1 and *Hs*SARD4 (Xu et al., 2018). Genes encoding homologs of ALD1 and SARD4 are also be found in the *Physcomitrella patens* genome (Holmes et al., 2019). These findings suggest that Pip is a common metabolite in plants and the Pip biosynthesis pathway likely has an ancient origin.

In addition to *Arabidopsis*, several other plant species, including *N. benthamiana*, tomato, soybean, and corn, were recently shown to produce NHP (Holmes et al., 2019). Blast analysis of genome sequences of 50 plant species showed that close homologs of FMO1 exist in all the plant species except *P. patens*, suggesting that the NHP biosynthetic pathway is also conserved in plants and NHP could be a universal plant defense signaling molecule (Holmes et al., 2019).

Regulation of NHP Biosynthetic Genes

Similar to SA, NHP biosynthetic genes are also coordinately regulated by SARD1 and CBP60g in *Arabidopsis*. ChIP analysis revealed that SARD1 and CBP60g target not only genes involved in SA biosynthesis, but also the NHP synthetic genes *ALD1*, *SARD4,* and *FMO1* (Sun et al., 2015, 2018a). In the *sard1 cbp60g* double mutant, the induction of *ALD1*, *SARD4,* and *FMO1* by *Psm* ES4326 is greatly reduced. On the other hand, overexpression of *SARD1* leads to increased *ALD1* and *SARD4* expression and elevated Pip levels (Sun et al., 2018a). In addition, Pip and NHP levels following infection by *Psm* ES4326 are much lower in the *sard1 cbp60g* double mutant than in wild-type plants (Sun et al., 2018a) (Sun et al., 2019), suggesting that SARD1 and CBP60g activate Pip and NHP biosynthesis through upregulation of their biosynthetic genes (Figure 2).

WRKY33 is another transcription factor that positively regulates Pip and NHP biosynthesis (Wang et al., 2018). In *wrky33* mutant plants, *Pst* DC3000 *avrRpt2*-induced *ALD1* and *FMO1* expression, as well as Pip accumulation, is dramatically reduced. *Psm* ES4326-induced Pip and NHP accumulation was also reduced in *wrky33*. ChIP-PCR analysis revealed that WRKY33 binds to the promoter of *ALD1*, suggesting that it directly regulates the expression of *ALD1* to promote Pip and NHP biosynthesis. Loss of WRKY33 was shown to result in elevated SA levels during *Botrytis cinerea* infection (Birkenbihl et al., 2012). It is possible that WRKY33 also indirectly promotes NHP accumulation by affecting SA level.

Regulation of SARD1 and CBP60g

Upon pathogen infection, the expression of *SARD1* and *CBP60g* is dramatically induced (Wang et al., 2009; Zhang et al., 2010). Overexpression of *SARD1* is sufficient to activate genes involved in SA and NHP biosynthesis, such as *ICS1*, *ALD1,* and *SARD4* (Sun et al., 2018a; Zhang et al., 2010). Two closely related transcription factors, TGACG-BINDING FACTOR 1 (TGA1) and TGA4, are involved in the induction of *SARD1* and *CBP60g* (Figure 2) (Sun et al., 2018a). In the *tga1 tga4* double mutant, the induction of *SARD1* and *CBP60g*, as well as SA and Pip accumulation, are greatly reduced at the early stage of plant defense. ChIP-PCR analysis showed that TGA1 binds to the promoter of *SARD1* but not *CBP60g*, suggesting that the TGA transcription factors directly regulate *SARD1* and indirectly regulate *CBP60g* expression. Another transcription factor GT2-like 1 (GTL1) was shown to directly regulate pathogen-induced *CBP60g* expression (Figure 2) (Volz et al., 2018). GTL1 binds to the promoter region of *CBP60g*, and the expression level of *CBP60g* is significantly reduced in the *gtl1* mutant after flg22-treatment. Consistently, *ICS1* expression and SA accumulation are also compromised in the *gtl1* mutant.

Several transcription factors, including WRKY70 and CAMTA1/CAMTA2/CAMTA3, are involved in the negative regulation of *SARD1* and *CBP60g* expression (Figure 2). WRKY70 binds to a GACTTTT motif in the *SARD1* promoter, and basal *SARD1* expression level is elevated in *wrky70* mutant plants, suggesting that it may inhibit *SARD1* expression in the absence of pathogen infection (Zhou et al., 2018). In the *camta3-1* knockout mutant, the SA level is elevated and the expression levels of *SARD1* and *CBP60g* are dramatically increased at low temperature (Du et al., 2009; Kim et al., 2013). On the other hand, the induction of *SARD1* and *CBP60g* by flg22 and *Psm* ES4326 is significantly reduced in the gain-of-function *camta3-3D* mutant (Jacob et al., 2018) (Sun et al., 2019). Consistent with expression levels of *SARD1* and *CBP60g*, *camta3-1* accumulates elevated levels of SA, Pip, and NHP in the absence of pathogen infection, and *Psm* ES4326-induced SA, Pip, and NHP is significantly reduced in *camta3-3D* (Sun et al., 2019). CAMTA3 directly binds to the promoter of *CBP60g,* but not *SARD1, in planta*, suggesting that it directly regulates *CBP60g* expression, and its effect on *SARD1* is most likely indirect (Sun et al., 2019). The close homologs of CAMTA3, CATMA1 and CAMTA2, have partially redundant functions with CAMTA3 (Kim et al., 2013). The elevated *SARD1* and *CBP60g* expression, and autoimmune phenotypes in *camta3-1,* are dramatically enhanced in the *camta1 camta2 camta3* triple mutant.

Post-transcriptional Regulation of SA and NHP Biosynthesis

Decoding Ca^{2+} signatures by Ca^{2+}-binding or CaM-binding transcription factors is critical for generating specific gene expression patterns during signal transduction (Liu et al., 2019). Although SARD1 and CBP60g belong to the same protein family, CBP60g but not SARD1 carries an N terminal CaM-binding domain, which is required for the function of CBP60g in promoting *ICS1* expression and SA accumulation (Wang et al., 2009). Unlike *SARD1*, overexpression of *CBP60g* is not sufficient to induce the expression of SA and NHP biosynthetic genes (Zhang et al., 2010), supporting that the activity of CBP60g is modulated by Ca^{2+}/CaM at the post-transcriptional level (Figure 3). At the C terminus of CBP60a, there is also a CaM-binding domain. CBP60a mutants without CaM-binding ability failed to complement the enhanced disease-resistance phenotype of *cbp60a*, suggesting that the activity of CBP60a is also modulated by Ca^{2+}/CaM (Figure 3) (Truman et al., 2013).

Several Ca^{2+}-dependent protein kinases (CPKs) are involved in promoting SA and NHP biosynthesis. Overexpression of the

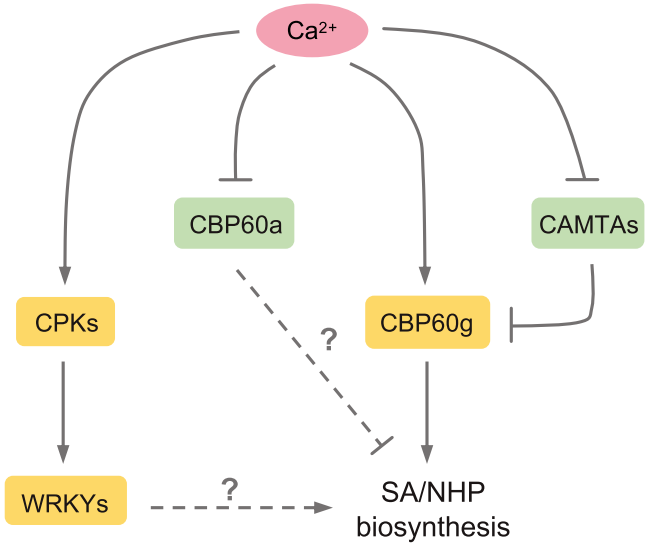

Figure 3. Regulation of SA and NHP Biosynthesis by Ca^{2+} Signaling.
Ca^{2+} regulates SA and NHP biosynthesis by modulating the activities of CPKs, CBP60a/CPB60g, and CAMTA1/2/3. Positive regulation is indicated with arrows and negative regulation with blocks.

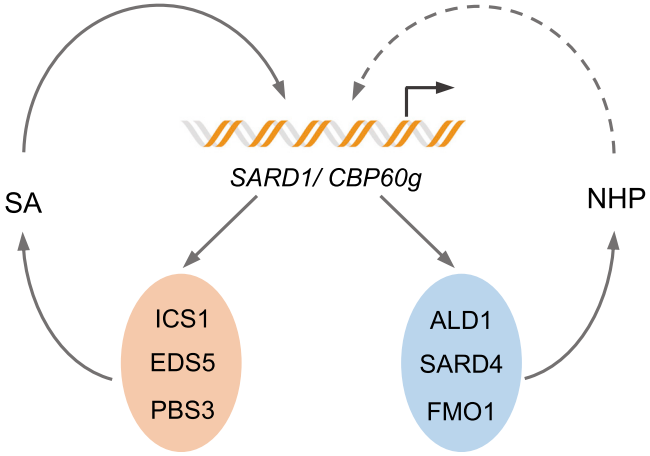

Figure 4. A Model Illustrating Positive Feedback Amplification of SA and NHP Biosynthesis.
Upon activation of plant immunity, transcription factors SARD1 and CBP60g activate the expression of biosynthetic genes of SA (*ICS1/EDS5/PBS3*) and NHP (*ALD1/SARD4/FMO1*), leading to increased SA and NHP levels, which positively regulate *SARD1* and *CBP60g* to further amplify immune responses.

CPK5-GFP fusion protein results in constitutive activation of *SARD1* and genes involved in SA and NHP biosynthesis, leading to dramatically increased SA and NHP levels (Guerra et al., 2019). How CPK5 regulates SA and NHP biosynthesis is unclear. As CPK5 interacts with the TIR-NB protein, TN2, and the autoimmune phenotype in the CPK5-GFP overexpression line is dependent on TN2 (Liu et al., 2017), increased expression of SA, and NHP biosynthetic genes caused by overexpression of CPK5-GFP could be due to activation of TN2-mediated immunity. CPK4, CPK5, and CPK11 were also shown to phosphorylate WRKY8 and WRKY48, which are involved in the induction of *ICS1* in ETI (Figure 3) (Gao et al., 2013). *In vitro* phosphorylation of the WRKYs by the CPKs is Ca^{2+}-dependent and it enhances the binding activities of the WRKYs to the W boxes. Whether these activities contribute to SA accumulation *in vivo* awaits further investigation.

Activation of MAPKs was found to induce the expression of NHP biosynthetic genes and result in elevated Pip and NHP levels (Wang et al., 2018). Infection by *Pst* DC3000 *avrRpt2* results in sustained activation of MPK3 and MPK6, which are required for the full induction of *ALD1* and *FMO1,* as well as Pip accumulation. WRKY33 is a substrate of MAPKs that is activated by MPK3 and MPK6 (Mao et al., 2011). In the *wrky33* mutant plants, MAPK-induced *ALD1* expression and Pip accumulation is significantly reduced, suggesting that phosphorylation of WRKY33 contributes to MAPK-mediated activation of Pip biosynthesis (Wang et al., 2018). PCRK1 and PCRK2 are two receptor-like cytoplasmic kinases involved in connecting PRRs to downstream MAP kinase cascades (Kong et al., 2016). In the *pcrk1 pcrk2* double mutant, the induction of *SARD1*, *CBP60g*, *ICS1*, *ALD1,* and *FMO1* by *Pst* DC3000 *hrcC*$^-$, as well as *Psm* ES4326, is compromised (Kong et al., 2016), suggesting that PCRK1 and PCRK2 function redundantly to promote the expression of SA and NHP biosynthetic genes in plant defense.

Currently, very little is known on post-transcriptional regulation of the biosynthetic enzymes of SA and NHP. ICS1 was shown to be stabilized through association with PROHIBITINs (PHBs), a group of scaffold proteins with diverse biological functions (Seguel et al., 2018). Following UV-C treatment, lower ICS1 protein, but not transcript level, was observed in *phb3* plants, suggesting that ICS1 is subjected to post-transcriptional regulation. It remains to be determined whether post-transcriptional regulation also plays a role in controlling the levels and enzymatic activities of the other proteins in the SA and NHP biosynthetic pathways.

Feedback Regulation and Crosstalk between SA and NHP

SA levels in plants are under both positive and negative feedback regulation. SA treatment rapidly induces the expression of genes involved in SA biosynthesis (Ding et al., 2018). In addition, *SARD1* is also rapidly induced by SA treatment. Activation of *SARD1* expression by SA is facilitated by the SA receptors NPR1 and NPR3/NPR4 (Ding et al., 2018). The induction of *SARD1* most likely contributes to the upregulation of *ICS1*, *EDS5,* and *PBS3* in response to SA treatment (Figure 4). On the other hand, both basal and pathogen-induced SA levels are significantly higher in *npr1* mutant plants, suggesting that the accumulation of SA is also negatively regulated by NPR1 (Delaney et al., 1995). The exact mechanism of how NPR1 negatively regulates SA accumulation is currently unknown.

Watering plants with Pip induces the expression of *ALD1*, *SARD4,* and *FMO1* (Hartmann et al., 2018). Infiltration of NHP also induces the expression of these three genes in both local and distal tissue (Chen et al., 2018), suggesting that there is a positive feedback loop to stimulate NHP biosynthesis (Figure 4). Furthermore, both *SARD1* and *CBP60g* are strongly upregulated in plants treated with Pip (Hartmann et al., 2018). Since SARD1 and CBP60g are required for the induction of NHP biosynthetic genes, it will be interesting to determine

whether the induction of *ALD1*, *SARD4,* and *FMO1* by NHP is dependent on them.

Interestingly, SA biosynthetic genes *ICS1*, *EDS5,* and *PBS3* are induced by Pip (Hartmann et al., 2018), whereas the Pip biosynthetic genes *ALD1* and *SARD4* are upregulated upon SA treatment (Ding et al., 2018), suggesting that there are mutual amplifications between SA and Pip/NHP (Figure 4). Such mutual amplifications of SA and Pip/NHP signaling are required for the autoimmunity of the *camta1 camta2 camta3* triple mutant (Kim et al., 2019; Sun et al., 2019). Since SARD1 and CBP60g are essential positive regulators of SA and NHP biosynthesis, and are both induced by SA as well as NHP, they may serve as major facilitators for the mutual amplification between SA and Pip/NHP (Figure 4).

In *ald1* mutant plants, pathogen-induced SA accumulation is only slightly reduced in local tissue but completely blocked in the distal leaves (Song et al., 2004b). Induction of SA accumulation in the distal leaves by *Psm* ES4326 is also blocked in *sard4* mutant plants (Ding et al., 2016), suggesting that Pip/NHP is required for activation of SA biosynthesis in the systemic tissue during the establishment of SAR. Surprisingly, upon infection by *Psm* ES4326, NHP level is much higher in the *sid2* mutant than in wild type, suggesting that SA also negatively regulates the accumulation of NHP (Hartmann et al., 2018). Since SA treatment does not inhibit the expression of *FMO1*, the increased NHP accumulation in *sid2* mutant plants could be due to reduced NHP catabolism or general compensation mechanisms of the whole immune system upon loss of SA.

Conclusion Remarks and Future Perspectives

During the past few years, tremendous progress has been made in SA and NHP biology. However, there are still some major questions remaining to be addressed. Most of the research on SA biosynthesis has been focused on the ICS pathway in *Arabidopsis*. It is unclear to what extent what we have learned in *Arabidopsis* can be applied to plants outside of Brassicaceae. The key enzyme that catalyzes the conversion of BA to SA in the PAL pathway is still missing, and very little is known about how this pathway is regulated. In addition to SARD4, there should be another reductase working together with ALD1 to produce Pip, the identity of which is currently unknown. Furthermore, it is unclear whether it is NHP or its derivatives serving as a signaling molecule in SAR. Whether NHP is transported to the distal tissue under physiological conditions should also be determined through grafting experiments. Although many transcription factors have been identified to regulate genes involved in SA and NHP biosynthesis, how they are connected to the upstream defense signaling components are often unclear and remain to be deciphered.

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