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RESEARCH REVIEW

Progress in research on dry afterripening†

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Abstract

The transition from the dormant to the non-dormant state of a viable and mature seed can take place at low hydration by exposure to air-dry storage conditions (dry afterripening; AR). The events occurring during this loss of dormancy are of considerable physiological, ecological and agricultural interest. AR may be attributable to increased sensitivity to germination-stimulating factors and a widening of the temperature window for germination. Genetic, –omics and physiological studies on this mode of dormancy breaking provide support for a key role of the balance between gibberellin (GA) and abscisic acid (ABA) metabolism and sensitivity. Recent evidence also supports a possible role for ethylene (ET) in this complex signalling network that is necessary for AR implementation. However, hormone-independent signals, such as reactive oxygen species (ROS), nitrate (NO_3^-) or nicotinamide adenine dinucleotide (NAD^+), also appear to be involved. The way in which hormone- and non-hormone-signalling pathways affects each other (cross-talk) is still under study. This review provides updated information on the programmes that overcome seed dormancy. Thus, we have reviewed: (1) the –omic status in dry seeds; (2) the relationship between temperature and nitrate signalling and AR; (3) alterations in ABA/GA synthesis and signalling; (4) the action of hormone molecules other than ABA and GA (i.e. ET, salicylic and jasmonic acids); and (5) participation of reactive oxygen species (ROS), NAD^+ and protein carbonylation. Taken together, the acquisition and implementation of dry AR involve a complex signalling network that is difficult to disentangle.

Keywords: abscisic acid, after-ripening, dry-seed, ethylene, gibberellins, hydrothermal time model, nitrate, reactive oxygen species

Introduction

The development of seeds involves several phases: morphogenesis, growth and reserve deposition, maturation and desiccation, prior to dispersal from the mother plant. Development is regulated by a complex network of endogenous signalling pathways which respond to environmental cues (Kermode, 2005; Gutiérrez *et al.*, 2007; Matilla, 2007; Bentsink and Soppe, 2008; Sorefan *et al.*, 2009; De Smet *et al.*, 2010). The result is a viable and autonomous organism which will germinate when internal and environmental conditions are suitable (Black *et al.*, 2006; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008a; Preston *et al.*, 2009; Nonogaki *et al.*, 2010).

The transition from dormancy to germination is a critical control point leading to the initiation of vegetative growth. Dormancy is an important trait that was altered during domestication of wild species (Vaughan *et al.*, 2007) and its physiology appears to be similar among different species (Bentsink *et al.*, 2010; Linkies *et al.*, 2010). It has been proposed that natural variation in seed dormancy in Arabidopsis is

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Abbreviations: ABA, abscisic acid; AR, afterripening; DOG, delay of germination; HCN, cyanide; ET, ethylene; GA, gibberellins; JA, jasmonic acid; NAD^+, nicotinamide adenine dinucleotide; NOX, NADPH oxidase; NICase, nicotinamidase; NO_3^-, nitrate; ROS, reactive oxygen species; SA, salicylic acid; NRT, tonoplast nitrate transporter.

controlled by different genetic and molecular pathways rather than by epistatic interactions (Bentsink *et al.*, 2010). Although the adaptive significance of dormancy is evident in wild plants, this state poses a practical problem in seeds of commercial crops (e.g. cereals; reviewed by Matilla, 2007; Fang and Chu, 2008). Dormant seeds can remain alive in a state of very low hydration for extended periods without losing vigour, until environmental cues such as cold stratification (a pre-chilling treatment of fully imbibed seeds) or afterripening (AR) (a period of dry storage also called post-harvest storage) trigger the release from dormancy (Bentsink and Koornneef, 2008).

Although seed dormancy has been studied extensively during the past century, there is still limited information available on the physiological and molecular mechanisms that break it. Understanding dormancy or its loss in model plants is fundamental to developing new strategies to control the trait in commercial crops. This review focuses on novel knowledge acquired during the past decade on the molecular and physiological mechanisms involved in the breaking of seed dormancy by dry AR.

Dry AR, a means to dormancy loss under low moisture conditions

AR is the loss of the dormancy achieved by exposure of a seed to a specific set of environmental conditions after maturation and separation from the mother plant (Foley, 2001; Black *et al.*, 2006). The conditions that facilitate AR vary with the species. For example, some species respond to warm, dry conditions (i.e. dry AR), others to cool, moist conditions (i.e. stratification or chilling) and some respond to both sets of conditions (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al.*, 2008). The time required to complete AR is also variable between species. Dry AR does not bring about an abrupt change from a dormant to a fully germinable state; rather, seeds in a population become more responsive to a set of conditions within which they are able to germinate (Finch-Savage and Leubner-Metzger, 2006; Carrera *et al.*, 2008; Holdsworth *et al.*, 2008a, b).

Dry AR occurs in non-imbibed viable seeds under very low water potentials (i.e. less than $0.1\,g\,H_2O$ (g dry weight)$^{-1}$; Oracz *et al.*, 2007). In tobacco and pine seeds there appear to be cells with different degrees of hydration in the dry, mature state (Manz *et al.*, 2005; Terskikh *et al.*, 2005). Consequently, the moister cells could be involved in the alterations promoted by dry AR. Oil content of a dry seed is a parameter that seems to influence dry AR because it affects water potential (Manz *et al.*, 2005). Dry AR does not occur in very dry seeds because it requires seed moisture above a threshold value. This threshold moisture content is species-specific and is lower in oil-storing seeds than in starchy seeds because the former contain less bound water at any particular relative humidity. The conditions that generate optimal low-hydration values for dry AR of several species have been determined (Leubner-Metzger, 2005).

Modelling of germination

Seeds may persist in the soil for months or even years until the environmental conditions, interacting with the seeds' physiological sensory mechanisms, trigger germination. Some mathematical models have been developed to describe the germination and dormancy behaviour of seeds on a population basis (Bradford, 2002). Their application has provided a comprehensive physiological explanation for the responses of seed germination to temperature and other physiological parameters. Hydrotime (Ht) and hydrothermal-time (HTt) models can be used to study the dry AR status (Bradford, 2002; Bradford *et al.*, 2008). Both provide information about how physiological and environmental factors interact to regulate dormancy release as well as speed of germination (Alvarado and Bradford, 2002; Allen, 2003; Bradford, 2005; Allen *et al.*, 2007). Although the HTt model does not identify the biochemical mechanism(s) by which water potential (Ψ) of a seed is regulated, it clearly points to necessary biochemical and molecular investigations. That is, the parameters of the HTt model can be used to quantitatively characterize and compare the physiological status of seed populations under different environmental conditions or from different genetic backgrounds (Chantre *et al.*, 2009, 2010). Ht and HTt have revealed that, at a given temperature, the timing and percentage of germination in a seed population are controlled by the difference between a physiologically determined Ψ threshold and the seed Ψ. Seed dormancy is a reflection of high values of the Ψ threshold, and conditions that break dormancy (e.g. dry AR) shift the Ψ distribution to lower values (Allen *et al.*, 2007). Progressive loss of dormancy in a seed population may be related to a progressive decrease in the seed $\Psi(50)$ (Bradford, 1995). Dormancy release due to AR in seeds of the winter annual weeds *Bromus tectorum* and *Elymus elymoides* (Meyer *et al.*, 2000; Bair *et al.*, 2006) has been described through a reduction in $\Psi(50)$ values. Hydrothermal AR time models for *Bromus tectorum* (Bair *et al.*, 2006) and *Lolium rigidum* (Steadman *et al.*, 2003a, b) indicate that AR occurs at a moisture-content range of 5–20% (fresh weight basis) (Fig. 1). The AR conditions for *Arabidopsis thaliana* Cabo Verde Island (Cvi) accession seeds are 20°C and 33% relative humidity, with a measured moisture content of 7% (Finch-Savage *et al.*, 2007). The discovery that dry AR rate is a linear function of storage temperature

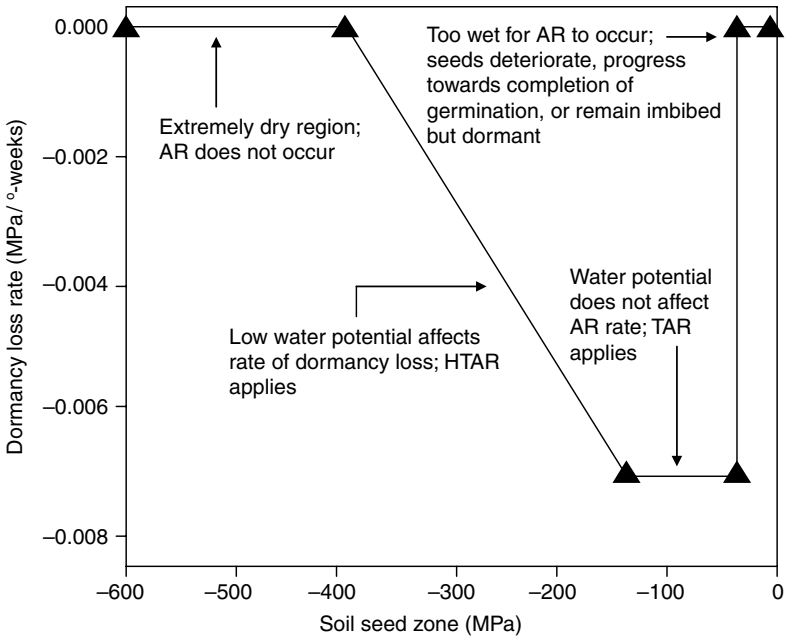

Figure 1. Conceptual diagram proposing how storage water potential influences dry afterripening (AR) in *Bromus tectorum* seeds as indicated by changes in $\Psi_b(50)$ per unit thermal time. HTAR, hydrothermal AR time; TAR, thermal AR time. (Adapted from Bair *et al.*, 2006).

above a specific base temperature led to the use of thermal-time (Tt) to describe rates of dormancy loss. Using a Tt approach, Steadman *et al.* (2003a, b) found that the rate of dormancy loss in *L. rigidum* seeds is a positive linear function of dry AR temperature. Dry AR was characterized by a progressive increase in the mean maximum germination temperature and a reduction in the Tt requirements for germination at sub-optimal temperature (Chantre *et al.*, 2009). Recently, it was demonstrated that dry AR can be adequately described as a Tt response, and that this AR–Tt developed model can be used for predicting both the extent and timing of *Lithospermum arvense* germination under field conditions (Chantre *et al.*, 2010).

AR alters gene expression in dry viable seeds

At the end of maturation, the seed has residual low moisture content (i.e. ~5–15%, depending on the species). Whether or not there is transcriptional activity in these seeds is debatable. Reorientation of the genetic programme during dry AR occurs in dry seeds, but its significance or importance in dormancy relief by dry AR, is unknown (Chibani *et al.*, 2006; Finch-Savage *et al.*, 2007; Carrera *et al.*, 2008; Holdsworth *et al.*, 2008b; Holman *et al.*, 2009). Using cDNA amplified fragment length polymorphism (AFLP) to identify transcripts differentially expressed in the dry seeds before and after dry AR, it appears that both gene transcription and translation are possible in *Nicotiana plumbaginifolia* and *Hordeum vulgare* (Bove *et al.*, 2005; Leymarie *et al.*, 2007). Because the *AtDOG1* gene decreases in abundance during dry AR and this gene expression is abscisic acid (ABA)-dependent, it was hypothesized that AtDOG1 is associated with the induction of primary dormancy during seed development (Finch-Savage *et al.*, 2007). *AtDOG* transcripts are present in dormant and AR dry seeds and decline upon imbibition of the latter (Bentsink *et al.*, 2006, 2010; Finch-Savage *et al.*, 2007; Graeber *et al.*, 2010). In dry seeds of *Sisymbrium officinale,* which are highly susceptible to AR, dry AR increased the expression of *SoGA3ox2* transcripts, and greatly reduced those of *SoACS7* (Iglesias-Fernández and Matilla, 2009). This indicates that the preparation for radicle protrusion during AR requires strong stimulation of gibberellic acid (GA) synthesis and has a lesser requirement for the stimulation of ethylene (ET) synthesis. Cell wall modifying enzymes like endo-β-mannanases (MAN) are also involved in germination of seeds such as *Datura ferox* (Nonogaki *et al.*, 2010) and *A. thaliana*, in which transcripts of three (*AtMAN5*, *6* and *7*) of the eight members of the *AtMAN* family are localized in the micropylar endosperm during germination of these seeds. Moreover, AR seeds of knock-out lines of these genes showed a lower germination rate than the wild type (Wt) (Iglesias-Fernández *et al.*, 2010). Leubner-Metzger (2005) demonstrated that there is a transient low level of transcription and translation of the β-1,3-glucanase gene during tobacco seed AR, leading to the release of dormancy.

Local hydrated pockets within cells or tissues of dormant seeds might allow changes in gene expression in the dry state during AR (Leubner-Metzger, 2005). However, it remains to be confirmed that gene activity exists in these particular localized areas.

Signalling of ROS is related to dry AR

Expression analyses have shown that the transition from late embryogenesis to germination occurs gradually and therefore characteristics of both co-exist during the transition (Nakabayashi *et al.*, 2005). Seed development, germination and post-germination seedling growth are well-regulated processes that involve high metabolic activity and generation of reactive oxygen species (ROS) (Bailly, 2004). During desiccation seeds must face severe oxidative stress and show particularly high levels of expression of genes involved in ROS detoxification, such as catalases and superoxide dismutases (SOD) (Bailly, 2004; Bailly *et al.*, 2008). It has been suggested that during storage seeds accumulate free radicals, which must be detoxified during dormancy, dry AR and germination to prevent damage to proteins, membranes and DNA (Kibinza *et al.*, 2006; Garnczarska *et al.*, 2009). ROS production during seed storage in the dry state was documented by Pukacka and Ratajczak (2005). ROS accumulation during seed maturation has been associated with seed desiccation and during germination with metabolic activity, gene expression and with programmed cell death (PCD) in the aleurone layer of wheat seeds (Pulido *et al.*, 2009). ROS could be one of the signals responsible for induction of antioxidant gene expression, causing a substantial increase of the respective enzymatic activities of SODs in developing and germinated maize seeds (Foyer and Noctor, 2005; Mylona *et al.*, 2007). Thus ROS production is likely continuous and in mature seeds is initiated immediately after harvest. Interestingly, the amount of *CATA1* transcript, one of the four genes encoding catalase in sunflower, declines during dry AR, raising the possibility that the H_2O_2-scavenging capacity of non-dormant seeds is low during early imbibition. Non-dormant imbibed seeds of sunflower contain much higher H_2O_2 amounts than the dormant ones (El-Maarouf-Bouteau *et al.*, 2007). In agreement with this, Oracz *et al.* (2007) have shown that dry AR in sunflower is associated with accumulation of ROS in the embryonic axes; they measured the content of H_2O_2 and O_2^- during dormancy release, recording increases of 100% and 50%, respectively. They suggested that AR is associated with lipid peroxidation. In *Bidens pilosa*, ROS appear to be implicated in the alleviation of seed dormancy, and reagents that generate hydroxyl radicals and superoxide partially replaced the requirement for AR (Whitaker *et al.*, 2010).

In addition to gene alterations promoted in the dry seed by AR, there may be protein modifications, as well. ROS can alter the function of seed proteins through modification of their redox state, or, for example, carbonylation (El-Maarouf-Bouteau *et al.*, 2007; Oracz *et al.*, 2007; El-Maarouf-Bouteau and Bailly, 2008). Carbonylation is the most commonly occurring oxidative protein modification (for a review, see Møller *et al.*, 2007) and inhibits or alters protein activities and intensifies their susceptibility to proteolytic attack. There are no indications that carbonylation is reversible. The task of identifying the causal factors behind age-dependent increased carbonylation has proved difficult. In sunflower seeds, ROS accumulation clearly leads to carbonylation of specific embryo proteins, not only during dry AR (Oracz *et al.*, 2007), but also during artificial breaking of dormancy by cyanide application (HCN; a compound that triggers ROS accumulation because it is an inhibitor of SOD, catalase and carbonylation) or methylviologen (a ROS-generating compound) (Bethke *et al.*, 2006; Oracz *et al.*, 2007, 2008; El-Maarouf-Bouteau and Bailly, 2008). The hypothesis that HCN interacts with ROS-producing pathways has been supported by data on intracellular ROS production in response to HCN treatment (Oracz *et al.*, 2009). In dry, mature Arabidopsis seeds, most of the carbonylation occurred in the storage proteins, and carbonylation of a number of other proteins increased strongly during germination although the germinated seeds still gave rise to vigorous plantlets (Job *et al.*, 2005; Møller *et al.*, 2007). Interestingly, the carbonylation level of some proteins decreased during AR, e.g. a 20S proteasome α-subunit (Oracz *et al.*, 2007). This observation is consistent with previous data showing a requirement for proteasome activity in sunflower embryos for both the breaking of dormancy by ethylene (ET) and the progression of germination. In summary, Oracz *et al.* (2007) proposed a novel mechanism for seed dormancy release involving a change in proteome oxidation, resulting from an accumulation of ROS during dry AR.

NADPH oxidases (NOX) catalyse the production of ROS (Swanson and Gilroy, 2009) and in plants the NOX homologues have been named 'respiratory burst oxidases' (Rboh) (Sagi and Fluhr, 2006). However, it is not known what roles Rboh, a major producer of superoxide in germinating Arabidopsis seeds, play in seeds. It has been demonstrated, however, that the *A. thaliana AtRbohB* gene, which is expressed in the embryo and not in the endosperm, plays a role in AR and seed germination, AR being prevented in the *AtrbohB* mutant which also shows reduced protein carbonylation (Müller *et al.*, 2009). The *AtrbohB* pre-mRNA is alternatively spliced in fresh and AR seeds and this is ABA-dependent. It was hypothesized that the alternative splicing is used by seeds to react quickly to changes in their environment, especially to

stress, in which ABA and ROS signalling play a major role (Müller *et al.*, 2009).

In addition to its role as a cofactor, nicotinamide adenine dinucleotide (NAD^+) is involved in signalling and gene-regulation pathways in eukaryots (Hunt *et al.*, 2004). Nicotinamide (NIC) is transformed to nicotinic acid by nicotinamidase (NICase; EC 3.5.1.19) and participates in the Arabidopsis salvage pathway of NAD biosynthesis (Wang and Pichersky, 2007). NICase activity has also been detected in embryos of germinating mungbean; both exogenous NIC and NAD^+ inhibit seed germination, and a link between depth of seed dormancy and NAD^+ content has been demonstrated, using a null mutant for NICase (Zheng *et al.*, 2005; Hunt *et al.*, 2007). Arabidopsis *NIC2* is expressed at relatively high levels during seed maturation but is low at 24 h after imbibition (HAI) (Nakabayashi *et al.*, 2005). In addition, the NAD^+ concentration in fresh seeds of different Arabidopsis ecotypes with low, intermediate and high levels of dormancy, respectively (Cvi, Col-0 and Ws), was proportional to the degree of seed dormancy, whereas that of $NADP^+$ was inversely proportional (Alonso-Blanco *et al.*, 2003; Hunt and Gray, 2009). While the amount of $NADP^+$ does not change between fresh and AR seeds; that of NAD^+ declines significantly during AR in the Cvi accession. Thus in Arabidopsis it appears that the depth of seed dormancy is also related to $NAD^+/NADP^+$ or $NAD^+/NADH^+$ ratios (Hunt *et al.*, 2007; Hunt and Gray, 2009). Analysis of the Arabidopsis *NICase* mutant (*nic2-1*), whose seeds contain a higher amount of $NADP^+$, showed reduced NICase activity and ABA hypersensitivity as compared to Wt seeds. This suggests a role for NICase activity in regulating seed germination potential. Seeds of the Cvi accession, which have deep seed dormancy, had a relatively high NICase activity, which appeared to be reduced by AR (Hunt *et al.*, 2007; Hunt and Gray, 2009). This suggests that NICase activity is not directly correlated with depth of seed dormancy.

The relationship between dry AR, temperature and nitrate signalling

Higher plants, and some organs and cell components, exhibit a range of responses to the temperature of their environment (Penfield, 2008; Penfield and Hall, 2009), although the temperature sensors are unknown. It is widely accepted that temperature is the major environmental factor governing changes in the depth of dormancy in seeds in temperate environments (Benech-Arnold *et al.*, 2000; Probert, 2000; Baskin and Baskin, 2004; Black *et al.*, 2006). Dry AR is highly temperature-dependent (Donohue, 2002; Steadman *et al.*, 2003a; Bair *et al.*, 2006). In species that produce seeds in the spring/summer and that germinate in autumn, a period of prolonged desiccation often leads to a loss of primary dormancy that is present when the mature seed is shed. Although soil temperature is generally considered to be the primary environmental factor regulating dormancy, the effect of temperature on dormancy release and induction may also be modulated by soil moisture (Bair *et al.*, 2006; Batlla and Benech-Arnold, 2006). *Bromus tectorum*, a winter annual, requires dry AR for dormancy release, but very low soil moisture contents slow the rate at which dormancy is lost (Bair *et al.*, 2006). Ecologically, a dry AR requirement prevents seeds from germinating during the hottest periods of the summer when there is a risk of seedling death due to inadequate rainfall. In some species, dry AR must precede stratification in order to break dormancy for at least a proportion of the seed population (Garvin and Meyer, 2003). From this perspective, dry AR broadens the optimal temperature range for germination, accelerating radicle protrusion as compared with non-ripened seeds (Foley, 2001; Corbineau and Côme, 2003; Bair *et al.*, 2006; Oracz *et al.*, 2007; Iglesias-Fernández and Matilla, 2009). Thus, temperature, together with seed moisture status, affects dry AR (Foley, 2008).

Nitrate (NO_3^-) is an important nitrogen source for plants, so much so that some (i.e. nitrophilous plants) live in soils enriched in this anion. NO_3^- is also involved in plant signalling and controlling various aspects of development (Alboresi *et al.*, 2005; Krouk *et al.*, 2010), but its mechanism of action is still largely unclear. NO_3^- releases dormancy of some seeds (Alboresi *et al.*, 2005; Bethke *et al.*, 2006), negatively affecting early ABA synthesis (Ali-Rachedi *et al.*, 2004; Matakiadis *et al.*, 2009) or light requirement (Batak *et al.*, 2002). When applied to dormant seeds of Arabidopsis Cvi accession it results in a decrease in ABA content and prevents its *de novo* synthesis, which normally occurs in the absence of NO_3^- and in the presence of light (Ali-Rachedi *et al.*, 2004). Cross-talk between light and NO_3^- signalling has also been related to the breaking of seed dormancy (Alboresi *et al.*, 2005). NO_3^- nutrition of the mother plant affects dormancy of seeds, and their depth of dormancy is inversely correlated to endogenous maternal NO_3^- content (Alboresi *et al.*, 2005). In Arabidopsis seeds, the gene *AtNRT2.7*, which encodes a tonoplast nitrate transporter, is involved in NO_3^- accumulation (Chopin *et al.*, 2007). This gene is expressed in developing seeds, particularly at the end of seed maturation, its transcripts peaking in dry seeds. Physiological analyses of Arabidopsis plants defective in the dual-affinity nitrate transporter *NRT1.1* (i.e. *nrt1.1*) and *NRT2.7* (i.e. *nrt2.7*), have revealed a possible involvement of these transporters in NO_3^- accumulation and in the intensity of seed dormancy and signalling (Alboresi *et al.*, 2005; Chopin *et al.*, 2007). Recently, it was demonstrated that an intact two-component

complex of AtNRT2.1 and AtNAR2.1 (AtNRT3.1), localized in the plasma membrane, is the form that is active in the inducible high-affinity NO_3^- transport system (Yong *et al.*, 2010). During stratification, Arabidopsis Cvi accession seeds first become sensitive to NO_3^-, then to cold, and finally to light (Finch-Savage *et al.*, 2007). Results by Iglesias-Fernández and Matilla (2009) on the germination rate in seeds of the nitrophilous plant *Sisymbrium officinale* have shown that NO_3^- requirements diminished with AR; this suggests cross-talk between dry AR and NO_3^- signalling. Exogenous NO_3^- increased the induction of *AtCYP707A* transcript and accelerated the ABA decline at 6 HAI, conferring to the *CYP707A2* gene a central role in controlling seed dormancy in response to NO_3^- (Matakiadis *et al.*, 2009). This work highlights the importance of the regulation of ABA degradation rather than ABA synthesis in controlling the Arabidopsis seed dormancy response to NO_3^-. Likewise, transcript profiling of imbibed seeds with or without NO_3^- showed that exogenous NO_3^- led to a higher expression of NO_3^--responsive genes, whereas endogenous NO_3^- led to a profile similar to that of stratified or AR seeds (Matakiadis *et al.*, 2009).

AR acquisition triggers alterations in ABA metabolism

Once a viable seed is afterripened, it is ready to germinate. This is considered to be determined by the balance of negative and positive effects of the hormones ABA and GA, respectively (Yamaguchi *et al.*, 2007; Bentsink and Koornneef, 2008; Yamaguchi, 2008; Rodríguez-Gacio *et al.*, 2009; Seo *et al.*, 2009; Nambara *et al.*, 2010). It has been suggested that dry AR and loss of dormancy are two distinct processes, because ABA-deficient, non-dormant Arabidopsis seeds still displayed transcriptome changes characteristic of dry AR after several months of storage at room temperature. Thus, dry AR and dormancy were considered genetically separate pathways and ABA contributed only to the induction and maintenance of dormancy of imbibed seeds, and was not required for AR (Carrera *et al.*, 2008; Holdsworth *et al.*, 2008a).

The *NCED6* and *NCED9* genes, which are involved in ABA synthesis, play a major role in the control of seed development, including dormancy, while the *CYP707A* gene family is involved in ABA inactivation, playing a critical role in dormancy release in several species (Gubler *et al.*, 2005; Rodríguez-Gacio *et al.*, 2009; Seo *et al.*, 2009; Nambara *et al.*, 2010). Reverse-genetic analyses in Arabidopsis seeds (Kushiro *et al.*, 2004; Okamoto *et al.*, 2006) indicate that (1) *AtCYP707A2* expression in the dry seed, controls the rapid decline in ABA during imbibition (between 6 and 12 HAI); (2) *AtCYP707A1* and *AtCYP707A3* are involved in germination; (3) the *Atcyp707a2* mutant overaccumulates ABA in dry seeds and the high ABA content is maintained after seed imbibition; and (4) freshly harvested *Atcyp707a2* seeds exhibit hyperdormancy.

The ABA content of dry seeds of the Arabidopsis line Col (non-dormant) is much lower than that of Cvi (highly dormant), which in turn is only slightly higher than in those of dry afterripened Cvi seeds (non-dormant) (Ali-Rachedi *et al.*, 2004). The ABA content of dormant tobacco seeds was strongly reduced during AR (Grappin *et al.*, 2000). Dry AR itself had little effect on the ABA content of embryos of dry barley seeds. However, a decrease in ABA content and a rise in phaseic acid occurred during imbibition of AR seeds as compared to those that were not AR (Jacobsen *et al.*, 2002). Phaseic and dihydrophaseic acids also accumulated in dry AR Arabidopsis (Col) seeds at 24 HAI (Kushiro *et al.*, 2004). In barley, *HvNCED1* (an ABA biosynthesis gene) was not affected by dry AR but is probably involved in preventing germination in non-optimal conditions of light and temperature (Gubler *et al.*, 2008). Millar *et al.* (2006) showed in barley and in the Arabidopsis ecotype C24 that (1) *AtCYP707A2* and *ABA 8′-hydroxylase* (*HvABA8′OH-1*) genes were induced in dry AR seeds as compared to dormant seeds; (2) *HvABA8′*OH-1-mRNA was preferentially localized in the coleorhiza; it occurred in barley AR seeds but not in 12-h-imbibed dormant ones. Therefore, ABA signalling appears to take place in the coleorhiza and is affected by dry AR (Barrero *et al.*, 2009). These authors point to a pivotal role for the *HvABA8′OH-1* gene in controlling dormancy and that the action of its enzyme product may be confined to the coleorhiza. Thus this region of the embryo may play a major role in maintaining dormancy by acting as a barrier to root emergence, and AR potentiates molecular changes related to ABA metabolism and sensitivity that ultimately lead to degradation of the coleorhiza, and completion of germination as radicle emergence. On the other hand, ABA metabolism occurred also in the endosperm of Arabidopsis (Okamoto *et al.*, 2006) and constitutive overexpression of a *CYP707A* gene in transgenic Arabidopsis resulted in a decreased ABA content in mature dry seeds and a much shorter AR period to overcome dormancy. Conversely, mutating the *CYP707A2* gene resulted in seeds that required longer AR. On the other hand, greater induction of the *AtCYP707A* gene in Col than in Cvi may be related to the greater reduction of ABA content in the former (Preston *et al.*, 2009). Finally, by using two Arabidopsis ABA metabolism mutants (i.e. ABA-deficient mutant *aba2* and ABA overaccumulating *cyp707a1a2a3* triple mutant) Okamoto *et al.* (2010) demonstrated that a cluster of ABA-upregulated genes partially resembled those of dormant genes, whereas ABA-downregulated genes partially

overlapped with dry AR-regulated genes. This elegant work shows the complexity of cross-talk between ABA and AR in seeds. Gerjets *et al.* (2010), analysing six wheat varieties, also indicated no obvious relationship between ABA function and dry AR in wheat.

An ABA-insensitive seed-germination mutant *chotto1* (*cho1*) has been identified and characterized (Nambara *et al.*, 2002). It carries a mutation in a gene encoding a double APETALA2 repeat transcription factor. *CHO1* is expressed in imbibed seeds and requires *ABI4* (Nambara and Marion-Poll, 2005). Imbibed dry AR seeds were affected in their content of ABA, of GA_4, jasmonate, salicylate and N6-(D2-isopentenyl) adenine (iP). Dry AR also affected the expression of genes involved in ABA and GA metabolism beyond seed dormancy release, and the pattern of phytohormone and transcript contents indicated that briefly stored *cho1* mutant seeds mimicked fully dry AR Wt-seeds (Yano *et al.*, 2009). These results indicate that *CHO1* acts downstream of ABA to repress GA biosynthesis during seed germination.

AR also affects ethylene responses

ET also positively influences germination potential (Matilla and Matilla-Vázquez, 2008). However, the role of this hormone is less evident than that of ABA and GA (Vandenbussche and Van der Straeten, 2007; Stepanova and Alonso, 2009). ET is not the hormone that triggers the decisive steps during the appearance and elimination of dormancy, but rather is part of a complex network of interacting signals involved in dormancy, the details of which are currently being investigated. ET seems to act antagonistically with ABA during dormancy termination, but acts in concert with GA to promote germination (Kucera *et al.*, 2005; Bentsink and Soppe, 2008; Holdsworth *et al.*, 2008a; Matilla and Matilla-Vázquez, 2008). Recently, it has been suggested that in afterripened *S. officinale* seeds GA is more important than ET during early imbibition (up to 6 HAI), while ET is more important later during germination (Iglesias-Fernández and Matilla, 2010).

Concluding remarks

Seed dormancy can be broken by dry storage. This process, called dry AR, is genetically determined and enables the seed to germinate upon subsequent imbibition. Some mechanisms that regulate dry AR have been investigated in depth, but many gaps still remain. The alterations triggered by dry AR itself already occur in the dry seed, but its effects also continue during and after imbibition. Dry AR increases germination potential by enhancing the sensitivity of seeds to factors that promote germination, such as light and GA, and reduce sensitivity to those that inhibit germination, such as ABA. The lowering of ABA content in imbibed seeds is strongly influenced by dry AR and the length of its application, thus promoting germination. In barley and *A. thaliana*, AR treatments led to an up-regulation of *CYP707A* expression, resulting in a decline of ABA in the imbibed seed. In addition, transcriptome analysis of *A. thaliana* Cvi-0 seeds has revealed that the breaking of seed dormancy by dry AR is associated with changes in the expression of ABA- and GA-metabolism genes, such as the *CYP707A2* and the *GA3ox1* genes (Fig. 2). However, transcriptome analysis of non-dormant *aba-deficient1* or *aba-insensitive1* mutants has demonstrated that dry AR functions independently of the ABA signalling pathway. In Arabidopsis (Col) dry AR affects the transcript levels of genes involved in ABA and GA metabolism beyond seed dormancy release, i.e. dry AR affects the expression of a large number of genes even in the non-dormant mutants and continues to reduce ABA responsiveness even though the seed is completely released from primary dormancy.

Quantitative trait locus (QTL) analysis of Arabidopsis has identified the *DOG1* gene, a major player in the control of seed dormancy that requires ABA for its expression (Fig. 2). *DOG1* transcripts, that decrease during dry AR more strongly than in non-AR germinating seeds, are localized in the radicle and micropylar endosperm of *Lepidium sativum* and, as in Arabidopsis, the transcripts are rapidly degraded upon imbibition. This strongly suggests a novel role(s) for DOG1 in germination timing (e.g. negatively regulating AR).

In addition, ROS (i.e. $O_2^{\cdot-}$ and H_2O_2) have been proposed to produce important effects on seed germination and dormancy. Some of these involve the modification of proteins (e.g. carbonylation), and their relationship with dry AR is at present under study. A large number of hormonal (i.e. ET) and non-hormonal (i.e. HCN, NAD^+) signals are involved in seed ROS production. NAD^+ content appears to be a good indicator of the depth of seed dormancy and *nic2-1* mutant seeds contain high NAD^+ amounts, diminished NICase activity, ABA hypersensitivity and delayed dry AR. Synthesis of ROS during dry AR causes a notable degradation of key proteins by carbonylation. Seeds of the Arabidopsis NADPH-oxidase mutant (*AtrbohB*) germinate more slowly when subjected to dry AR, and the ROS-production genes (i.e. *NADPHox* and *NOX*) are differentially affected during dry AR.

C*ho1* mutant seeds are more sensitive to dry AR than Wt seeds. The *cyp707a2-1 cho1-3* double mutant (as well as *35S::CHO1* lines) indicate that the primary action of *CHO1* is to regulate ABA responsiveness.

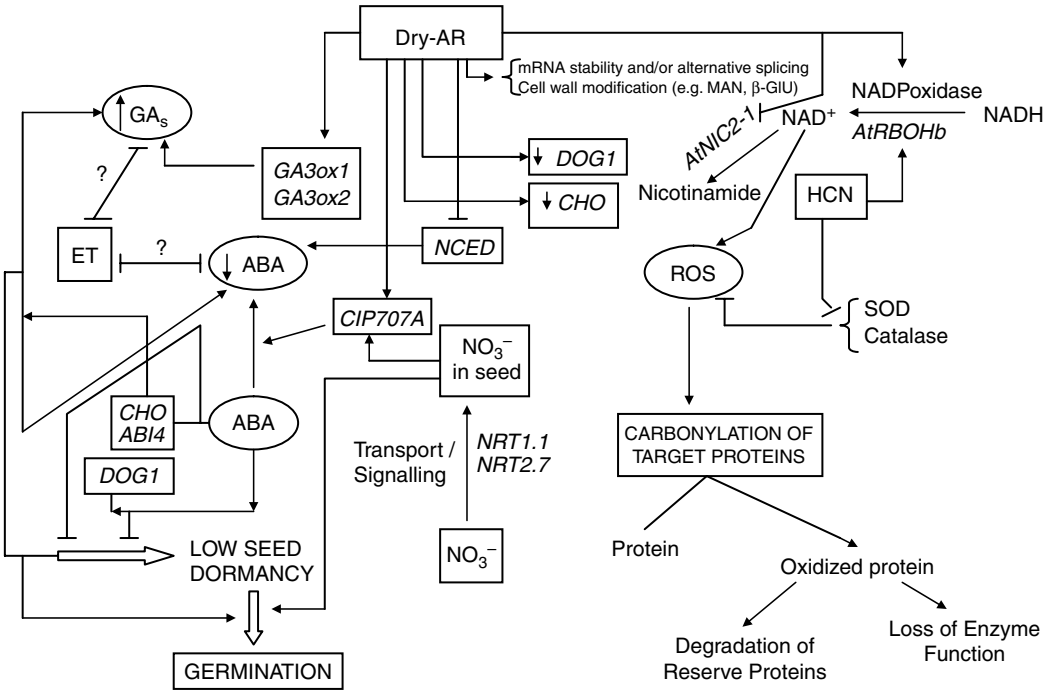

Figure 2. Known implications of dry afterripening on the breaking of seed dormancy, and germination.

Likewise, in both Wt and *cho1* mutants, dry AR affects the transcript levels of ABA metabolism (*ZEP, NCED9*, *CYP707A2*, *CYP707A3*) and GA metabolism (*GA3ox1*, *GA3ox2*) genes after imbibition, even after seed dormancy is completely broken by dry AR. This indicates that, in dry-stored seeds, dry AR progresses beyond seed-dormancy release. Dry AR affects gene expression in non-dormant *aba1* mutants. Moreover, *CHO1* participates in the regulation of endogenous ABA and GA balance by affecting transcription of these hormone-metabolism genes in imbibed seeds (Fig. 2).

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