ABA responses during seed development and germination

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Contents

Abstract

ABA regulates various biological programs in seeds. This article initially introduces the basic information about the regulation of seed reserve accumulation and desiccation tolerance by ABA, and then delves into the emerging mechanisms of ABA signaling during embryo de-greening, seed dormancy and germination. Pioneering research on seed reserve accumulation and desiccation tolerance many years ago identified

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the major ABA signaling components, such as the purine (R) and pyrimidine (Y) motif (RY motif) and ABA response/responsive element (ABRE), which established the foundation of our current understanding of ABA signaling. Recent research is further advancing our knowledge on the involvement of ABA in other seed programs. ABA INSENSITIVE3 (ABI3) regulates embryo de-greening through STAY-GREEN (SGR), a rate-limiting enzyme for chlorophyll degradation. ABI1 and ABI2, protein phosphatases 2C (PP2C), are involved in ABA signaling associated with seed dormancy and germination. Unlike ABI1 and ABI2, ABA HYPERSENSITIVE GERMINATION1 (AHG1), another PP2C involved in germination, is resistant to the inhibition by the ABA receptor, which highlights a unique ABA signaling pathway in seeds. This pathway seems to create a byway that allows the germination program to operate even in the presence of ABA. On the other hand, the long-awaited biochemical function of DELAY OF GERMINATION1 (DOG1), a master regulator of seed dormancy, is now understood to sequestrate AHG1 to shut down this pathway, enhance ABA sensitivity in seeds and impose dormancy. Interesting regulatory mechanisms of *DOG1* and antisense *DOG1* expression, which are also relevant to ABA signaling in plant responses to drought, are also discussed in this article.

1. Introduction

The mechanisms of ABA signaling in seeds are essentially similar to those found in the core ABA signaling pathway (Cutler, Rodriguez, Finkelstein, & Abrams, 2010) that drives stomata closure in leaves during drought responses and other cellular responses in plant organs during salinity and temperature responses. However, seeds go through the specific program of germination that is not observed in other plant organs, except pollen and spores. Seed dormancy, in particular, which is the temporal suppression of germination under the conditions otherwise favorable for embryo emergence, plays a critical role in the plant life cycle and is regulated primarily by ABA. Seed dormancy research has identified the ABA signaling mechanisms and key regulatory proteins that are unique to seeds. In this article, ABA signaling during seed development and imbibition will be discussed with the specific focus on the mechanisms of seed dormancy and germination.

2. Seed development

Seed development can be divided into the two phases - embryogenesis and maturation (Bewley, Bradford, Hilhorst, & Nonogaki, 2013). It is hard to define these two phases completely because embryogenesis primarily

refers to morphological changes, such as tissue and organ development, whereas maturation mainly refers to physiological and biochemical events. These two phases have overlapping stages where part of the maturation program (e.g. expression of maturation genes, reserve accumulation) commences while the embryo is still transforming its morphology.

During early embryogenesis, particularly in the globular to heart stage embryos, the plant hormone auxin plays the major role in histodifferentiation, which specifies distinct tissue domains in the embryo, such as the hypophysis, the adaxial and abaxial sides of cotyledons and the shoot apical meristem (Bowman & Floyd, 2008; Chandler, 2008). In a narrow definition, embryogenesis can be considered complete by the heart stage, in which the cell fates have already been determined (Mayer, Ruiz, Berleth, Miséra, & Jürgens, 1991; Raz, Bergervoet, & Koornneef, 2001). In this narrow definition, further embryo development is referred to as growth stage, which ends with the formation of a full-size embryo (Goldberg, de Paiva, & Yadegari, 1994; Raz et al., 2001). In general, the torpedo, linear and bent-cotyledon embryos are also included in embryogenesis stages (Bewley et al., 2013; Meinke, 1994). These stages are still earlier than so-called physiological maturation that occurs in (morphologically) fully-developed embryos.

While the primary role of auxin in the establishment of the plant body plan during embryogenesis is well known (Bowman & Floyd, 2008; Chandler, 2008), information about ABA involvement in early embryogenesis is limited. Since *aba2-1*, an ABA-deficient mutant, exhibits delayed development of the globular, heart and torpedo embryos during embryogenesis (Cheng et al., 2014), ABA signaling seems to play some role also in early embryogenesis. Nevertheless, its predominant role during seed development resides in the maturation stage. The major events during seed maturation that are regulated by ABA include de-greening of the embryo, seed reserve accumulation, acquisition of desiccation tolerance and induction of seed dormancy (Bewley et al., 2013). Since the regulation of seed reserve accumulation and desiccation tolerance by ABA has been well summarized in textbooks (e.g. Bewley et al., 2013) and previous reviews (Fatihi et al., 2016; Lepiniec et al., 2018; Leprince, Pellizzaro, Berriri, & Buitink, 2017), only brief history of the identification of the major ABA signaling components, such as the purine (R)- and pyrimidine (Y)-rich repeat sequence (RY motif) and ABA response/responsive element (ABRE), will be discussed for reserve accumulation and desiccation tolerance. Then, a specific focus will be placed on the relatively new research developments on embryo de-greening, which is also regulated by ABA

signaling. A revised concept of ABA production in the maternal and zygotic tissues during seed development will also be introduced here in this section.

2.1 Reserve accumulation and desiccation tolerance

Developing seeds continue to receive nutritional supply from the maternal plant, which accumulates initially in the endosperm and is then transferred to the embryo (Bewley & Black, 1985). The extent of this transfer and the ratio between the endosperm and embryo reserves and tissue mass in the mature seeds differ depending on species (Nonogaki, 2006). Cereal seeds contain a substantial amount of storage materials (mainly starch) in the relatively large endosperm while *Brassica* seeds contain most of the reserves (lipid and proteins) primarily in the embryo in the mature seeds. In cereal seeds, the embryo is much smaller than the endosperm while the embryo becomes the predominant tissue in the mature *Brassica* seeds. In general, ABA stimulates the seed tissues to initiate an anabolic or storage mode of action while gibberellins (GA) promote catabolism of seed storage compounds or reserve mobilization at the post-germinative stages (Bewley et al., 2013).

Interestingly, ABA appears to function also in mammals in somewhat analogous way to its mode of action in seeds. ABA is produced in and released from human islets and stimulates secretion of insulin, an anabolic hormone that promotes the synthesis of glycogen, fat and proteins in liver and muscles (Bruzzone et al., 2008). Based on these functions and its structural similarities to thiazolidinediones (TZD), which reduce insulin resistance (Guri, Hontecillas, Si, Liu, & Bassaganya-Riera, 2007), ABA is considered a potential drug for diabetes (Bassaganya-Riera et al., 2010; Zocchi et al., 2017). It is interesting that ABA favors an anabolic mode of action over catabolism also in mammals, since ABA clearly serves as an anabolic hormone in developing seeds.

Extensive research has been performed on the mechanisms of storage protein synthesis in seeds, especially for edible species, which has greatly contributed to the current understanding of the general mechanisms of ABA responses. Here, we can recall some seminal works in seed research, which led to the discoveries of the key ABA signaling components and their binding *cis*-elements. ABA induces genes encoding for major seed storage proteins, such as albumins and globulins (Wilen, Mandel, Pharis, Holbrook, & Moloney, 1990). A number of studies on the structure and expression of the genes encoding for seed storage proteins during 1980s and 1990s identified the common DNA sequences in their promoter regions, such as the

legumin box (Shirsat et al., 1989, 1990 and prolamin box (Vicente-Carbajosa, Moose, Parsons, & Schmidt, 1997). The discovery of the purine (R)- and pyrimidine (Y)-rich repeat sequences in the 5′-flanking sequences of the seed lectins phytohemagglutinins (*PHAs*) in *Phaseolus vulgaris* (Hoffman & Donaldson, 1985) and the legumin genes (Dickinson, Evans, & Nielsen, 1988) led to an understanding of the function of the RY motif as a seed-specific *cis*-element (Bäumlein, Nagy, Villarroel, Inzé, & Wobus, 1992). The RY motif is now well understood as the binding site of the B3 domain-containing transcription factors, such as FUSCA3 (FUS3) and LEAFY COTYLEDON2 (LEC2). ABI3, another B3 domain-containing transcription factor, does not directly bind to the RY motifs in the Arabidopsis *2S ALBUMIN* promoter but co-induces this gene with a basic leucine zipper (bZIP) protein, which recruits ABI3 to the promoter (Kroj, Savino, Valon, Giraudat, & Parcy, 2003). (*see* Bewley et al., 2013 for more information on seed storage proteins.)

ABA plays a critical role also in establishing desiccation tolerance of seeds, which is mediated through the induction of a specific group of proteins (genes), such as *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) proteins including *Early Methionine-labelled* (*Em*) proteins (Bewley et al., 2013). While the RY motif was discovered from the investigation of seed storage proteins, research on seed desiccation tolerance identified another conserved sequence that is essential for ABA responsive gene expression. The analysis of wheat *Em* promoter led to the identification of an 8-base pair sequence (CACGTGGC), which was originally termed ABA response element, and its binding factor EmBP1 (Guiltinan, Marcotte, & Quatrano, 1990; Marcotte, Bayley, & Quatrano, 1988). These are the pioneering studies that later led to the broader recognition of ABRE and ABRE-biding factor (ABF), including ABI5, which are conserved in various species of plants. Thus, intensive studies on seed reserve accumulation and desiccation tolerance many years ago greatly contributed to the identification of the major ABA signaling components such as RY, ABRE, ABF and ABI, which will be discussed for embryo-greening and seed dormancy mechanisms below.

2.2 Embryo de-greening

The morphogenesis during seed development can be visually followed until the embryo reaches the mature architecture. In contrast, the transitions occurring in the physiological state of the growth-phase and mature embryos are not generally obvious. Exceptions that apparently reflect the status of maturing seeds are greening and de-greening of the embryo (Fig. 1A), which are typically observed in developing seeds of Arabidopsis

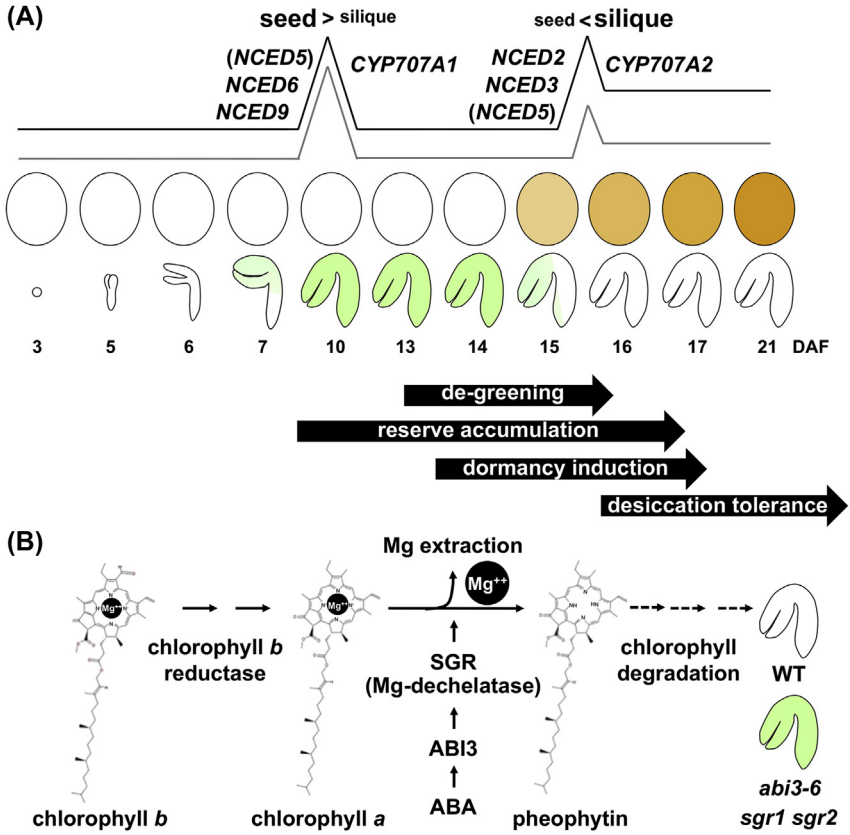

Fig. 1 ***Seed developmental programs including ABA metabolism and chlorophyll degradation.*** (A) The timing of embryo greening and de-greening during seed development. Approximate windows of the major seed developmental programs (embryo de-greening, reserve accumulation, dormancy induction and desiccation tolerance) are also shown. The two peaks of ABA during seed development, which were reported by the pioneering studies (*gray line*) and the latest studies (*black line*), are indicated together with the ABA biosynthesis (*NCED*) and catabolism (*CYP707A*) genes expressed around the two peaks (*see* text for details). The predominance of ABA in seed (seed>silique) or silique (seed<silique) is also shown. DAF, days after flowering. *CYP*, *cytochrome P450*; *NCED*, *nine-cis-epoxycarotenoid dioxygenase*. (B) Chlorophyll degradation. Chlorophyll *b* is converted to chlorophyll *a* by chlorophyll *b* reductase. The rate-limiting step of chlorophyll *a* degradation is catalyzed by Mg-dechelatase, which is encoded by the *STAY-GREEN* (*SGR*) genes. *SGRs* are in turn regulated by ABA INSESITIVE 3 (ABI3). The sever alleles of *abi3* mutants (e.g. *abi3-6*) and the *sgr1 sgr2* double mutants produce seeds containing green embryos. *(A) Based on Bentsink et al. (2006), Delmas et al. (2013), González-Morales et al. (2016), Kanno et al. (2010), Karssen et al. (1983), Lefebvre et al. (2006), and Okamoto et al. (2006). (B) Based on Delmas et al. (2013), Sato, Shimoda, Matsuda, Tanaka, and Ito (2018b), and Shimoda et al. (2016).*

(and other species). Embryo greening is observed during mid embryogenesis while a full-size embryo at the late stages starts to lose chlorophyll during maturation. A mutation in the latter process results in green seeds, which was originally described in Mendel's seminal paper as yellow (*I*) and green (*i*) cotyledons in peas (*Pisum sativus*) (Mendel, 1866). The *STAY-GREEN* (*SGR*) gene was mapped to the Mendel's *I* locus (Armstead et al., 2007). The *SGR* orthologs in Arabidopsis, meadow fescue (*Festuca pratensis*) and rice (*Oryza sativa*) were originally characterized for leaf senescence and cotyledon greening (Armstead et al., 2007). Arabidopsis *SGR* was identified also as *NON-YELLOWING* (*NYE*) (Ren et al., 2007). Arabidopsis *SGR1* and *SGR2* encode for Mg-dechelatase, an enzyme catalyzing the first rate-limiting step of chlorophyll *a* degradation (Shimoda, Ito, & Tanaka, 2016) (Fig. 1B). A defect in the rate-limiting reaction for chlorophyll degradation prevents normal de-greening of the embryo, which could damage germination capacity of the mature seeds under excessive light. Germination of the *nye1 nye2* (i.e. *sgr* double mutant) seeds is impaired by prolonged light exposure to the seeds, which is probably due to the photosensitizing properties of chlorophyll to cause a burst of reactive oxygen species (Li et al., 2017). De-greening of the embryo seems to be a required process for developing seeds to establish the protection against intensive light at least in Arabidopsis, although pigmentation of the testa, a seed coat (Fig. 1A), must also play a significant role in photo protection (and sensing).

The severe *abi3* alleles produce green seeds at maturity (Koornneef, Hanhart, Hilhorst, & Karssen, 1989), indicating that ABA signaling is involved in the de-greening process. Seeds of wildtype and the weak alleles of *abi3* (e.g. *abi3-8*) complete embryo de-greening normally by 16 days after flowering (DAF) (Fig. 1A). In contrast, the *abi3-6* embryos stay green at maturity (Delmas et al., 2013). Ectopic expression of either *SGR1* or *SGR2* rescues the *abi3-6* stay-green defect, suggesting that ABI3 regulates embryo de-greening through the SGR function downstream (Fig. 1B). SGRs are regulated by ABI3 in developing seeds probably through its direct transcriptional control because the B3 DNA-binding domain of ABI3 physically interacts with the *cis*-elements found in the *SGR* promoters (Delmas et al., 2013).

Mutations in ABI3 affect other seed maturation programs, including seed reserve accumulation, dormancy and desiccation tolerance (Koornneef et al., 1989). The *abi3* defects in these maturation programs are not rescued by ectopic expression of *SGRs* (Delmas et al., 2013; Ren et al., 2007;), suggesting that the other seed maturation programs, which are also regulated by

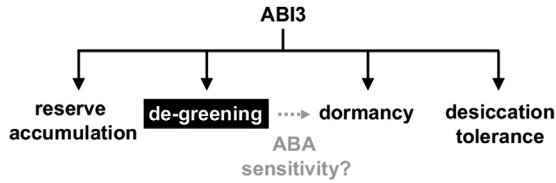

Fig. 2 ***Regulation of seed developmental programs by ABA INSENSITIVE 3.*** ABA INSENSITIVE 3 (ABI3) is involved in the regulation of the major seed developmental programs, such as embryo de-greening, reserve accumulation, dormancy and desiccation tolerance. The *STAY-GREEN* (*SGR*) genes play an essential role for embryo de-greening downstream of ABI3. While the de-greening defect in the *abi3-6* mutant seeds is complemented by ectopic expression of *SGRs*, it does not rescue *abi3-6* from other defects in reserve accumulation, seed dormancy and desiccation tolerance. Consistently, seeds of the *sgr1 sgr2* double mutant, which stay green at maturity, exhibit normal reserve accumulation, dormancy and desiccation tolerance. Therefore, the embryo de-greening pathway, which is regulated by SGRs, is thought to be independent of other seed developmental programs. An exception is that the *sgr2* mutants exhibit slightly reduced ABA sensitivity, suggesting a possible link of embryo de-greening to seed dormancy (*gray dotted line*). *Based on Delmas et al. (2013).*

ABA, are independent of the embryo de-greening pathway, or *vice versa* (Fig. 2). This notion is also supported by the observation that the *sgr1 sgr2* double mutant seeds with the stay-green defect exhibit normal reserve accumulation, dormancy and desiccation tolerance (Delmas et al., 2013). However, there remains a possibility that *SGR* might partially influence seed dormancy (Fig. 2) because the *sgr2-2* and *sgr1-1 sgr2-2* mutant seeds exhibit significantly higher germination in the presence of 2 μM ABA compared to the performance of the *sgr1-1* mutant and wild-type seeds under the same condition (Delmas et al., 2013). These results suggest reduced ABA sensitivity in the *sgr2* (and related) mutant seeds (ABA sensitivity will be discussed more in the DORMANCY section.)

The function of SGR2 in chlorophyll degradation seems to be more specific to seeds compared to the SGR1 function since misexpression of *SGR2* in the *abi3-6* mutant specifically restores embryo de-greening while *SGR1* expression causes pleiotropic shoot phenotypes also (Delmas et al., 2013). The reduction in ABA sensitivity in the *sgr2-2* and *sgr1-1 sgr2-2* mutant seeds may not be a consequence of chlorophyll retention because the chlorophyll levels in the *sgr2-2* seeds is similar to those found in wild-type and the *sgr1-1* seeds, although the *sgr1-1 sgr2-2* seeds contain a significantly higher level of chlorophyll compared to wild-type and the *sgr* single mutant seeds (Delmas et al., 2013). The chloroplast localized Mg-chelatase H subunit (CHLH) is involved in ABA responses, however, its action is also independent of

chlorophyll biosynthesis (Wu et al., 2009). It is not clear how SGR2 affects ABA sensitivity of the mature seeds.

The residual chlorophyll in seeds negatively affects seed quality, including oil quality, germination and storability of seeds (Cicero, Schoor, & Jalink, 2009; Jalink, Frandas, van der Schoor, & Bino, 1998a; Jalink, van der Schoor, Frandas, van Pijlen, & Bino, 1998b; Li et al., 2017; Nakajima, Ito, Tanaka, & Tanaka, 2012). Therefore, it is economically important to address the defects in the de-greening process in agricultural species, such as soybean (*Glycine max*) and rapeseed (*Brassica napus*). Frost could result in the production of green seeds in agricultural species, which can be mimicked in Arabidopsis by exposing the maturing seeds to sublethal frost. Freeze-induced green seeds in Arabidopsis can be rescued by overexpressing *ABI3* (Delmas et al., 2013), confirming the significance of ABA signaling in embryo de-greening during seed maturation. Thus, understanding the mechanisms of ABA signaling for embryo de-greening during seed maturation has great potential for applied science as well.

2.3 ABA levels during seed development

While this article focuses on ABA responses, it is probably essential to touch on ABA biosynthesis and catabolism in developing seeds here for a full understanding of ABA responses in seeds. In Arabidopsis, ABA increases during seed development with two distinct peaks around 9−10 DAF and 15−16 DAF (Gazzarrini, Tsuchiya, Lumba, Okamoto, & McCourt, 2004; Kanno et al., 2010; Karssen, Brinkhorst-van der Swan, Breekland, & Koornneef, 1983; Okamoto et al., 2006; Takeuchi et al., 2014), which approximately correspond to the timings of embryo greening and de-greening, respectively (Fig. 1A). The pioneering study, using reciprocal crosses between the ABA-deficient mutant (*aba2*) and wildtype, suggested that the first peak of ABA was primarily originated from the maternal plant while the relatively small second peak of ABA (Fig. 1A) was of zygotic origin, which was thought to play a critical role for seed dormancy (Karssen et al., 1983).

However, ABA quantification in the initial studies was performed using the siliques containing developing seeds in most cases. More accurate analysis, for which developing seeds were separated from the silique envelop (including the pedicels, receptacles, valves, replums, septa and funiculi) (Fig. 3), indicated that most of the ABA detected in the whole siliques at the first peak was present in seeds and that the majority of ABA at the second peak was attributed to the silique ABA (Kanno et al., 2010) (Fig. 1A). Thus,

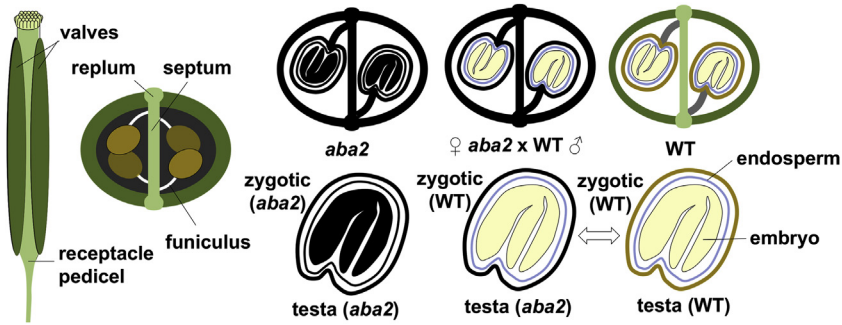

Fig. 3 ***The maternal and zygotic ABA in wild-type and mutant seeds.*** Separation of seeds and the silique tissues (funiculus, septum, valves, receptacle and pedicel) does not necessarily separate the maternal and zygotic tissues because seeds contain the testa, a maternal tissue originated from the integuments. To examine the contribution of the maternal ABA in the testa to the ABA levels in seeds, the ABA-deficient mutant (♀ *aba2*) was crossed with wild-type (♂ WT), which produced the F-1 seeds with the mutant testa and WT zygotic tissues (*aba2*/WT heterozygous endosperm and embryo). The ABA levels in the mutant seeds were comparable (open double headed arrow) to those in WT seeds, suggesting that ABA detected in seeds is mostly attributed to the zygotic tissues. *See* text for details. *Based on Kanno et al. (2010).*

the seed-focused, precise quantification of ABA seems to have revised the traditional view of the roles of ABA from the maternal and zygotic origins. However, the occurrence of ABA in seeds does not necessarily specify its origin because the hormone can be transported from the silique to the seed tissues. Besides, extracting seeds from the siliques does not allow complete separation of the zygotic and maternal tissues because seeds still contain the testa, a maternal tissue that originates from the integuments and affects seed dormancy characteristics genetically (e.g. gene expression in the testa) and physiologically (e.g. chemical transport through, or retention in, the testa).

To address this issue, heterogeneous seeds carrying wild-type endosperm and embryo with the ABA-deficient testa were produced on the *aba2* plants crossed with wild-type pollen (Fig. 3) and were subjected to ABA quantification. The results of the analysis indicated that the ABA content in the F-1 seeds with the mutant testa and wild-type zygotic tissues (endosperm and embryo) was comparable to that detected in wild-type seeds (Kanno et al., 2010) (Fig. 3), suggesting that the ABA deficiency in the maternal plant, including the testa, does not significantly affect ABA levels in seeds. Therefore, ABA in seeds is mainly attributed to that of zygotic origin. It should still be noted, however, that the maternal ABA is translocated to

the *aba2* embryo (in F-2) in which zygotic ABA is missing (Kanno et al., 2010). More research seems to be necessary to clarify the importance of the maternal vs. zygotic origin of ABA that accumulates in seeds.

The expression of the rate-limiting ABA biosynthesis genes *nine-cis-epoxycarotenoid dioxygenase 6* (*NCED6*) *and NCED9* during the mid seed development coincides with the first peak of ABA while *NCED2* and *NCED3* are expressed around the second peak (Okamoto et al., 2006) (Fig. 1A). *NCED6* is expressed mainly in the endosperm while *NCED9* is expressed in the testa/endosperm layer and the peripheral layers of the embryo (Frey et al., 2012; Lefebvre et al., 2006). The *nced6 nced9* double mutant seeds exhibit reduced dormancy (Lefebvre et al., 2006), suggesting that the expression of *NCED6* and *NCED9* in developing seeds is a critical part of the dormancy mechanisms. Residual dormancy is still observed in the *nced6 nced9* mutant seeds (Lefebvre et al., 2006), which is significantly reduced in the *nced5 nced6 nced9* triple mutant (Frey et al., 2012). Thus, these three *NCEDs* function redundantly in the seed dormancy mechanisms. The exact timing of *NCED5* expression is not clear, however, it seems to start around the first peak and increase toward the second peak (Frey et al., 2012).

NCED2 and *NCED3* are expressed during late seed development mainly in the siliques (Kanno et al., 2010; Okamoto et al., 2006) (Fig. 1A), which is consistent with ABA being detected predominantly in the silique tissues during the second peak of ABA (Okamoto et al., 2006). *NCED3* is a typical *NCED* responsible for ABA biosynthesis in leaves upon drought stress (Iuchi et al., 2001). The expression of *NCED3* (and *NCED2*) in the siliques might mimic their induction in leaves during drought responses because the silique is made of the two fused carpels (unit leaves), which go through maturation drying, although maturation drying is a spontaneous event and part of the developmental program of seeds, unlike occasional drought.

Hormonal levels are determined by the balance of biosynthesis and catabolism. The expression of *CYP707A1*, a gene encoding for the ABA catabolism enzyme 8′-hydroxylase, accounts for the decline of ABA after the first peak. This role of *CYP707A1* is taken over by *CYP707A2* toward the end of seed maturation (Fig. 1A). In the mature Arabidopsis seeds, the expression of *CYP707A2* approximately 6 h after the start of imbibition is an important determinant of dormancy release. The *cyp707a2* mutant seeds, which lack ABA catabolism during this window, exhibit profoundly deep dormancy (Kushiro et al., 2004). In contrast, mutations in *CYP707A1* does not significantly affect ABA catabolism during imbibition, highlighting

the predominant role of *CYP707A2* in imbibed seeds (and late maturation) (discussed more in the GERMINATION section).

However, the *cyp707A1* mutant seeds still exhibit enhanced dormancy (Okamoto et al., 2006). Therefore, the expression of *CYP707A1* during seed development, which causes the decline in ABA levels from the first peak during seed development (Fig. 1A), is also an essential part of seed dormancy control. The physiological role of *CYP707A1* expression around 10 DAF is probably to limit the depth of dormancy in the mature seeds at a certain level. This role of *CYP707A1* during seed development is interesting because the depth of dormancy in imbibed seeds is controlled by the ABA levels during imbibition, which means that the ABA levels during mid seed development could determine the ABA levels (to be synthesized and catabolized) in the mature seeds upon imbibition. It is well known that germination of the mature seeds is controlled by the environmental conditions that the maternal plants (and seeds) experience during the developmental stages (Cumming, 1963; Evenari, Koller, & Gutterman, 1966; Gutterman, 1978). Therefore, it is possible that *CYP707A1* takes an important part in recording the history of seed development to establish the developmental *"memory"* in the mature seeds. It is interesting that the ABA levels (and signaling?) during seed development could determine the rate of ABA synthesis and catabolism in the mature seeds upon rehydration, although this hypothesis still needs to be supported by more evidence, and even when it is valid, the cryptic mechanisms still need to be fully explained. Further analyses of the expression of *CYP707A1* during seed development under various environmental conditions and its consequences in the context of dormancy levels in the harvested seeds will probably address this hypothesis, which will also be informative for seed production in agriculture. The predominant role of *CYP707A2* in ABA catabolism in imbibed seeds is obvious and often emphasized for dormancy release, however, the importance of *CYP707A1* expression during seed development as a determinant of dormancy levels should not be underestimated.

3. Seed dormancy

Seeds become capable of germinating during development. The germinability of developing seeds reaches the peak at a certain DAF, which depends on species, and then declines thereafter (Sidhu & Cavers, 1977). This decline of germinability during seed development reflects the induction

of dormancy (Bewley et al., 2013). Dormant seeds do not germinate even when a suitable condition for germination is provided. Dormancy in the mature seeds will be released over time (generally weeks to months), which is called after-ripening (Bewley et al., 2013; Brown, 1939). The mechanisms of dormancy and after-ripening have been elusive for a long period of time. Nonetheless, there were significant breakthroughs in recent seed dormancy research, which shed light on the novel mechanisms of ABA signaling. In this section, the function of *DELAY OF GERMINATION1* (*DOG1*), which is a master regulator of seed dormancy and a novel component of ABA signaling in seeds, will be focused. Irregular ABA signaling in seeds that employs a unique protein phosphatase 2C (PP2C) in the DOG1 pathway will be highlighted for the mechanisms of seed dormancy and after-ripening. The regulatory mechanisms of *DOG1* expression, which are also relevant to ABA signaling in drought responses of plants and predict a potentially broader role of *DOG1* in the biology of plants, will also be discussed intensively.

3.1 DOG1 and seed dormancy

The early studies of Arabidopsis research using ABA-deficient and -insensitive mutants had already highlighted the importance of ABA for the regulation of seed germination and dormancy (Koornneef et al., 1989; Nambara, Akazawa, & McCourt, 1991), which could have been applied to agricultural crops to prevent unwanted germination, such as preharvest sprouting (PHS). However, the pioneers were concerned that altering ABA levels or signaling in plants could cause unwanted phenotypes in plants, due to pleiotropic effects. Therefore, they believed that dormancy-specific genes would have to be identified. Seed dormancy-specific genes that are devoid of hormone or pleiotropic effects were sought for based on this concept. The most prominent and successful case of this type of study is probably the identification of *DOGs* (Alonso-Blanco, Bentsink, Hanhart, Blankestijn-de Vries, & Koornneef, 2003), although some of them turned out to be associated with ABA signaling and not to be specific to dormancy in a strict sense (discussed below).

DOGs were identified by taking advantage of natural variation of seed dormancy between different accessions of Arabidopsis and by using the introgression or near isogenic lines (NILs) created from them. The analyses of the recombinant inbred line (RIL) population, which was generated from a cross between a weakly dormant Landsberg *erecta* (L*er*) and deeply dormant Cape Verde Islands (Cvi), identified multiple seed dormancy-associated

QTLs termed *DOGs* (Alonso-Blanco et al., 2003). *DOG1* that is the best characterized *DOG* gene to date was found from a NIL containing a Cvi introgression at the position of a dormancy QTL in a L*er* genetic background. Seeds of the L*er* that contains *DOG1-Cvi* (originally termed NIL-DOG17−1; now called NILDOG1) (Alonso-Blanco et al., 2003; Bentsink et al., 2006, 2010) are more dormant than wild-type L*er* seeds. The *dog1* mutation in Columbia-0 (Col), which has a 1-bp deletion at position 914 and produces a truncated protein (due to frameshift and a premature stop codon), results in non-dormancy phenotypes. Unlike hormone-deficient and -insensitive mutants, the *dog1* mutant does not exhibit obvious phenotypes in plant growth under normal conditions (Bentsink et al., 2006), suggesting that *DOG1* is a seed dormancy-specific gene (exceptions discussed below). Transformation of the Col *dog-1* mutant with a 5.6 kb fragment of the Cvi allele complements the mutation and recovers dormancy in the Col mutant (Bentsink et al., 2006). Taken together, *DOG1* is a master regulator of seed dormancy.

DOG1 is expressed mainly in developing seeds around 14−16 days after pollination (Bentsink et al., 2006), suggesting its involvement in the induction of dormancy during seed maturation (Fig. 1A). While the mature Arabidopsis seeds still contain significant amount of the *DOG1* transcripts (approximately 80% compared to the peak expression), it is reduced during imbibition even when dormancy is still observed (Nakabayashi et al., 2012). In contrast, DOG1 protein accumulates during seed maturation and remains stable during imbibition. It has been proposed that the DOG1 protein serves as a "timer" for after-ripening in the mature seeds (Nakabayashi et al., 2012). However, there are some occasions where the accumulation of the DOG1 protein is not consistent with dormancy levels. For instance, a relatively high level of DOG1 protein, which typically occurs in the freshly harvested dormant seeds, can still be found in the 13-week-old, after-ripened seeds, in which dormancy has already been alleviated (Nakabayashi et al., 2012). Therefore, it is likely that the function of DOG1 protein is regulated by the alteration of its biochemical property by post-translational modification(s), rather than the rate of *de novo* synthesis or destabilization of the protein. The changes in the isoelectric point (pI) of the DOG1 peptide are observed during after-ripening (Nakabayashi et al., 2012), which suggests that the posttranslational modification (i.e. inactivation) of this protein is involved in dormancy release. However, the nature of DOG1 modification (e.g. phosphorylation/de-phosphorylation, redox changes, etc.) in dry or imbibed seeds has not been demonstrated explicitly.

DOG1 was discovered from the efforts to isolate dormancy-specific genes that are independent of the hormonal pathway, especially from the ABA-dependent dormancy pathway, as discussed above. However, there were some observations to imply that the *DOG1* pathway interacts with the ABA pathway of dormancy. The most critical evidence that highlights this possibility is that the integration of NILDOG1 with the ABA-deficient mutant (*aba1-1*), which does not exhibit seed dormancy, still results in the production of non-dormant seeds (Bentsink et al., 2006). This finding suggests that ABA is indispensable for the DOG1 function in seed dormancy, although it is fair to remind that ABA cannot impose seed dormancy in the absence of *DOG1* (Bentsink et al., 2006). Thus, ABA and DOG1 seemed to present the two separate pathways of dormancy, which are somehow interdependent on each other.

3.2 DOG1 and ABA signaling

The enigmatic mechanisms of seed dormancy, which were represented by the DOG1 and ABA pathways, had left many puzzling questions in seed dormancy research over a decade. The long-awaited question to be answered was the biochemical function of DOG1. DOG1, a plant-specific protein, was predicted to be a regulatory protein based on its localization in the nucleus (Nakabayashi et al., 2012, 2015). The *DOG1* cDNA showed a similarity to a *B. napus* EST from an embryo library, however, it was an unannotated gene and therefore did not provide a clue for DOG1 function (Bentsink et al., 2006). While the wheat transcription factor Histone gene Binding Protein-1b (HBP-1b) also showed a similarity to DOG1, the sequence similarity between the two genes was very limited and hardly predicted DOG1 function (Bentsink et al., 2006; Nonogaki, 2014). There were some reports about dormancy regulation by DOG1 through flowering mechanisms (Huo, Wei, & Bradford, 2016) or endosperm weakening (Graeber et al., 2014) and its involvement in dormancy cycling (Footitt, Douterelo-Soler, Clay, & Finch-Savage, 2011). However, these studies did not address the crucial question about biochemical function of DOG1 and the exact link between the ABA and DOG1 pathways of seed dormancy.

A breakthrough came from recent investigation for DOG1-interacting proteins, which finally shed light on the merging point of the DOG1 and ABA pathways. Two research groups independently identified ABA HYPERSENSITIVE GERMINATION1 (AHG1) and AHG3 as DOG1-interacting proteins (Née et al., 2017; Nishimura et al., 2018).

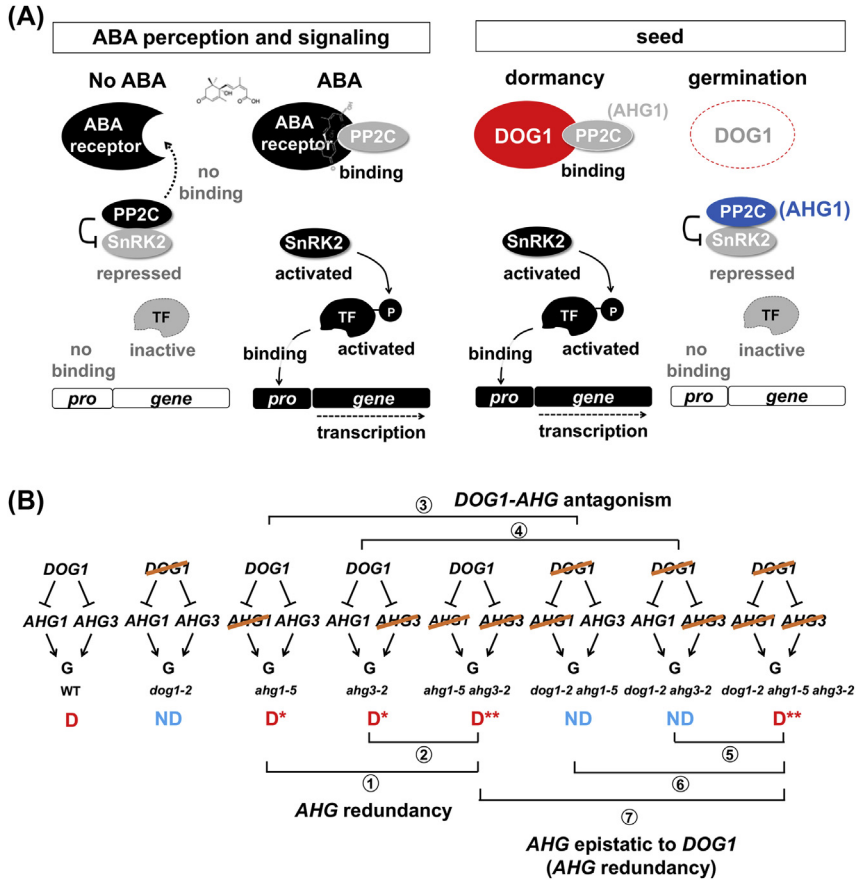

Fig. 4 ***ABA signaling in seed dormancy and germination.*** (A) General ABA pathway in plants and unique ABA signaling in seeds. *Left,* ABA perception and signaling, which is normally observed in plants. Upon ABA perception, the receptor changes its conformation and binds to protein phosphatase 2C (PP2C), which results in the inhibition of PP2C activity. In this way, the original suppression of SNF1-related protein kinase 2 (SnRK2) by PP2C is eliminated. The de-repressed kinase then phosphorylates (P) the transcription factor (TF) downstream, which in turn binds to the promoter (*pro*) region of the target genes to induce expression. *Right*, unique ABA signaling in seeds. ABA HYPERSENSITIVE GERMINATION1 (AHG1) is also a PP2C involved in ABA signaling. However, AHG1 is resistant to the inhibition by ABA receptor and still capable of suppressing the kinase in the presence of ABA, which could maintain the germination program still operative even in the presence of high levels of ABA in developing seeds. DELAY OF GERMINATION1 (DOG1) physically interacts with and inactivates AHG1. The sequestration of AHG1 by DOG1 establishes the dormant state of seeds. *See* text for details. (B) Genetic analysis of *DOG1* and *AHGs*. Seed dormancy phenotypes of wild-type (WT) and the mutants lacking *DOG1*, *AHG1* and/or *AHG3* are shown as: no dormancy (ND), dormancy (D), enhanced dormancy (D*), and further enhanced dormancy (D**) in

AHG1 (Nishimura et al., 2004, 2007) and AHG3 (Nishimura et al., 2004; Yoshida et al., 2006) are PP2Cs, which are involved in ABA signal transduction. Therefore, the possible link of DOG1 to the ABA pathway, which had been implied from the genetic studies (i.e. NILDOG1 in *aba1-1* discussed above), has now been clearly demonstrated.

In the general ABA perception and signaling pathway, ABA is received by its receptor, such as PYRABACTIN RESISTANCE1 (PYR1) or PYR1-LIKEs (PYLs), which changes its conformation and makes the pocket where the ABA INSENSITIVE1 (ABI1) subfamily PP2Cs, including ABI1, ABI2, HYPERSENSITIVE TO ABI1 (HAB1) and HAB2, bind (Cutler et al., 2010; Ma et al., 2009; Park et al., 2009) (Fig. 4A). The ABA-bound receptor inhibits PP2C activity through this binding. In this way, the original repression of SNF1-related protein kinase 2 (SnRK2) by PP2C is eliminated, which de-represses and activates the kinase (Fig. 4A). The active kinase then targets the downstream transcription factors, such as ABRE (ABA responsive element)-binding factors (ABFs), to phosphorylate them. The activated transcription factors in turn bind to ABRE in the promoter regions of the target genes, the consequence of which is the activation of the downstream flow in the ABA signaling cascades (Nakashima & Yamaguchi-Shinozaki, 2013) (Fig. 4A).

AHG1 and AHG3 are also PP2Cs involved in the ABA signaling pathway (Nishimura et al., 2004, 2007; Yoshida et al., 2006). Both of them are expressed in seeds, with AHG1 being more specific to seeds than AHG3, according to the eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). AHG1, particularly, has an irregular and interesting feature as a PP2C involved in ABA signaling. As mentioned above, the biochemical event that is central to ABA perception and signaling is the inhibition of PP2C activity by the receptor (binding) upon its perception of ABA, which de-represses the kinase from the PP2C (Fig. 4A). When the kinase is co-incubated with PP2C in in-vitro experiments,

double mutants. The comparisons of the mutant phenotypes highlight the redundancy between AHG1 and AHG3 (①, ②), the antagonistic function between AHGs and DOG1 (③,④), and AHG epistatis to DOG1 (and AHG redundancy) (⑤, ⑥, ⑦). *See* text for details. G, germination. *(A) Modified from Nonogaki (2019). Based on Antoni et al. (2012), Cutler et al. (2010), Ma et al. (2009), Nakabayashi et al. (2012), Nakabayashi et al. (2015), Nakashima and Yamaguchi-Shinozaki (2013), Née et al. (2017), Nishimura et al. (2004), Nishimura et al. (2007), Nishimura et al. (2018), Park et al. (2009), and Yoshida et al. (2006). (B) Based on Née et al. (2017) and Nishimura et al. (2018).*

autophosphorylation of the kinase is repressed by PP2C while this inhibitory effect of PP2C on the kinase is abolished by the addition of the ABA receptor together with ABA to the same reaction, resulting in autophosphorylation of the kinase (Antoni et al., 2012). Intriguingly, co-incubation of the kinase, the ABA receptor and ABA with AHG1 still prevents autophosphorylation of the kinase (Antoni et al., 2012), suggesting that AHG1 (PP2C activity) is resistant to the ABA receptor and still capable of repressing the kinase (Fig. 4A) even in the presence of ABA. This feature of AHG1 presents a remarkable exception to the well-known ABA signal transduction pathway and indicates that there is a sideway to block the ABA signaling cascades even when ABA is received by the receptor.

From a seed biology point of view, this irregular nature of AHG1 as an ABA signaling component is even more interesting since its resistance to the ABA receptor means that the seed germination program (an ABA-sensitive process otherwise) can still operate even in the presence of high levels of ABA (which is the case in developing seeds). In other words, the AHG1 window has to be completely shut down for the induction and maintenance of seed dormancy. The major biological role of DOG1 is probably to shut down the AHG1 (and AHG3) pathways. In fact, DOG1 inhibits PP2C activity of AHG1 in *in-vitro* experiments (Nishimura et al., 2018). There is a conflicting observation in a separate study that AHG1 still exhibited PP2C activity when it was co-incubated with DOG1, however, this could be due to some factors missing in *in-vitro* experiments (Née et al., 2017) or other unknown reasons. The physical interaction of DOG1 with AHG1 and the inactivation of its PP2C activity suggest that DOG1 sequestrates AHG1 and induces dormancy. Taken together, DOG1 can be considered a *"watchdog"* to arrest the ABA escapee (AHG1) from the remaining window of germination to ensure the dormant status of seeds.

The discoveries of the biochemical function of the DOG1 protein are well-supported by the genetic analyses of *AHG1*, *AHG3* and *DOG1* using the corresponding mutants with seed germination phenotypes. *AHG1* and *AHG3* are positive regulators of seed germination. As their names indicate, seeds of the loss-of-function mutants (*ahg1-1* or *ahg3-1*) become hypersensitive to ABA (Nishimura et al., 2004, 2007; Yoshida et al., 2006) and exhibit enhanced dormancy compared to wild-type seeds (Née et al., 2017). The enhanced dormancy phenotypes of the single mutants (*ahg1-5* or *ahg3-2*) are intensified in the double mutants (*ahg1-5 ahg3-2*) (Née et al., 2017), which is also observed in different combinations of double mutants (*ahg1-1 ahg3-1*) (Nishimura et al., 2018). These results suggest

that *AHG1* and *AHG3* function redundantly in the seed dormancy pathway (Fig. 4B). The enhanced dormancy observed in the *ahg1* (*ahg1-5*) and *ahg3* (*ahg3-2*) single mutants is completely removed when they are integrated with the *dog1* (non-dormancy) mutation (*dog1-2 ahg1-5* or *dog1-2 ahg3-2*) (Née et al., 2017), demonstrating that *DOG1* counteracts *AHG1* and *AHG3* (Fig. 4B). In contrast, seeds of the triple mutant (*dog1-2 ahg1-5 ahg3-2*) are still highly dormant as those of the *ahg* double mutant (*ahg1-5 ahg3-2*) (Née et al., 2017) (Fig. 4B). These results suggest that *DOG1* requires *AHG1* and *AHG3* for its function and that *AHG1* and *AHG3* act redundantly downstream of *DOG1*. Thus, genetic data support the idea that the DOG1 protein sequestrates the AHG1 and AHG3 PP2Cs to establish seed dormancy.

3.3 *DOG1* regulation

DOG1 was known to be expressed predominantly in seeds (Bentsink et al., 2006; Cyrek et al., 2016; Nakabayashi et al., 2015). However, its expression and involvement in drought responses of Arabidopsis plants have also been discovered (Yatusevich et al., 2017). This finding suggests that the irregular ABA signaling pathway that was found in seed dormancy is not limited to seeds but also takes part in ABA signaling in Arabidopsis plants. To examine the broader function of *DOG1* outside seeds, it is critical to understand the complex regulatory mechanisms of *DOG1* expression in seeds, including alternative-splicing and alternative-polyadenylation site selection (Cyrek et al., 2016; Dolata et al., 2015; Fedak et al., 2016; Nakabayashi et al., 2015). Therefore, discussion here will initially focus on the biogenesis of *DOG1* mRNA in seeds and then will be extended through a possible involvement of the DOG1-dependent ABA signaling in Arabidopsis plants during drought responses.

3.4 The biogenesis of *DOG1* RNA in seeds

The *DOG1* gene produces five transcript variants (α, β, γ, δ, ε) through alternative splicing (Nakabayashi et al., 2015) and other mechanisms (see alternative polyadenylation below), with *DOG1-ε* producing the predominant form of active protein (Fig. 5). The details of alternative splicing of *DOG1* have been summarized in the previous review (Nonogaki, 2017). *DOG1* expression eventually results in the production of *short DOG1* (*shDOG1*; identical to *DOG1-ε*) or *long DOG1* (*lgDOG1*), through alternative polyadenylation (Di Giammartino, Nishida, & Manley, 2011), which selects the proximal transcription termination site (pTTS) or the distal

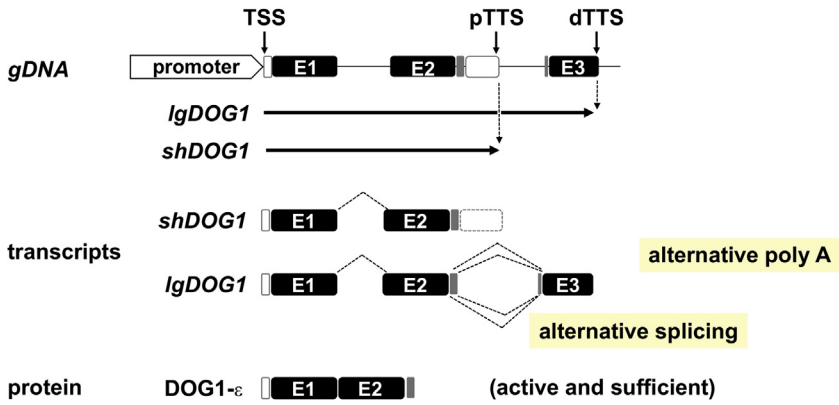

Fig. 5 ***The biogenesis of*** **DELAY OF GERMINATION1** ***mRNA.*** The *DOG1* gene produces five transcript variants (*α, β, γ, δ, ε*) through alternative splicing (and alternative polyadenylation; *see* below), from which DOG1-ε is produced as the predominant form of active protein. Alternative polyadenylation generates essentially the two major forms of mRNA, *short DOG1* (*shDOG1*; identical to *DOG1-ε*) and *long DOG1* (*lgDOG1*) through the selection of the proximal transcription termination site (pTTS) and the distal TTS (dTTS), respectively. The protein encoded by *shDOG1* (DOG1-ε) is sufficient for seed dormancy. E, exon; TSS, transcription start site. *Modified from Nonogaki (2017). Based on Bentsink et al. (2006), Cyrek et al. (2016), Di Giammartino et al. (2011), Fedak et al. (2016), and Nakabayashi et al. (2015).*

TTS (dTTS), respectively (Cyrek et al., 2016) (Fig. 5). The extended C-terminal region of the DOG1 protein that is translated only from the *lgDOG1* mRNA (Fig. 5) is not conserved or completely absent in the DOG1 proteins in various species (Cyrek et al., 2016; Fedak et al., 2016), suggesting that this region of lgDOG1 is dispensable for the biochemical function of the DOG1 protein. *shDOG1* is capable of complementing the *dog1-3* mutation and recovers seed dormancy in the non-dormant mutant (Cyrek et al., 2016), which suggests that *shDOG1* is sufficient also for the biological function of DOG1 in seed dormancy. Thus, selective expression of *shDOG1* or *lgDOG1* does not differentiate protein function itself.

3.5 Regulation of *DOG1* by antisense *DOG1*

The DNA sequence of the extended 3′ end of *lgDOG1*, however, is highly conserved in various species, despite the random occurrence of the extended C-termini in the DOG1 proteins in various species, or low conservation of amino acid sequences in the cases where they are expressed (Cyrek et al., 2016; Fedak et al., 2016). The low evolutionary pressure on the amino

acid sequences and high conservation of the DNA sequences in the extended region of *lgDOG1* predict the expression of functional long non-coding RNA (lncRNA) from this region or the vicinity. Indeed, *antisense DOG1* (*asDOG1*) is expressed from the pTTS region, with the extended region serving as a promoter in an antisense orientation (Fig. 6). Seeds of the *dog1* mutants, such as *dog1-1*, *dog1-3*, and *dog1-4* exhibit reduced dormancy, confirming the importance of *DOG1* in dormancy. In contrast, the *dog1-5* T-DNA insertion mutation, which disrupts the extended 3′ end of *lgDOG1* and reduces *asDOG1* expression, enhances *DOG1* expression and seed dormancy (Fedak et al., 2016) (Fig. 6). These results suggest that *asDOG1* is a negative regulator of *DOG1* and seed dormancy, and that the genomic region corresponding to the 3′ end of *lgDOG1* is crucial for *DOG1* regulation.

It is known that the expression of lncRNA from part of a gene could silence its own transcription, with the lncRNA serving as a functional RNA. LncRNA could interact with short interfering RNA (siRNA) on ARGONAUTE4 (AGO4) and cause histone and DNA methylation to silence genes (Jackson, Lindroth, Cao, & Jacobsen, 2002; Nonogaki, 2014; Qi et al., 2006; Wierzbicki, 2012; Wierzbicki et al., 2008, 2009; Zilberman et al., 2004). Alternatively, lncRNA could bind to and/or stimulate Polycomb Repressive Complex 2 (PRC2) to trigger histone 3 lysine

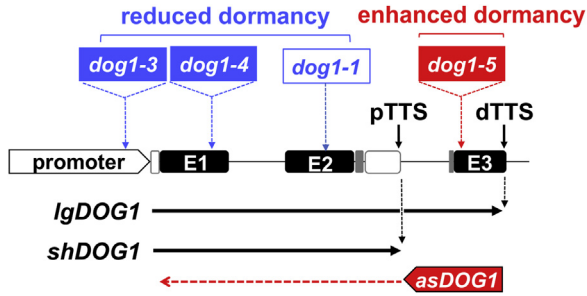

Fig. 6 ***Expression and function of*** **antisense DOG1.** *Antisense DOG1* (*asDOG1*) is expressed from the genomic region corresponding to the proximal transcription termination site (pTTS) of *short DOG1* (*shDOG1*). The genomic region corresponding to the extended 3′ end of *long DOG1* (*lgDOG1*) through the distal TTS (dTTS) contains the promoter in an antisense orientation. The *dog1-5* mutant with a T-DNA insertion in the *asDOG1* promoter region exhibits enhanced seed dormancy, unlike other *dog1* mutants (*dog1-1*, *dog1-3*, *dog1-4*) that show reduced seed dormancy, suggesting that *asDOG1* expression negatively affects *DOG1* expression and dormancy. E, exon. *Modified from Nonogaki (2017). Based on Cyrek et al. (2016) and Fedak et al. (2016).*

27 trimethylation (H3K27me3) (Davidovich, Zheng, Goodrich, & Cech, 2013; De Lucia & Dean, 2011; Heo & Sung, 2011; Simon & Kingston, 2009; Swiezewski, Liu, Magusin, & Dean, 2009; Wu, Murat, Matak-Vinkovic, Murrell, & Balasubramanian, 2013) (Fig. 7) and the subsequent monoubiquitination of H2A, which results in gene silencing (Simon & Kingston, 2009). It is possible that these *trans*-regulation mechanisms caused by functional RNA trigger *DOG1* silencing (Bouyer et al., 2011; Molitor, Bu, Yu, & Shen, 2014; Nonogaki, 2014) (Fig. 7). However, evidence has not been obtained to indicate that *asDOG1* actually functions through the *trans*-regulation mechanisms.

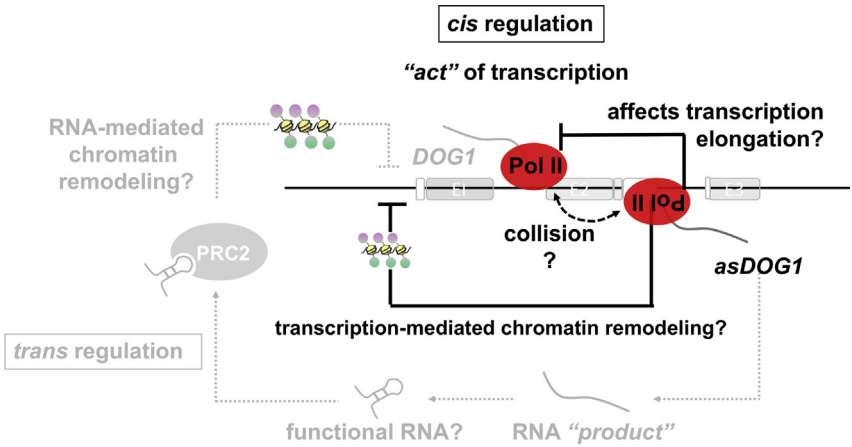

Fig. 7 ***Possible mechanisms of the negative regulation of*** **DOG1** ***expression by*** **antisense DOG1** ***transcription.*** The expression of *antisense DOG1* (*asDOG1*) negatively affects *DOG1* expression and dormancy (Fig. 6). It is known that long non-coding RNA (lncRNA) expressed from a gene silences its own transcription, which is mediated by chromatin remodeling, such as histone 3 lysine 27 trimethylation (H3K27me3) by Polycomb Repressive Complex 2 (PRC2). In this scenario, the *"product"* of transcription lncRNA binds to PRC2 and/or stimulates chromatin remodeling to silence the gene (called *trans* regulation). However, genetic analysis with allele-specific *asDOG1* expression suggests that *asDOG1* does not function in this *trans* regulation but does act in *cis*, which means that the *"act"* of *asDOG1* transcription itself, rather than its RNA products, directly affects *DOG1* expression in *cis*. While the mechanisms of the *cis* regulation are not known, it is possible that *asDOG1* expression affects transcription elongation of *DOG1* through transcription interference due to RNA polymerase II (Pol II) collision or promoter competition while transcription-mediated chromatin remodeling is also possible. See text for details. *Modified from Nonogaki (2017). Based on Fedak et al. (2016), Geisler and Coller (2013), Hongay et al. (2006), Kornienko et al. (2013), Pelechano and Steinmetz (2013), Quinn and Chang (2016), and Shearwin et al. (2005).*

The genetic analysis using allele-specific *asDOG1* expression suggests that *asDOG1* does not exert its effects on *DOG1* through *trans*-regulation but does function through *cis*-regulation (Fedak et al., 2016) (Fig. 7). That is, the *"act"* of transcription itself (Kornienko, Guenzl, Barlow, & Pauler, 2013; Pelechano & Steinmetz, 2013), rather than its product (RNA), is thought to be the primary cause of the negative regulation of *DOG1* expression and seed dormancy by *asDOG1*. This process is still largely unknown. However, it is possible that co-transcription of sense and antisense strands causes transcriptional interference due to direct collision of RNA polymerases or promoter competition (Pelechano & Steinmetz, 2013; Quinn & Chang, 2016; Shearwin, Callen, & Egan, 2005) (Fig. 7). Transcription elongation, which is critical for the efficiency of gene expression (discussed below) and also known to be important for the *DOG1* function in seed dormancy (Liu et al., 2007, 2011; Mortensen & Grasser, 2014; Nonogaki, 2014), could also be disturbed by the antisense-mediated transcriptional interference (Hongay, Grisafi, Galitski, & Fink, 2006; Pelechano & Steinmetz, 2013) (Fig. 7). In any event, it is interesting that the *"act"* of transcription of *asDOG1* regulates *DOG1* expression to affect seed dormancy negatively. This mechanism adds a new dimension to *DOG1* regulation and ABA signaling (*asDOG1* regulation by ABA discussed below).

3.6 Biological significance of alternative polyadenylation

The presence of the antisense promoter in the *DOG1* genomic region, which corresponds to the extended 3′ end of *lgDOG1* (Fig. 6), highlighted the role of this region in *DOG1* expression and explained the conservation of the DNA sequences in various species over the course of evolution. However, the expression of *asDOG1* does not still explain the biological significance of differential expression of *shDOG1* and *lgDOG1* in the control of seed dormancy. Another important question is the mechanistic significance of differential expression of the two forms of *DOG1* mRNA. Does alternative polyadenylation site selection have anything to do with *asDOG1* expression? These questions need to be answered to achieve a better understanding of *DOG1* regulation for seed dormancy (and ABA signaling in drought responses of plants discussed below).

Information about the biological significance of *shDOG1* and *lgDOG1* is emerging. The selection of pTTS or dTTS by alternative polyadenylation differentiates *shDOG1* or *lgDOG1* (Fig. 8). There are mutants, which cause the preferential selection of pTTS or dTTS in *DOG1* expression. A mutation in *C-terminal domain* (*CTD*) *phosphatase-like 1* (*CPL1*)

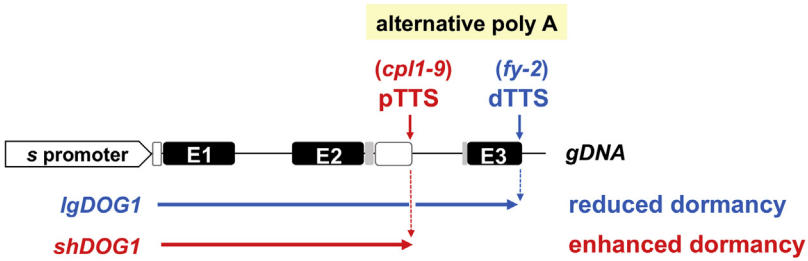

Fig. 8 ***The biological significance of alternative polyadenylation of* DOG1 *in seed dormancy.*** The selection of the proximal transcription termination site (pTTS) results in the expression of *short DOG1* (*shDOG1*) while the expression through the distal TTS (dTTS) produces *long DOG1* (*lgDOG1*). The *c-terminal domain phosphatase-like 1–9* (*cpl1-9*) mutant, which preferentially selects pTTS and expresses *shDOG1*, exhibits enhanced seed dormancy. In contrast, the flowering time control mutant *fy-2*, in which dTTS is selected and *lgDOG1* is predominantly expressed, shows reduced seed dormancy. These results indicate that alternative polyadenylation at pTTS (*shDOG1*) or dTTS (*lgDOG1*) exerts positive or negative effect on seed dormancy, respectively. E, exon; *s* promoter, sense promoter. *Modified from Nonogaki (2019). Based on Cyrek et al. (2016), Fedak et al. (2016), and Kowalczyk et al. (2017).*

(Koiwa et al., 2004) causes the preferential selection of pTTS and *shDOG1* expression. The *shDOG1*-enriched *cpl1* mutant (*cpl1-8*, *cpl1-9*) seeds exhibit enhanced dormancy (Kowalczyk et al., 2017) (Fig. 8). To the contrary, the preferential selection of dTTS and preferential expression of *lgDOG1* are observed in the flowering time control mutant *fy-2*, which has a defect in a component of the Cleavage and Polyadenylation Specificity Factor (CPSF, an Arabidopsis ortholog of the yeast RNA 3′ processing factor Pfs2p) (Simpson, Dijkwel, Quesada, Henderson, & Dean, 2003). The *lgDOG1*-enriched *fy-2* mutant shows reduced seed dormancy (Kowalczyk et al., 2017) (Fig. 8). Thus, alternative polyadenylation at pTTS or dTTS, which determines the predominant form of *DOG1*, has a significant biological role in seed dormancy. Although the shDOG1 protein (or DOG1-ε) was described to be sufficient for dormancy in the discussion above, *shDOG1* expression is actually more important than *lgDOG1* expression, or even antagonizes *lgDOG1*, in terms of seed dormancy regulation.

3.7 Regulation of antisense *DOG1* by sense *DOG1*

The second question (or hypothesis) mentioned above in regard to the possible mechanistic link between alternative polyadenylation and *asDOG1* expression also seems to be valid because the 3′ end of *lgDOG1*, which is expressed by extended read through with dTTS selection, corresponds to

the region of the *asDOG1* promoter (Fig. 6). Interestingly, *asDOG1* expression is reduced in the *fy-2* (i.e. dTTS-favoring and *lgDOG1*-enriched) mutant seeds (Kowalczyk et al., 2017), suggesting that *asDOG1* expression is indeed affected by the read through of the pTTS-dTTS region (Fig. 9). These results highlight that the transcription of sense *DOG1* negatively affects *asDOG1* expression, just like *asDOG1* expression causes transcriptional interference to sense *DOG1* expression (Fig. 7). In other words, the intriguing mechanism of *"reciprocal regulation of the DOG1 and asDOG1 pair"* (Kowalczyk et al., 2017) was discovered for dormancy regulation. This interdependent regulation between sense and antisense expression might be a universal or widespread mechanism of gene expression (Nguyen et al., 2014; Rosa, Duncan, & Dean, 2016).

The downregulation of *asDOG1* expression suggests the suppression of the *asDOG1* promoter, of which region is read through during *lgDOG1* expression in a sense orientation. Transcription of promoter regions could suppress their activity through chromatin remodeling (Mellor, Woloszczuk, & Howe, 2016), including H2B monoubiquitination (H2Bubq) (Batta et al., 2011). The expression of *asDOG1* is upregulated in the *hub1-5* mutant (Kowalczyk et al., 2017), which has a defect in a H2Bubq enzyme (Liu et al., 2007). Therefore, H2Bubq of this region during sense *DOG1* expression is probably associated with the suppression of the *asDOG1* promoter (Kowalczyk et al., 2017) (Fig. 9).

H2Bubq during sense *DOG1* expression, on the other hand, is known to be an important factor for transcription elongation of *DOG1* and seed dormancy (reviewed in Nonogaki, 2014). Transcription elongation is one of the critical factors for the efficiency of gene expression, which is affected by the temporal arrest of RNA polymerase II (Pol II) and its recovery from the arrest (Saunders, Core, & Lis, 2006), through the function of Transcription elongation factor S-II (TFIIS) (Kim, Guermah, & Roeder, 2010) (Fig. 9). Mutations in TFIIS and other components associated with transcription elongation, such as Pol II-Associated Factor 1 Complex (PAF1C), H2B MONOUBIQUITINATION1 (HUB1) and ARABIDOPSIS TRITHORAX-RELATED 7 (ATXR7) (Fig. 9), result in reduced expression of *DOG1* and dormancy (Grasser et al., 2009; Liu et al., 2007, 2011). TFIIS and HUB1 are known also as REDUCED DORMANCY 2 (RDO2) (Liu et al., 2011) and RDO4 (Liu et al., 2007) (Fig. 9), respectively. HUB1/RDO4 is an Arabidopsis ortholog of yeast Bre1, a ubiquitin ligase (Liu et al., 2007), which interacts with Set1 (Sun & Allis, 2002), a histone methyltransferase in yeast, while ATXR7 is an

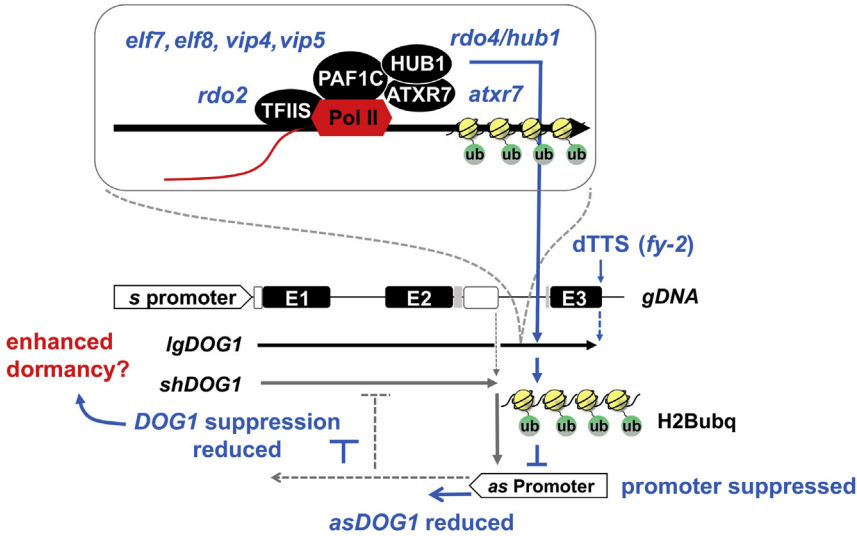

Fig. 9 ***Possible mechanisms of the negative regulation of*** **antisense DOG1** ***expression by*** **DOG1** ***transcription.*** Transcription elongation is a critical factor for *DOG1* expression and seed dormancy. The components of the protein complex associated with RNA polymerase II (Pol II), including Transcription elongation factor S-II (TFIIS), Pol II-Associated Factor 1 Complex (PAF1C), H2B MONOUBIQUITINATION1 (HUB1) and ARABIDOPSIS TRITHORAX-RELATED 7 (ATXR7) (*inset*), play important roles in transcription elongation. Mutations in these factors (indicated by *blue italic symbols* in inset) negatively affects seed dormancy. While histone 2B monoubiquitination (H2Bubq) by HUB1 positively affects transcription elongation of *DOG1* during sense expression, H2Bubq at the extended 3′end of *DOG1*, could serve as a repressive mark on the *asDOG1* promoter. This is probably how *lgDOG1* expression in a sense orientation suppresses *asDOG1* expression. The regulation of *DOG1* expression by *asDOG1* transcription (Fig. 7) and the regulation of *asDOG1* expression by *DOG1* expression (this figure) present an interesting mechanism of interdependent regulation between sense and antisense expression. However, the outputs of phenotypes seem to be counterintuitive. The consequence of dTTS selection, *lgDOG1* expression, H2Bubq on the *asDOG1* promoter and reduced expression of *asDOG1* is anticipated to de-repress *DOG1* and result in enhanced dormancy. However, dTTS selection and *lgDOG1* expression in the *fy-2* mutants cause reduced dormancy (Fig. 8). The reason for this discrepancy between the molecular model prediction and the phenotypic outputs is not known. It is possible that *asDOG1* regulation by *DOG1* is a negative feedback and a secondary mechanism. *See* text for details. *atxr7*, *arabidopsis trithorax-related7; elf*, *early flowering*; *hub1*, *h2b monoubiquitination1*; *rdo*, *reduced dormancy*; *vip*, *vernalization independence*. E, exon; *s* Promoter, sense promoter. *Modified from Nonogaki (2014, 2017). Based on Cyrek et al. (2016), Fedak et al. (2016), Kim et al. (2009), Kim et al. (2010), Kowalczyk et al. (2017), Liu et al. (2011), Porter, Penheiter, and Jaehning (2005), Saunders et al. (2006), Sun and Allis (2002), and Zhu et al. (2005).*

Arabidopsis ortholog of Set1 (Liu et al., 2011). H2Bubq by HUB1 and the subsequent H3K4 and H3K79 methylation (Nakanishi et al., 2009) by ATXR7 are thought to affect transcription elongation of sense *DOG1* positively (Liu et al., 2007, 2011; Nonogaki, 2014) (Fig. 9). Therefore, it is conceivable that H2Bubq deposited at the pTTS-dTTS region during sense *DOG1* expression in turn serves as a repressive mark on the antisense promoter (Fig. 9), although H2Bubq, which was assessed by chromatin immunoprecipitation, was not specific to this region but spread over throughout the *DOG1* locus (Kowalczyk et al., 2017). More research may be necessary for the mechanisms of the suppression of the *asDOG1* promoter.

The seemingly separate stories of alternative polyadenylation and *asDOG1* expression have now been integrated with each other, providing more information for the regulatory mechanisms of *DOG1* expression. However, the biological significance of the interdependent regulation between *DOG1* and *asDOG1* in seed dormancy is still a mystery. The most puzzling part is the output of dormancy phenotypes. The selection of dTTS and more read through of the extended region during *lgDOG1* expression put the repressive mark of H2Bubq on the *asDOG1* promoter, which results in reduced expression of *asDOG1* (Fig. 9). Since *asDOG1* expression negatively affects *DOG1* expression and dormancy (Fig. 6), the consequence of reduced expression of *asDOG1* is expected to be enhanced dormancy (Fig. 9). However, the *fy-2* mutant seeds, in which *lgDOG1* is preferentially expressed and *asDOG1* is reduced, exhibit reduced dormancy instead (Kowalczyk et al., 2017) (Fig. 8). Besides, the *cpl1-9* mutant seeds, where *shDOG1* is selectively expressed and *asDOG1* expression is reinforced, show enhanced dormancy (Kowalczyk et al., 2017) (Fig. 8). These phenotypic results are counterintuitive while the molecular data themselves are consistent at all levels (i.e. dTTS selection → *lgDOG1* expression → enhanced H2Bubq → reduced *asDOG1*) (Fig. 9). The reason for these discrepancies between the molecular and phenotypic data is not known. It is possible that the expression of *shDOG1* or *lgDOG1* is the primary determinant of seed dormancy and the consequential suppression of the *asDOG1* promoter by H2Bubq is a negative feedback regulation, which serves as a relatively minor mechanism (Nonogaki, 2019). More information from future research will probably address the cryptic mechanisms of *DOG1* regulation, just like the puzzling questions about *DOG1* in the past research (e.g. the merging point of the DOG1 and ABA pathways, the significance of transcript variants) were answered by the elegant experiments.

3.8 *DOG1* and antisense *DOG1* in ABA signaling in plants

The *asDOG1* function is best understood for seed dormancy, however, the highest expression of *asDOG1* is detected in seedlings (Fedak et al., 2016). Although this article focuses on ABA responses is seeds, discussing possible function of *asDOG1* in seedlings is also relevant to the general understanding of the DOG1 involvement in ABA signaling. Therefore, new developments about the *asDOG1* role in drought tolerance will also be introduced here.

The *asDOG1* promoter is activated in the apical meristem, flowers and young leaves while its activity is reduced in older leaves (Yatusevich et al., 2017). The expression of *asDOG1* in leaves is reduced by exogenous ABA, with *DOG1* showing the complementary patterns of increased expression (Yatusevich et al., 2017). These results suggest that *DOG1* might be involved in ABA-regulated biological responses in seedings. In fact, the *dog1-3* and *dog1-4* knockout mutants exhibit drought-sensitive phenotypes, with diminished expression of the typical ABA-induced stress response markers, such as *KIN1*, *RD29A*, *RD29B* and *RAB18*, compared to wildtype (Yatusevich et al., 2017). These results suggest that *DOG1* expression in leaves is associated with the ABA-dependent pathway of drought responses in seedlings (Nakashima, Yamaguchi-Shinozaki, & Shinozaki, 2014). Thus, unexpected function of DOG1 outside seed dormancy mechanisms has also been discovered.

The activity of the *DOG1* promoter-luciferase (*LUC*) reporter gene, which contains the full-length *DOG1* (p_{DOG1}*:LUC::DOG1*) or *shDOG1* lacking the 3′ region corresponding to the *asDOG1* promoter (p_{DOG1}*:shDOG1::LUC*) (Fig. 10A), has been examined (termed P_{DOG1}*:DOG1* vs. p_{DOG1}*:shDOG1* for convenience by omitting *LUC* hereafter). The reporter gene containing the full length *DOG1* (p_{DOG1}*:DOG1*) was not expressed in the absence of ABA while it was clearly activated by ABA (Fig. 10A), confirming the upregulation of *DOG1* by ABA. In contrast, the reporter gene containing *shDOG1* (p_{DOG1}*:shDOG1*) was expressed in seedlings regardless of ABA application (Yatusevich et al., 2017) (Fig. 10A). These results support the idea that *DOG1* expression in seedlings is repressed by *asDOG1* (promoter and expression) in the absence of ABA and that *DOG1* is de-repressed from *asDOG1* when antisense expression is downregulated by ABA (Fig. 10B). When the TATA elements in the *asDOG1* promoter is mutated in the full length *DOG1* gene (p_{DOG1}*:DOG1xx*), the reporter gene (which is otherwise suppressed by *asDOG1*),

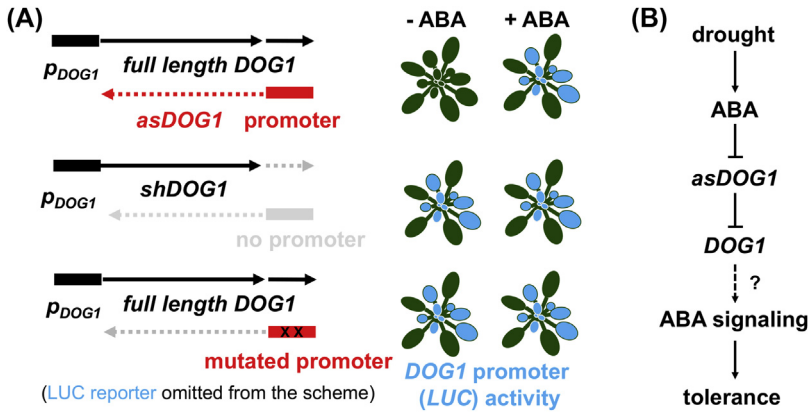

Fig. 10 ***Possible DOG1-dependent ABA signaling in plants.*** (A) Expression of a *luciferase* (*LUC*) reporter driven by the *DOG1* promoter (p_{DOG1}), fused with the full length *DOG1* containing the 3′ extended region corresponding to the *antisense DOG1* (*asDOG1*) promoter (*top* and *bottom* (p_{DOG1}*LUC::DOG1* called p_{DOG1}*:DOG1* for convenience)) or *short DOG1* (*shDOG1*) lacking the *asDOG1* promoter (*middle*) (p_{DOG1}*:shDOG1::LUC* called *pDOG1:shDOG1*). In the case of the full length *DOG1* (*top*, p_{DOG1}*:DOG1*), reporter gene expression is induced by ABA while it is expressed regardless of ABA in the case of *shDOG1* (*middle*, p_{DOG1}*:shDOG1*) or the full length *DOG1* with mutations (x x) in the *asDOG1* promoter region (*bottom,* p_{DOG1}*:DOG1xx*). These results suggest that the expression of *asDOG1* suppresses *DOG1* expression in the absence of ABA while ABA inhibits *asDOG1* expression and induces *DOG1* to increase drought tolerance. Note that the *LUC* is omitted from the schematics of the reporter genes. (B) Diagram illustrating the suppression of *asDOG1* by ABA upon drought stress, which de-represses *DOG1* from *asDOG1* and increases drought tolerance of the plants. If and how DOG1 modulates ABA signaling during drought responses are not known. *Based on Fedak et al. (2016), Nakashima et al. (2014), and Yatusevich et al. (2017).*

was expressed in seedlings even in the absence of ABA. These results provide a proof for the involvement of the *asDOG1* promoter (i.e. *asDOG1* expression) in the regulation of *DOG1* by ABA and drought (Fig. 10B). These results demonstrate a broader role of DOG1 in the biology of plants and ABA signaling outside seeds.

4. Seed germination

The key to inducing seed germination from dormant seeds is the inactivation of the DOG1 protein through (yet-to-be-determined) posttranslational modification, which occurs during after-ripening and reduces ABA sensitivity in seeds. Then, what is the key physiological event that is

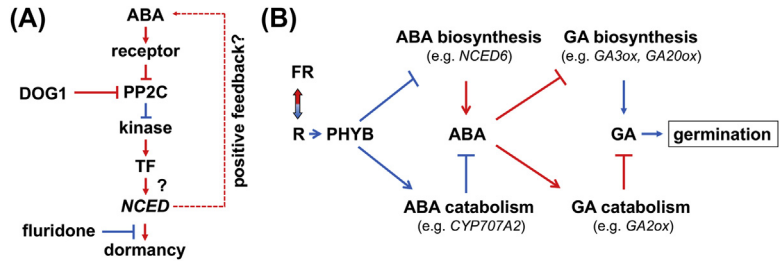

Fig. 11 ***ABA signaling and metabolism during seed dormancy and germination.*** (A) Possible positive feedback in the DOG1-dependent seed dormancy mechanism. The final output of DOG1-dependent seed dormancy seems to be ABA biosynthesis because deep dormancy caused by the *DOG1* transgene is still released by the application of fluridone, a carotenoid (hence ABA) biosynthesis inhibitor. *NCED*, *nice-cis-epoxycarotenoid dioxygenase*. *See* text for details. (B) The changes in ABA-GA balance during seed germination. Red light (R) promotes seed germination while far-red (FR) antagonizes it in Arabidopsis seeds. Phytochrome B (PHYB), upon the perception of red light, downregulates ABA biosynthesis and upregulates ABA catabolism, both of which reduce ABA levels in seeds. In this way, the original suppression of GA biosynthesis and the enhancement of GA catabolism by ABA are reduced, which results in the increase in GA levels and the induction of seed germination. Note that the scheme does not represent all mechanisms of ABA-GA antagonism but depicts only part of the reciprocal regulations between ABA and GA metabolisms in a unidirectional manner of germination induction in this case. *CYP*, *cytochrome P450*; *GA3ox*, *gibberellin 3-oxidase*; *NCED*, *nine-cis-epoxycarotenoid dioxygenase*. *(A) Based on Nonogaki (2014) and Née et al. (2017). (B) Modified from Martin, Pluskota, and Nonogaki (2010). Based on Seo et al. (2006) and (2009).*

suppressed by DOG1 and ABA sensitivity during dormancy? Another enigma in the seed dormancy and germination mechanisms is that the downstream of DOG1 and ABA signaling seems to be ABA biosynthesis (Fig. 11A). Deep dormancy imposed by both the native *DOG1* in Arabidopsis Cvi wild-type seeds (Ali-Rachedi et al., 2004) and the *DOG1-GFP* in transgenic seeds (Née et al., 2017) is released by fluridone, a carotenoid biosynthesis inhibitor that blocks ABA biosynthesis, suggesting that ABA biosynthesis is a final output of seed dormancy during imbibition. These results imply the presence of a positive feedback loop between ABA signaling and biosynthesis (Nonogaki, 2014) (Fig. 11A). While the upstream regulator NGATHA1 has been identified for *NCED3* during dehydration stress of plants (Sato et al., 2018a), a transcription factor in seeds, which is phosphorylated by a kinase and binds to the *NCED5*, *NCED6* or *NCED9* promoter upon AHG1 sequestration by DOG1 and kinase activation (Fig. 4A), has not been discovered yet. Therefore, how ABA

biosynthesis is maintained downstream of DOG1 and ABA signaling in dormant seeds and how it is reduced in after-ripened seeds are not known. In contrast, the mechanisms of ABA catabolism by *CYP707A2*, another final output of dormancy release that seems to be always coupled with the downregulation of *NCED* (Fig. 11B) are emerging. During this germination window, ABA sensitivity is also reduced through de-stabilization of ABI5, another signaling component of ABA in addition to DOG1. These emerging mechanisms to reduce ABA levels and signaling will be discussed for the mechanisms of seed germination induction below.

4.1 Reduction in ABA levels

ABA biosynthesis and catabolism seem to be always coupling to each other (inversely). When phytochrome B (PHYB) downregulates ABA biosynthesis, which reduces ABA levels in seeds, ABA catabolism is upregulated by PHYB, which further diminishes seed ABA levels (Fig. 11B). This type of double regulation is also observed in the control of GA biosynthesis and catabolism by ABA. ABA downregulates GA biosynthesis while it upregulates GA catabolism (Fig. 11B). As mentioned above, the regulatory mechanisms of the seed *NCEDs* are not clear yet, however, the regulation of ABA catabolism, particularly *CYP707A2* induction by nitrate (Matakiadis et al., 2009), a seed dormancy releasing factor, is becoming clear.

The promoter region of *CYP707A2* contains the nitrate-responsive-*cis*-element (NRE) (Fig. 12), which was originally found in the promoter region *of NITRITE REDUCTASE1* (*NIR1*) and mediates gene expression in a nitrate-dependent manner (Konishi & Yanagisawa, 2010). NREs are also found in the nitrate reductase *NIA1*, although they are not found in the promoter region but reside in the 3′ end of the gene (Konishi & Yanagisawa, 2011). Nodule Inception (NIN)-like protein 6 (NLP6) has been found as a NRE-binding protein for *NIR1* and *NIA* (Konishi & Yanagisawa, 2013). In imbibed Arabidopsis seeds, *NLP8* is expressed during a narrow window of early imbibition (Yan et al., 2016), which nearly coincides with the window of *CYP707A2* expression and ABA reduction (Kushiro et al., 2004). NLP8 physically binds to the NRE in the *CYP707A2* promoter and induces its expression (Yan et al., 2016). NLP6 is activated possibly by the modification of its N-terminal domain by nitrate. The same process could be involved in the activation of NLP8 in seeds (Fig. 12). Nitric oxide (NO), which can be produced from nitrate, stimulates *CYP707A2* expression (Arc, Galland, Godin, Cueff, & Rajjou, 2013; Liu et al., 2009), however, the induction of *CYP707A2* by NLP8 is not NO

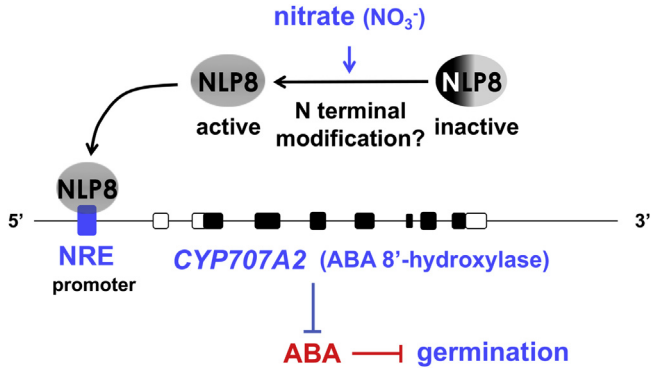

Fig. 12 ***The regulatory mechanism triggering ABA catabolism during seed germination.*** *CYP707A2*, which encodes for the ABA catabolism enzyme ABA 8′-hydroxylase, is induced by Nodule Inception (NIN)-like protein 8 (NLP8), which binds to the nitrate-responsive-*cis*-element (NRE) in its promoter region. NLP8 is possibly activated by nitrate through the modification of its N-terminal domain. *Modified from Nonogaki (2017). Based on Konishi and Yanagisawa (2010), (2011), (2013), Kushiro et al. (2004), and Yan et al. (2016).*

dependent but a direct response to nitrate (Yan et al., 2016) (*see* below for more discussion about NO). The findings about the regulatory mechanisms of *CYP707A2* greatly advanced our knowledge on ABA metabolism during seed germination. One of the next major goals in seed dormancy and germination research is to identify direct upstream regulators of the seed *NCED*s and information about their responses to environmental signals, including those in soil environment.

4.2 Alleviation of ABA sensitivity

4.2.1 Nitric oxide

Nitric oxide (NO), a reactive and gaseous free radical that functions as a signaling molecule. NO production is catalyzed by nitrate reductase (NR) or nitric oxide synthase (NOS) (Bethke, Libourel, & Jones, 2007). NO negatively affects ABA signaling by suppressing *ABI5*, a major regulator of seed germination and post-germination, at both the transcriptional and post-translational levels.

The transcription of *ABI5* is regulated by the group VII ethylene response factors (ERFVIIs), which bind to the EBP-box *cis*-elements (EBPs) in the promoter region of *ABI5* (Gibbs et al., 2014, 2015) (Fig. 13). ERFVIIs are subjected to the N-end rule pathway (Gibbs et al., 2014, 2015), which is a ubiquitin-dependent proteolysis pathway and

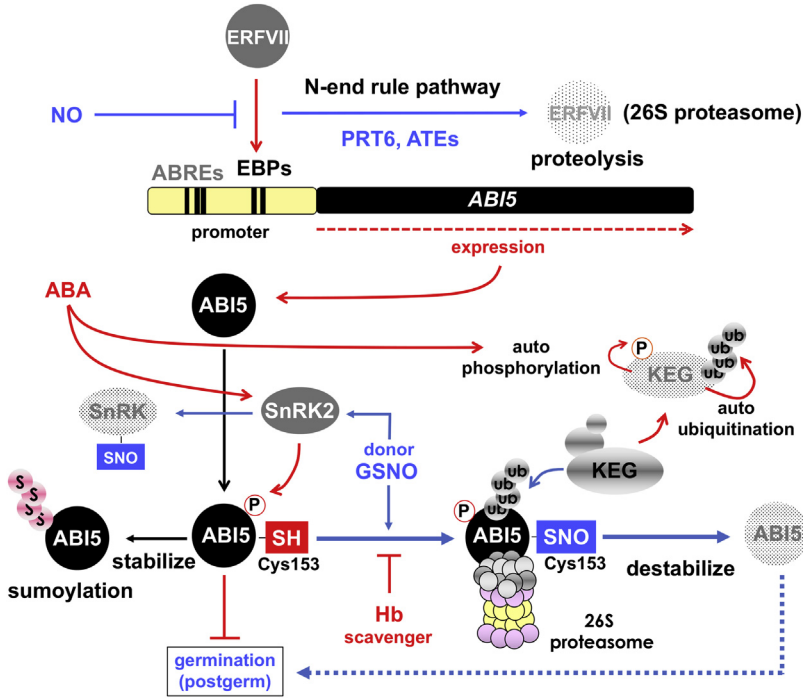

Fig. 13 ***Transcriptional and posttranslational regulation of ABI5.*** The expression of *ABI5* is promoted by the group VII ethylene response factors (ERFVIIs), which bind to the EBP-box *cis*-elements (EBPs) in the promoter region of *ABI5*. In the presence of nitric oxide (NO) signal, ERFVIIs are destabilized by the N-end rule pathway, which is mediated by PROTEOLYSIS 6 (PRT6), a RING-type E3 ligase, and arginyl-tRNA:protein arginyltransferase [ATE]), and the 26 proteasome pathway. At the posttranslational level, ABI5 is subjected to *S*-nitrosylation at the thiol side chain (—SH) of Cys153 (ABI5-SNO) possibly by *S*-nitrosoglutathione (GSNO), which triggers the ubiquitination of ABI5 by KEEP ON GOING (KEG), another RING-type E3 ligase, and the subsequent destabilization by the 26S proteasome pathway. Hemoglobin (Hb), an endogenous NO scavenger, could antagonize *S*-nitrosylation of ABI5. *S*-nitrosylation also occurs in SNF1-related protein kinase 2 (SnRK2), a positive regulator of ABA signaling (Fig. 4A) that phosphorylates and activates ABI5, and antagonizes ABA signaling. In contrast, ABA stabilizes ABI5 by promoting autoubiquitination and degradation of KEG, possibly through autophosphorylation of KEG. Sumoylation (S) of ABI5, which prevents ubiquitination and stabilizes the protein (in an inactive form) is also shown. ABRE, ABA responsive element. *Modified from Nonogaki (2017). Based on Albertos et al. (2015), Bachmair et al. (1986), Gibbs et al. (2014), Gibbs et al. (2015), Hill (2012), Holman et al. (2009), Liu and Stone (2010), Perazzolli et al. (2004), Piskurewicz et al. (2008), Stone et al. (2006), Tasaki and Kwon (2007), and Tasaki et al. (2012).*

determines half-life of proteins based on the N-terminal residues (N-degrons) (Bachmair, Finley, & Varshavsky, 1986; Tasaki & Kwon, 2007; Tasaki, Sriram, Park, & Kwon, 2012). The ubiquitin-dependent proteolysis in the N-end rule pathway is mediated by PROTEOLYSIS 6 (PRT6), a RING-type E3 ligase and arginyl-tRNA:protein arginyltransferase [ATE]) (Gibbs et al., 2014; Holman et al., 2009) (Fig. 13). NO destabilizes ERFVIIs through the N-end rule and 26S proteasome pathways, thereby suppressing *ABI5* expression (Gibbs et al., 2014, 2015).

The expression of *ABI5* does not solely determine the level of ABA signaling by this factor in seeds because the rates of transcript accumulation, translation into protein, and protein turnover also affect the level of ABI5 in the seed cells. The stabilization or de-stabilization of the ABI5 protein in seeds is also important. Interestingly, NO exerts the negative effects on ABI5 also through post-translational regulation, which affects germination and post-germination more directly than the transcriptional control of the gene.

The stability of the ABI5 protein is determined by its ubiquitination by KEEP ON GOING (KEG), another RING-type E3 ligase, and its degradation by the 26S proteasome pathway (Stone, Williams, Farmer, Vierstra, & Callis, 2006) (Fig. 13). The ubiquitination and subsequent destabilization of ABI5 by the protein degradation machinery in the cell is triggered by *S*-nitrosylation of the thiol side chain (−SH) of Cys153 in ABI5 by NO, which results in the formation of nitrosothiol (ABI5-SNO) (Albertos et al., 2015) (Fig. 13). NO can be provided by *S*-nitrosoglutathione (GSNO), an endogenous donor, and removed by hemoglobin (Hb), an endogenous scavenger (Albertos et al., 2015; Hill, 2012; Perazzolli et al., 2004), which can be replaced by the exogenous donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and scavenger 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), respectively under experimental conditions (Albertos et al., 2015).

S-nitrosylation and the resulting degradation of ABI5 present an interesting mechanism, through which NO counteracts ABA signaling. SnRK2, which phosphorylates and activates ABI5 (Piskurewicz et al., 2008), is also subject to *S*-nitrosylation (Wang, Zhu, & Lang, 2015) (Fig. 13), highlighting another layer of counteraction of NO against ABA signaling. ABA, on the other hand, promotes autoubiquitination and destabilization of KEG, by enhancing the autophosphorylation of KEG, which probably triggers its autoubiquitination (Liu & Stone, 2010) (Fig. 13). These findings about *S*-nitrosylation of ABI5 and the counteracting mechanisms

broaden our understanding about ABA signaling in seeds in response to NO (and nitrate in the soil environment).

4.2.2 Biotic responses

There was another interesting discovery about ABI5 involvement in seed germination recently, which is possibly associated with seed responses to rhizosphere bacterial pathogens. Bacterial cell communicates with each other through chemical signals called autoinducer to synchronize the activities of the population, which is known as quorum sensing (QS) (Waters & Bassler, 2005). The consequences of the responses to autoinducers by an individual bacterium, including gene expression, may be negative to the individual but beneficial to the population as a whole (Waters & Bassler, 2005). The QS system in bacteria itself is interesting enough because it suggests that bacterial cells are communicating and prokaryotes could behave like multicellular organisms (Waters & Bassler, 2005). What is even more interesting is that recent developments in germination research imply that seeds might be *"listening"* to this bacterial communication.

The autoinducer L-2-amino-4-methoxy-trans-3-butenoic acid (AMB) produced by *Pseudomonas aeruginosa*, which lives in a broad spectrum of environments including soil, causes sever defects in Arabidopsis seedlings (Chahtane et al., 2018). Arabidopsis seeds do not germinate in the presence of *P. aeruginosa* while germination is not suppressed by *P. fluorescens*, *P. putida*, *P. syringae* or *Escherichia coli* (Chahtane et al., 2018), demonstrating the specific seed responses to *P. aeruginosa*. The suppression of seed germination by AMB may not be due to random toxicity of the chemical to seeds because the *rgl2* mutant seeds, which are defective in the DELLA protein RGA-LIKE2, can germinate in the presence of AMB (Chahtane et al., 2018). RGL2 is a repressor of seed germination, which is ubiquitinated by the E3 ubiquitin ligase complex and degraded (or inactivated) through the 26S proteasome pathway upon GA perception by the GA receptor GA INSENSITIVE DWARF1 (GID1) (Ariizumi & Steber, 2007; McGinnis et al., 2003; Tyler et al., 2004). The resistance of the *rgl2* seeds to AMB suggest that (wild-type) seeds become dormant through the specific pathway of RGL2, a negative regulator of GA signaling, when they sense AMB. The suppression of germination by RGL2 is mediated by ABI5 (Piskurewicz et al., 2008). The inhibition of germination by the QS autoinducer AMB is drastically reduced in the *abi5* mutant seeds (as well as *abi3* and *abi1*) (Chahtane et al., 2018), suggesting that ABA signaling plays a critical role in responding to AMB. These results suggest that seeds might be sensing

the presence of pathogenic bacteria by monitoring the QS autoinducer to avoid anticipated attack by the bacteria to their seedlings after germination.

The interpretation of the bacterial QS signal detection by seeds still needs to be challenged by further experiments to make a conclusion about its significance in seed ecology. However, these findings also offer great potential for technology development, including herbicide applications (Chahtane et al., 2018) and seed pelleting and film coating (when the mechanisms are used for seed enhancement).

5. Perspectives

Seed dormancy research is entering a very exciting phase of a series of new discoveries, which is advancing a general understanding of ABA signaling. The unexpected discovery of the DOG1's role in drought responses of seedlings expands our view of the biological role of this protein in ABA signaling in plants. Even within the field of seed biology itself, the DOG1 function does not seem to be confined to the dormancy mechanisms. A number of genes, including *LEA*, *HEAT SHOCK PROTEIN* and their upstream regulator *ABI5*, are downregulated in the *dog1-1* mutant seeds, suggesting that *DOG1* modulates desiccation tolerance, another major program during seed maturation (Fig. 2). The normal embryo de-greening phenotype of the weak allele *abi3-1* can be converted to a stay-green phenotype by integrating the *dog1-1* mutation with it (*dog1-1 abi3-1*) (Dekkers et al., 2016), indicating that *DOG1* is an enhancer of *ABI3*. These results suggest that DOG1 is not limited to seed dormancy but is a master regulator of seed maturation program in general, which is important for our understanding of the origin and evolution of the DOG1 family proteins, the maturation programs and ABA signaling itself in seed plants (Nonogaki, 2019).

While seed dormancy is a specific program to gymnosperm and angiosperm, ABA-GA antagonism, which is the core mechanism of seed dormancy, is present in ferns, seedless vascular plants (McAdam et al., 2016). Therefore, it has been proposed that the seed dormancy mechanisms might date back through non-seed plants (McAdam et al., 2016). Mosses do not seem to synthesize an active GA (Miyazaki et al., 2018), which suggests that ABA-GA antagonism is limited to vascular plants. However, the ABA perception and signaling pathways are evolutionarily conserved in the liverwort *Marchantia polymorpha*. This liverwort ABA signaling plays a role in

gemma dormancy (Bowman et al., 2017; Eklund et al., 2018; Jahan et al., 2019), which suggests that a prototype of the ABA-dependent dormancy (or growth arrest) might have already been present in the common ancestor of land plants.

The information about the Arabidopsis DOG1 function also provides an implication for the evolution of ABA signaling. While DOG1 was discussed mainly for its function as an AHG1-binding protein in ABA signaling, DOG1 was also found to bind to heme, an iron-containing porphyrin, through its C-terminal histidines (Nishimura et al., 2018). How heme is involved in ABA signaling in seeds is not known. However, ABA induces a tryptophan-rich sensory protein O (TSPO), which binds to heme and controls heme levels, in the Arabidopsis suspension-cultured cells (Vanhee, Zapotoczny, Masquelier, Ghislain, & Batoko, 2011), highlighting a possible link between heme and ABA signaling, hence a possible role of the DOG1-heme binding in ABA signaling (Nishimura et al., 2018). Intriguingly, ABA signaling that is mediated through TSPO and heme levels is observed also in the unicellular red alga *Cyanidioschyzon merolae*, which contains one plastid, one mitochondrion and one nucleus (Kobayashi, Ando, Hanaoka, & Tanaka, 2016). *C. merolae* synthesizes ABA and arrests cell cycle under stress conditions to avoid cell death, which is mediated by TSPO, a heme-scavenging protein (Kobayashi et al., 2016). These results imply an ancient origin of the ABA-dependent stress response and growth arrest mechanisms.

C. merolae and other algal species do not seem to contain the counterparts of ABA receptors found in land plants, however, other ABA signaling components, including PP2Cs, SnRK2 and bZIP transcription factors, and the ABA metabolism gene *NCED,* are found in *C. merolae* (Kobayashi et al., 2016). Heme is probably synthesized in mitochondrion of *C. merolae* (Kobayashi et al., 2016) because ferrochelatase, a heme biosynthesis enzyme, which inserts iron to protoporphyrin IX, is localized only in mitochondrion in this alga (Watanabe et al., 2013) while ABA is probably synthesized in chloroplast (Kobayashi et al., 2016). Therefore, ABA might have evolved as a chloroplast signal to interact with the mitochondrial heme signal (Kobayashi et al., 2016). It is interesting to examine whether this type of inter-organelle communication through ABA and heme is involved also in ABA signaling in the seed dormancy mechanisms. If it is the case, DOG1 might serve as a heme scavenger to cause growth arrest in seeds, just like TSPO in *C. merolae*. Alternatively, heme is required as a prosthetic group of ABA-8′-hydroxylase, a cytochrome P450 (Saito et al., 2004), and therefore, the heme increase could promote ABA catabolism in *C. merolae*

(Kobayashi et al., 2016). In seeds, DOG1-heme binding could scavenge heme and reduce ABA catabolism, which are anticipated to enhance dormancy. Understanding the role of DOG1 binding to heme, an iron-containing porphyrin, might also help elucidate the mechanisms underlying how DOG1 affects embryo de-greening (Mg extraction from porphyrin). More research on ABA and heme signaling in the algal ancestor and bryophytes will probably reveal hidden pathways of ABA signaling and their evolution, which may be the key to revealing the complex ABA signaling mechanisms in seed plants.

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