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REVIEW PAPER

Primary seed dormancy: a temporally multilayered riddle waiting to be unlocked

Hicham Chahtane*, Woohyun Kim* and Luis Lopez-Molina†

Department of Plant Biology and Institute for Genetics and Genomics in Geneva (iGE3), University of Geneva, Geneva, Switzerland

* These authors contributed equally to this work.
† Correspondence: Luis.LopezMolina@unige.ch

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Abstract

Primary seed dormancy is an important adaptive plant trait whereby seed germination is blocked under conditions that would otherwise be favorable for germination. This trait is found in newly produced mature seeds of many species, but not all. Once produced, dry seeds undergo an aging time period, called dry after-ripening, during which they lose primary dormancy and gradually acquire the capacity to germinate when exposed to favorable germination conditions. Primary seed dormancy has been extensively studied not only for its scientific interest but also for its ecological, phenological, and agricultural importance. Nevertheless, the mechanisms underlying primary seed dormancy and its regulation during after-ripening remain poorly understood. Here we review the principal developmental stages where primary dormancy is established and regulated prior to and during seed after-ripening, where it is progressively lost. We attempt to identify and summarize what is known about the molecular and genetic mechanisms intervening over time in each of these stages.

Key words: ABA, Arabidopsis, dormancy, endosperm, germination, seed.

Introduction

Mature Arabidopsis seeds are the endpoint of seed development, a process initiated after a double fertilization event characteristic of flowering plants. Two sperm cells are delivered by the pollen tube after it reaches the female gametophyte within the mature ovule. A sperm cell fuses with a haploid egg cell while another fuses with a diploid central cell, thus forming the diploid zygote and the triploid endosperm, respectively. These events trigger seed development where zygote and endosperm develop alongside to produce finally the Arabidopsis mature seed (Fig. 1).

Upon its completion, the Arabidopsis mature dry seed consists of an external layer of dead tissue, the testa, which is the remnant of maternal ovular integuments that collapsed during the seed maturation phase (Fig. 1). Beneath the testa lies the endosperm, which subsists as a single-celled layer surrounding the embryo (Fig. 1).

Arabidopsis seed development is roughly divided into two stages: embryogenesis and maturation (Baud *et al.*, 2002). Embryogenesis establishes the basic body plan of the embryo after the zygote grows through multiple cell division and differentiation programs. In parallel, the endosperm develops in four phases: syncytial, cellularization, differentiation, and degeneration, with the exception of a remaining single-cell layer surrounding the embryo in the mature stage (Berger, 1999). It is generally assumed that the degenerating Arabidopsis endosperm fulfills a nourishing role for the embryo during seed development. Sucrose transporters in the ovular integuments and endosperm are important

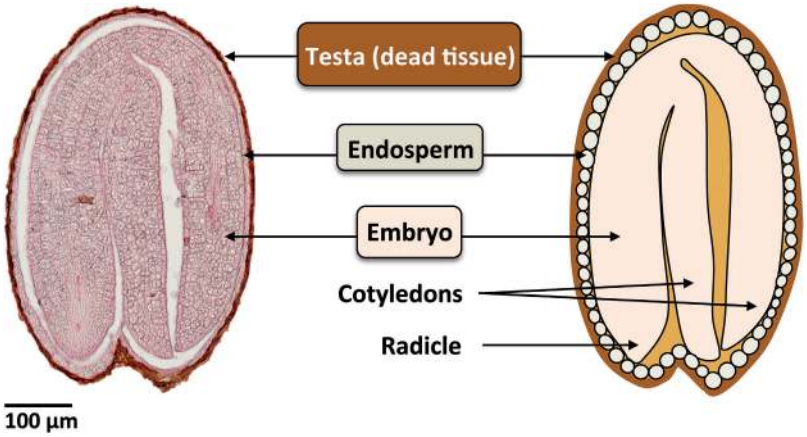

Fig. 1. Anatomy of the Arabidopsis mature dry seed. The mature dry seed is composed of three distinct tissues. The outer layer is the testa, a tissue made of collapsed maternal ovular integuments. Beneath the testa lies the endosperm, a single cell layer surrounding the embryo. The left side is a histological section of a Col-0 seed stained using the Sudan Red dye.

for providing maternal nutrients to the embryo (Baud *et al.*, 2005; Chen *et al.*, 2015). The expression of *AtSUC5*, encoding a sucrose transporter, is highly induced during early seed development specifically in the endosperm (Baud *et al.*, 2005). *atsuc5* mutant seeds display low levels of fatty acids and delayed embryo development, suggesting that nutrient delivery through AtSUC5 is crucial for normal embryo development. In addition, a recent report has provided direct evidence that maternal sucrose is deposited in developing seeds via the funicular phloem through specific SWEET transporters localized in the seed coat and endosperm (Chen *et al.*, 2015). Indeed, *sweet11*/*sweet12*/*sweet15* triple mutant seeds have a low lipid content and display high starch accumulation in the seed coat, whereas starch levels in the embryo are low. This indicated the occurrence of abnormal sugar efflux transport from the maternal ovular tissues towards the embryo through the endosperm (Chen *et al.*, 2015). Altogether, these recent reports suggest that the endosperm can also operate as a nourishment transmission tissue during Arabidopsis seed development. In the mature seed, the endosperm plays a central role, upon seed imbibition, in preventing the germination of primary dormant seeds (see below).

During the maturation stage, embryonic cells expand as they accumulate high levels of lipids and storage proteins. Lipids and proteins will end up accounting for up to 70% of the dry weight of the mature seed and will fuel future germination and seedling establishment (Baud *et al.*, 2002; Penfield *et al.*, 2004). Both the endosperm and embryo contain abundant food stores, although the majority are stored in the embryo. In the final stages of seed maturation, metabolism diminishes as the seed dries and acquires desiccation tolerance, which involves accumulation of late embryonic abundant (LEA) proteins conferring osmotolerance (Dekkers *et al.*, 2015).

The mature seed is a highly resistant entity that can withstand extended periods of time in its dry state awaiting proper conditions to germinate. Seeds transform plants into time and space travelers, which undoubtedly explains the success of angiosperms among terrestrial plants in colonizing numerous habitats.

Germination is a crucial developmental transition since it precedes the establishment of the seedling. Normal germination requires imbibition with water and first involves testa rupture, which is probably the result of micropylar endosperm cell expansion, followed by concomitant endosperm rupture and radicle protrusion out of the testa (Dekkers *et al.*, 2013; De Giorgi *et al.*, 2015; Scheler *et al.*, 2015).

Unlike the mature seed, the juvenile seedling is highly fragile. It is therefore unsurprising that germination control mechanisms have appeared in the course of evolution (Lopez-Molina *et al.*, 2001).

Primary seed dormancy in Arabidopsis

Seed dormancy is a broad phenomenon in plants, of which primary seed dormancy is a particular case. Seed dormancy can be generally defined as the blockade of the germination of a viable seed under favorable germination conditions. A non-dormant seed is a seed that is able to germinate under all the possible environmental conditions that are normally compatible with seed germination for a given plant species. Dormancy release is a term used to describe a dormant seed that lost its dormancy.

Different classes of seed dormancy have been described that include physiological dormancy (PD), morphological dormancy (MD), morphophysiological dormancy (MPD), physical dormancy (PY), and combinational dormancy (Nikolaeva, 1977; Baskin and Baskin, 2004, 2014; Finch-Savage and Leubner-Metzger, 2006).

Primary seed dormancy is the dormancy that develops during seed development in the mother plant (Hilhorst, 1995; Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). This review focuses on primary seed dormancy in *Arabidopsis thaliana*. *Arabidopsis thaliana* dormancy belongs to the PD class, which has three levels: deep, intermediate, and non-deep. The depth levels describe notably the growth behavior of the embryo excised from the dormant seed (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). The majority of seeds have a non-deep PD, which is also the case in *A. thaliana*. Seeds exhibiting non-deep PD are further

classified into five types notably according to the germination behavior in response to temperature (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). Secondary dormancy is found in non-deep PD and is a denomination used with seeds that lost primary dormancy to describe their behavior when exposed, notably in a prolonged manner, to environmental signals that are unfavorable for germination, such as darkness or low temperatures (Hilhorst, 1998). Indeed, in response to such unfavorable signals, the seed will enter a 'secondary dormant' state where germination is again repressed even under favorable germination conditions. In natural conditions, seeds may enter or leave secondary dormancy several times according to seasonal cues to optimize seedling establishment. Secondary dormancy has been less extensively studied than primary dormancy. Although both primary and secondary dormancy appear to share common underlying germination control mechanisms, notably the involvement of the phytohormones gibberellic acid (GA) and abscisic acid (ABA) (see below), whether they are similar at the molecular level remains poorly understood (Cadman *et al.*, 2006; Ibarra *et al.*, 2016).

Primary seed dormancy is a primal property of freshly produced seeds. The trait is functionally defined—a germination test is needed to assess whether a dry mature seed shows primary dormancy or not. Primary dormancy is released during dry after-ripening when seeds gradually acquire the capacity to germinate when exposed to favorable germination conditions and therefore become non-dormant (Holdsworth *et al.*, 2008). An unfavorable germination condition is a condition that invariably prevents seed germination, irrespective of the seed age, without killing the embryo within the seed. Germination conditions that are unfavorable for seed germination cannot therefore be used to assess primary dormancy. Examples of unfavorable seed germination conditions include seed imbibition in darkness followed a far-red (FR) pulse, imbibition in high temperatures, and high salinity conditions.

Primary seed dormancy is established during maturation. Indeed, Karssen *et al.* (1983) showed that seeds isolated at the mid-maturation stage from developing fruits germinated but not when isolated at later seed maturation stages. Similar observations were reported by Alboresi *et al.* (2005).

Primary dormancy, hereafter referred to as dormancy, can be illustrated when considering a population of Arabidopsis seeds harvested immediately upon completion of their maturation (Fig. 2). This initial population is divided into subpopulations, which will be subject to increasing amounts of dry after-ripening time (Fig. 2; Holdsworth *et al.*, 2008). A subpopulation of seeds of a given age is subject to a germination test, where the percentage of seed germination of the subpopulation is assessed after a certain time period upon seed imbibition. In the absence of after-ripening (i.e. when seeds newly terminated their maturation in the mother plant), a subpopulation is most likely to be unable to germinate (Fig. 2). This subpopulation is said to be dormant. As dry after-ripening time increases, the seed subpopulation germination is further tested and the percentage of seed germination gradually increases (Fig. 2). Thus, it is said that the depth of dormancy in these subpopulations decreases. Eventually, there will be a dry after-ripening time where the entire seed subpopulation will germinate, which is the time where seed dormancy is fully released (Fig. 2). The transient nature of seed dormancy can be used to define the depth of dormancy stored in a seed. The longer the after-ripening time needed to lose the dormancy of a seed population the higher the initial depth of dormancy in this population.

Dormancy is a potential property

The germination potential of a seed is its capacity to complete germination upon imbibition (Carrera *et al.*, 2008). The germination potential of a seed depends on both the particular extent of dry after-ripening and the particular environmental conditions used in the germination test. Thus, for a given intermediate after-ripening time, a subpopulation of seeds may fully germinate under favorable germination conditions (condition A) but may not germinate at all under less favorable germination conditions (condition B; Fig. 2). Since dormancy is functionally defined by a germination test,

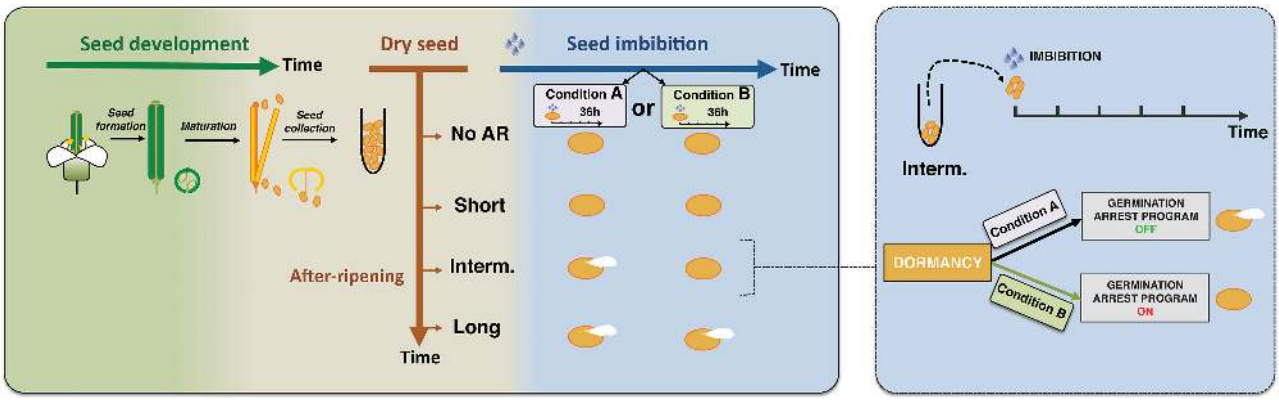

Fig. 2. Dormancy is a functionally defined trait and a potential dry seed property. Upon completion of seed development, mature dry seeds are harvested and dispatched in several tubes. Seeds are subjected to different after-ripening times. Seeds are then subjected to germination assays using two different favorable conditions called A and B. Condition B is favorable for germination but less so than condition A. Seed germination is assessed after a given amount of time upon imbibition. Seeds lost dormancy under conditions A but not under condition B. Thus, the same seed batch may or may not activate the germination arrest program upon seed imbibition depending on the particular favorable germination condition.

it follows that dormancy is a potential property of the seed. For example, a seed population may appear to lose dormancy rapidly under favorable germination conditions that include white light upon imbibition (condition A), whereas the same population may appear to retain dormancy under less favorable light conditions upon imbibition such as a red pulse followed by darkness (condition B) (Kim *et al.*, 2013).

These considerations led us to the following conclusions. (i) Dormancy is an intrinsic property of newly developed mature dry seeds. The trait can be considered as part of the normal seed maturation program. (ii) Dormancy is a dynamic property of dry seeds since dormancy is progressively released during seed dry after-ripening. (iii) Although an intrinsic property of mature dry seeds, seed dormancy cannot be currently assessed on mature dry seed material. Dormancy is only a potential property of a dry seed, which may or may not be actualized depending on the conditions used for the germination test (Fig. 2).

The developmental program responsible for preventing seed germination upon imbibition is distinct from the property of dormancy. Indeed, blockade of seed germination can still occur even in fully after-ripened seeds (i.e. in non-dormant seeds), upon seed imbibition under unfavorable seed germination conditions as defined above or upon exposure to sufficient concentrations of ABA, which invariably blocks germination.

Understanding seed dormancy therefore requires understanding how it is generated and released during several developmental stages over an extended period of time (Fig. 3): (i) during seed maturation (i.e. at the time when the depth of dormancy stored in seeds is determined); (ii) during dry seed after-ripening (i.e. when dormancy is progressively released); and (iii) upon imbibition (i.e. when the dormant state of the seed is able or unable to activate a germination arrest program, according to the extent of dry after-ripening and the particular favorable germination conditions used).

A genetic approach to understand the processes occurring during stages (i), (ii), and (iii) raises significant difficulties. The consequences of a mutation affecting any of these stages can only be detected when performing a germination test. Unambiguously attributing a given mutation to one or several of these stages can indeed be challenging.

Concerning the germination arrest program, one can in principle distinguish the program responsible for the germination arrest of non-dormant seeds under unfavorable conditions and that activated in dormant seeds under favorable germination conditions. It is highly likely that common downstream factors, such as the GA response factors and ABA response factors, repressing germination are utilized in both cases (see below).

In this review, we focus on describing what appears to be firmly established processes, or at least their regulation, sustaining seed dormancy during seed maturation and after-ripening. Events occurring upon seed imbibition are summarized but not reviewed in detail.

A core dormancy model

(i) Establishment and regulation of seed dormancy during seed maturation

The mechanisms underlying the establishment of seed dormancy during seed development are poorly understood. Although seed dormancy is established during seed maturation, the final depth of dormancy is not only determined during maturation or even during seed development. Indeed, exposure to environmental cues, such as cold temperature, during the vegetative stage, namely before first flowering (BFF), can markedly increase the final depth of seed dormancy (Chen *et al.*, 2014) (see below).

The role of ABA

ABA is a phytohormone that fulfills an orchestrating developmental role essential for proper seed maturation. The developmental role of ABA for seed maturation has been reviewed elsewhere (Vicente-Carbajosa and Carbonero, 2005;

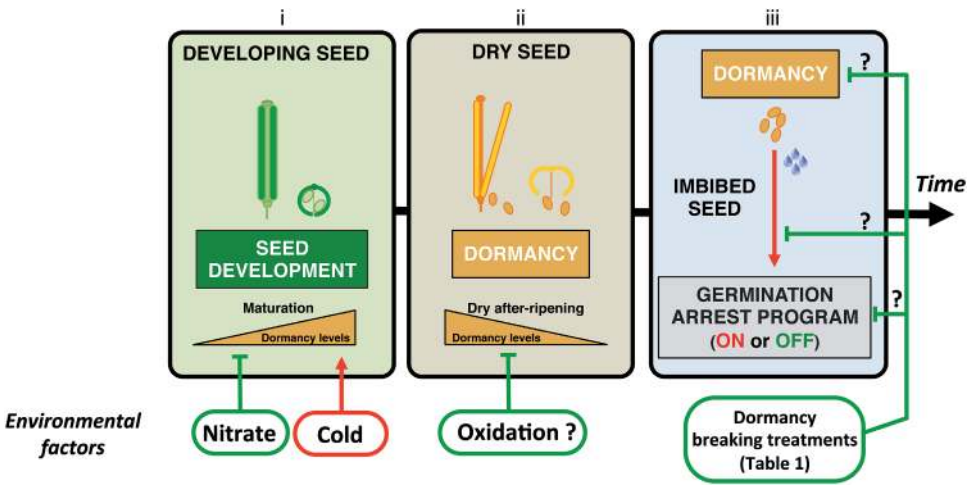

Fig. 3. A core primary dormancy model. Dormancy is a potential property, which may or may not be revealed under favorable germination conditions. This property is built during seed maturation. Depth of dormancy (also called dormancy levels) can be defined by the amount of dry after-ripening time required to lose dormancy. An after-ripening seed progressively loses its capacity to activate the germination arrest program upon imbibition. Environmental factors regulating dormancy levels at each of the developmental stages are shown.

Finkelstein, 2013). In a nutshell, *aba* mutants, unable to synthesize ABA, and ABA signaling mutants fail to develop the characteristic properties of mature seeds (Koornneef *et al.*, 1989; Parcy *et al.*, 1994; Lopez-Molina and Chua, 2000; Nakashima *et al.*, 2009; Delmas *et al.*, 2013). In these mutants the embryos remain green during seed development, have low *LEA* gene expression and low desiccation tolerance, and low levels of seed storage proteins.

Given the essential developmental role played by ABA during seed maturation and given that dormancy is a property of mature seeds, it is rather unsurprising that *aba* mutants and ABA signaling mutants are less dormant (Supplementary Table S1 at *JXB* online). It is therefore safe to assume that ABA promotes the establishment of the seed dormancy during seed development. However, unlike the role played by ABA upon seed imbibition to repress dormant seed germination (see below), the role of ABA during maturation to promote dormancy is less well understood. Indeed, the final ABA levels present in mature dry seeds are unrelated to the depth of dormancy of the dry seed (Ali-Rachedi *et al.*, 2004; Lee *et al.*, 2010). This could suggest that ABA abundance or signaling, or both, play an indirect role in promoting seed dormancy during seed development.

Concerning the origin of ABA acting to promote dormancy during seed development, elegant genetic experiments led Koornneef *et al.* (1989) to conclude that acquisition of seed dormancy involves ABA produced by fertilization-derived seed tissues rather than that produced by maternal tissues (Karssen *et al.*, 1983). These conclusions are further supported by a more recent study (Kanno *et al.*, 2010).

The role of environmental cues: cold

The depth of dormancy stored in the seed is not fixed since it can be significantly influenced by environmental conditions faced by the mother plant. Temperature is the most important known parameter to influence seed dormancy. Indeed, exposure of Arabidopsis plants to cold temperatures during seed development or even during their vegetative phase (BFF) can markedly increase final seed dormancy depth (Chen *et al.*, 2014). In contrast, exposure to warm temperatures tends to reduce the depth of seed dormancy (Schmuths *et al.*, 2006; Huang *et al.*, 2014; Burghardt *et al.*, 2016). How temperature regulates seed dormancy during seed set is poorly understood. Exposure of the mother plant to cold temperature led to changes in the expression of ABA biosynthesis genes such as *CYP707A2* during seed maturation (Kendall *et al.*, 2011). This was associated with higher endogenous ABA levels. Thus, cold temperatures may regulate the depth of seed dormancy by interfering with ABA biosynthesis or signaling during seed development.

Interestingly, Springthorpe and Penfield (2015) recently identified a threshold of temperature which serves as a switch for the final dormancy level: high dormancy is mostly found in seed populations that developed below 14 °C whereas the converse is found above 15 °C. These authors provided evidence that Arabidopsis plants living in different latitudes adjust their flowering time so that their seed development occurs within the 14–15 °C temperature range. This would produce seed populations with a mixture of dormant and non-dormant individuals, leading to a given seed population having the possibility to germinate at different times, thus minimizing risk and competition among seedlings (Springthorpe and Penfield, 2015). Furthermore, a recent study reported that under natural conditions a given plant may produce seeds with different depths of dormancy according to the particular temperature it experiences during seed development (Burghardt *et al.*, 2016). Both reports propose that regulation of the final depth of seed dormancy by temperature during seed set increases seed adaptability to future environments and therefore plant fitness.

A recent report has shown that cold affects dormancy through *FLOWERING LOCUS T* (*FT*) (Chen *et al.*, 2014). Indeed, *ft* mutant seeds are highly dormant when set at low (16 °C) or high (22 °C) temperatures (Chen *et al.*, 2014). Intriguingly, dormancy responses to temperature during seed setting appear to involve changes in testa composition (Chen *et al.*, 2014). The testa gives the brown pigmentation of Arabidopsis mature seeds, which arises from the end-products of the flavonoid biosynthetic pathway such as tannin and derivatives of quercetin (Debeaujon *et al.*, 2000; Chen *et al.*, 2014). As the testa is a purely maternal tissue, it is rather unsurprising that testa pigmentation is under maternal genetic control (Debeaujon *et al.*, 2000; Chen *et al.*, 2014). Previous reports showed that *transparent testa* (*tt*) mutants, deficient in flavonoid biosynthesis, exhibit a lower depth of dormancy (Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000). Conversely, the higher depth of dormancy of *ft* mutants is associated with an overaccumulation of tannin itself due to high expression of *TT2*, encoding a R2R3 MYB transcription factor promoting proanthocyanidin biosynthesis, in fruits (Supplementary Table S1; Nesi *et al.*, 2001; Chen *et al.*, 2014; MacGregor *et al.*, 2015).

How changes in tannin biosynthesis affect seed dormancy is unclear. Flavonoid synthesis may affect seed maturation and thus affect seed dormancy in an unforeseen manner. However, changes in testa pigmentation were shown to be inversely correlated with biophysical changes in seed permeability to tetrazolium dye (Debeaujon *et al.*, 2000; Chen *et al.*, 2014). Increased dry seed permeability to exogenous compounds, such as oxygen, may account for loss not only in final dormancy depth but also in seed viability (see below) (Fig. 4).

The role of environmental cues: nitrate

Nitrate and phosphate are major nutrient sources sustaining plant growth. Only nitrate appears to influence dormancy, although not as markedly as temperature (He *et al.*, 2014). Arabidopsis plants cultivated under high nitrate concentrations produce seeds with higher nitrate content and lower depth of dormancy (Alboresi *et al.*, 2005; He *et al.*, 2014).

High nitrate reduces the final ABA levels in dry mature seeds, which correlates with higher expression of the ABA catabolic gene *CYP707A2* (Matakiadis *et al.*, 2009; Kendall *et al.*, 2011). Thus, nitrate could affect final seed dormancy by modulating ABA metabolism or signaling in developing seeds.

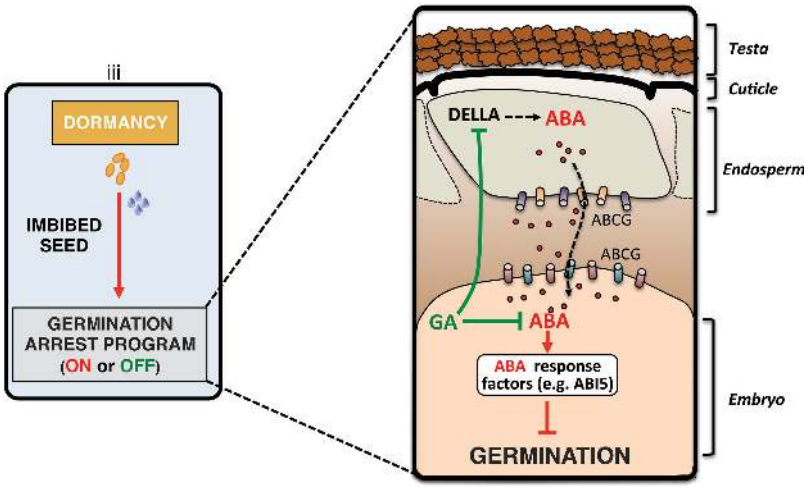

Fig. 4. A simplified model describing the germination arrest program. An unknown dry seed dormancy mechanism may activate the germination arrest program upon imbibition. This involves DELLA-dependent ABA production and release from the endosperm towards the embryo, which may involve ABCG transporters. ABA produced by embryonic tissues also contributes to the germination arrest. ABA response factors are ultimately responsible for blocking seed germination.

Interestingly, low temperature and low nitrate during seed development lead to similar changes in metabolites associated with nitrogen metabolism in dry seeds such as decreased contents in asparagine, γ-aminobutyric acid, and allantoin (He *et al.*, 2016). This is associated with changes in mRNA expression levels of nitrogen metabolism genes, including down-regulation of *NITRATE REDUCTASE 1* (*NIA1*) and *NITRITE REDUCTASE 1* (*NIR1*) expression and up-regulation of *NITRILASE 4* (*NIT4*) and *NITRATE TRANSPORTER 1.1* (*NRT1.1*) expression (He *et al.*, 2016). Kendall *et al.* (2011) also showed that warm temperatures during seed development, which decrease dormancy, were associated with high *NIA1*, *NIR1*, and *NITRATE REDUCTASE 2* (*NIA2*) expression in dry seeds. These data could suggest that temperature could regulate dormancy by influencing nitrate metabolism during seed development (Kendall *et al.*, 2011).

The role of environmental cues: light

Light is a fundamental environmental cue in plants, which has profound developmental consequences throughout their life cycle. The role of light quantity or quality in seed dormancy is not fully understood.

Concerning the role of light quantity, low light intensity during seed maturation results in higher seed dormancy (He *et al.*, 2014, 2016). However, long-day or short-day conditions during seed maturation have no significant influence on the final depth of seed dormancy (Donohue, 2005; Chiang *et al.*, 2009; Chen *et al.*, 2014). Thus, light intensity but not photoperiod appears to regulate dry seed dormancy significantly in Arabidopsis.

Concerning light quality, the depth of seed dormancy can be altered by the spectral properties of light during seed development. Arabidopsis plants cultivated in the presence of a high ratio of FR to red (R) light produce seeds with a greater depth of dormancy relative to that produced by plants grown under a low FR to R ratio (McCullough and Shropshire, 1970; Hayes and Klein, 1974). Thus, R/FR ratios can influence the depth of seed dormancy.

In summary, plants exposed to low temperature, nitrate, low light, and canopy light (high FR to R ratio) during seed development tend to regulate the dormancy of the seeds they produce (He *et al.*, 2016). Under natural conditions, these environmental factors probably congregate to modulate the final seed dormancy depth.

(ii) Loss of dormancy during dry after-ripening

Perhaps the most challenging unresolved question in seed biology is to (i) identify the molecular nature of the dormancy stored in the dry seed and (ii) understand how dormancy is released during dry after-ripening (Fig. 3). Indeed, the molecular nature of these processes at the heart of dry seed primary dormancy remains a mystery.

We review below the environmental parameters that could regulate the depth of dormancy of the mature dry seed as it ages during dry after-ripening.

Cellular metabolic processes most probably play a negligible role during after-ripening

The depth of dormancy of a mature dry seed diminishes over time during after-ripening. A previous report on tobacco seeds has suggested that dry seeds contain an uneven distribution of moisture (Leubner-Metzger, 2005). This could indicate that dry Arabidopsis seeds could also contain pockets of humidity where cellular metabolic processes could take place, such as gene transcription or enzyme-driven protein modifications. In turn, such processes could sustain the observed decay in dormancy depth during after-ripening. However, it remains to be shown unambiguously that such processes do indeed take place during Arabidopsis seed after-ripening.

There are few reports documenting whole-genome expression studies comparing mRNA levels between freshly harvested dormant dry seeds and after-ripened non-dormant dry seeds. Meimoun *et al.* (2014) performed such an experiment with sunflower seeds and concluded that there were no significant changes in mRNA abundance during after-ripening.

Finch-Savage *et al.* (2007) compared the transcriptome of dormant and after-ripened dry seeds in Arabidopsis and did not observe majors differences either. However, the authors mention that their use of different seed batches in their analysis is a potential caveat (Finch-Savage *et al.*, 2007).

Changes in mRNA abundance have been reported for some specific genes during dry after-ripening (Bentsink *et al.*, 2010; Rueda-Romero *et al.*, 2012). Intriguingly, Muller *et al.* (2009) provided evidence that *RESPIRATORY BURST OXIDASE HOMOLOG B* (*RBOHB*), encoding an NADPH-oxidase producing superoxides, is differentially spliced in fresh and after-ripened dry seeds. Whether differential splicing during after-ripening is a general phenomenon remains to be determined.

Altogether, available reports appear to indicate that very few changes in gene expression take place during dry after-ripening. This would suggest that dormancy release during dry after-ripening is the result of chemical transformations, unrelated to normal cellular metabolic processes, that affect the seed's metabolic products already present in freshly produced seeds.

Increased oxidation events during seed after-ripening

During dry after-ripening, seeds are exposed to air and are therefore expected to accumulate oxidative events (Job *et al.*, 2005; Sano *et al.*, 2016). Indeed, a number of reports have documented increases in protein carbonylation during after-ripening (Job *et al.*, 2005; Rajjou *et al.*, 2008). Furthermore, accumulation of lipid oxidation was also reported (Mene-Saffrane *et al.*, 2010; De Giorgi *et al.*, 2015). Therefore, authors have proposed that oxidation events could be responsible for the loss of dormancy (reviewed in Bailly *et al.*, 2008; El-Maarouf-Bouteau and Bailly, 2008). Consistent with this notion, accelerated aging procedures, where temperature and relative humidity are increased, tend to accelerate dormancy release but also, unsurprisingly, affect seed viability (Rajjou *et al.*, 2008; Basbouss-Serhal *et al.*, 2016).

Cruciferins (CRUs) are the most abundant proteins present in Arabidopsis seeds (Koornneef and Karssen, 1994). Previous reports have shown that CRUs are carbonylated during after-ripening (Rajjou *et al.*, 2008). Nguyen *et al.* (2015) recently reported that triple mutant seeds lacking three CRU isoforms (CRUa, CRUb, and CRUc) accumulate high overall levels of protein carbonylation and are less viable. This led the authors to propose that CRUs buffer seeds from oxidative stress (Nguyen *et al.*, 2015). Interestingly, Nguyen *et al*. did not find significant changes in the depth of dormancy of *crua*/*crub*/*cruc* triple mutant seeds despite their high oxidation state. These results therefore appear to be inconsistent with the hypothesis that oxidation is linked to loss of dormancy depth in Arabidopsis.

However, De Giorgi *et al.* (2015) recently described a thick cuticle covering the entire outer surface of the mature seed endosperm. Mutant seeds deficient in cutin biosynthesis have increased permeability to toluidine blue and accumulate higher levels of lipid oxidation (see below). Furthermore, cutin biosynthesis mutant seeds have a lower depth of dormancy (see below) (Supplementary Table S1; De Giorgi *et al.*, 2015).

Altogether, these results could suggest that increased oxidation during dry after-ripening is an attractive hypothesis to explain loss of the depth of dormancy. The specific molecular targets of these oxidation events and indeed the molecular nature of dormancy in dry seeds remain to be uncovered.

Genetic evidence suggests that ROS (reactive oxygen species) homeostasis mutants have alterations in seed dormancy. *CATALASE2* (*CAT2*) encodes a peroxisomal catalase that destroys the toxic H_2O_2 (Queval *et al.*, 2007). *VITAMIN E DEFICIENT 1* (*VTE1*) encodes a tocopherol cyclase essential for tocopherol synthesis (Sattler *et al.*, 2004). Tocopherol protects lipids from oxidation by scavenging ROS (Sattler *et al.*, 2004). The *RESPIRATORY BURST OXIDASE HOMOLOG D* (*RBOHD*) encodes a NADPH oxidase that catalyzes the production of apoplastic superoxide from oxygen and NADPH (Sagi and Fluhr, 2006).

CAT2, *VTE1*, and *RBOHD* are expressed during seed development and upon seed imbibition. *cat2* and *vte1* mutants accumulate high ROS levels and produce seeds with low dormancy (Supplementary Table S1; Leymarie *et al.*, 2012). In contrast, *rbohd* mutants accumulate low ROS levels and display high dormancy (Supplementary Table S1; Leymarie *et al.*, 2012).

Altogether, these results indicate that ROS homeostasis plays an important role in regulating seed dormancy. They are also consistent with the hypothesis that oxidative events play an important role in regulating the depth of dormancy.

Carrera *et al.* (2008) elaborated a genetic approach to identify after-ripening-dependent gene expression programs upon seed imbibition. ABA biosynthesis and signaling mutants lacking dormancy retain a high germination potential irrespective of the dry after-ripening time. Thus, transcriptome profiling upon imbibition of mutant seeds with or without previous after-ripening can be performed to identify gene networks specifically associated with seed after-ripening (Carrera *et al.*, 2008). These authors showed that these networks included genes encoding repressors and activators of ABA function. These results indicate that dormancy release during after-ripening is intimately connected with ABA function upon seed imbibition.

(iii) Events upon seed imbibition: germination arrest programs

Another unresolved question in seed biology is to determine how the intrinsic dormancy of a dry seed interacts with the germination arrest program upon seed imbibition (Fig. 3). We discuss here what appears to be established concerning the nature of the germination arrest program.

The role of ABA

Exposing non-dormant seeds to ABA blocks their germination. Furthermore, unfavorable germination conditions repress seed germination, which involves *de novo* endogenous ABA accumulation (Kim *et al.*, 2008; Toh *et al.*, 2008; Piskurewicz *et al.*, 2009). Thus, ABA appears to play an important role to repress non-dormant seed germination upon seed imbibition.

Concerning the role of ABA upon dormant seed imbibition, Ali-Rachedi *et al.* (2004) showed that dormant Cape Verde Islands (Cvi) seeds maintain high ABA levels upon seed imbibition, unlike after-ripened (i.e. non-dormant) seeds. Furthermore, treatment of dormant seeds with norflurazon or fluridone, inhibitors of ABA synthesis, triggers seed germination upon imbibition (Debeaujon and Koornneef, 2000; Ali-Rachedi *et al.*, 2004; Lee *et al.*, 2010). Altogether these experiments strongly indicate that dormant seeds activate ABA synthesis and signaling upon imbibition, which blocks germination.

The role of GA

GA and ABA are often viewed as hormones exerting an antagonistic role to control seed germination. However, exogenous GA poorly promotes the germination of highly dormant Arabidopsis accessions such as Cvi (Ali-Rachedi *et al.*, 2004; Lee *et al.*, 2010). Lee *et al.* (2010) showed that exogenous GA did not down-regulate the accumulation of the GA response factor RGA-LIKE 2 (RGL2), a repressor of seed germination, in highly dormant Cvi seeds, suggesting that Cvi dormant seeds do not have the capacity to respond to GA. This is consistent with a recent report showing that GA sensitivity increased during after-ripening (Hauvermale *et al.*, 2015). An interesting topic for future investigation will be to explore how dormant seeds acquire the capacity to signal in response to GA during after-ripening.

The role of the testa and endosperm

Removal of the testa and endosperm in imbibed dormant seeds triggers embryonic growth and greening (Bethke *et al.*, 2007; Lee *et al.*, 2010). Furthermore, Bethke *et al.* (2007) removed the testa of dormant seeds while maintaining intact the endosperm surrounding the embryo. Under these conditions, testa-less seeds did not germinate. These experiments show that the endosperm is necessary to repress seed germination of dormant seeds. Nevertheless, this does not rule out that the testa could contain or regulate the depth of dormancy of the mature dry seed. The former case could involve deposits that would migrate towards the endosperm upon imbibition to activate its germination repressive machinery. The ability of these deposits to do so would in turn decrease as the seed ages. Alternatively, the depth of dormancy could be contained in the endosperm but be further regulated by deposits in the testa.

Lee *et al.* (2010) further studied the germination-repressive activity of the endosperm by means of a seed coat bedding assay (SCBA). In this assay, embryos are dissected shortly upon seed imbibition and cultured under a layer of dissected endosperm still attached to the seed's testa. This allows monitoring the growth of the embryo under the influence of the underlying layer of endosperm tissue. Lee *et al.* (2010) could show that endosperm tissue isolated from dormant seeds was able to prevent the growth of embryos, unlike that isolated from non-dormant seeds. Furthermore, endosperm from dormant seeds was shown to release ABA in higher quantities relative to endosperm from non-dormant seeds (Lee *et al.*, 2010). The SCBA was recently used with non-dormant seed material to identify the ABC transporters of the ABCG subfamily, AtABCG25 and AtABCG31, as acting in concert to export ABA from the endosperm towards the embryo. The same study identified AtABCG30 and AtABCG40 as importers of ABA into the embryo (Fig. 4; Kang *et al.*, 2015). Thus, these transporters could serve similar functions to block the germination of dormant seeds, but whether this is indeed the case remains to be investigated.

Altogether, these reports conclusively point to the endosperm as a tissue serving a fundamental function in seed dormancy. First, its presence is essential to repress dormant seed germination, as shown by Bethke *et al.* (2007). Secondly, experiments using a SCBA show that the endosperm displays a germination-repressive activity whose strength is highest in freshly produced seeds. Thirdly, this higher activity is associated with higher release of endospermic ABA, which plays an essential role to repress seed germination of dormant seeds (Lee *et al.*, 2010). Thus, the SCBA experiments indicate that endosperm and testa contain the elusive dormancy mechanism present in dry seeds. Furthermore, the endosperm contains at least part of the germination-repressive machinery by means of its release of ABA, which is essential to block the germination of dormant seeds.

Treatments breaking seed dormancy upon seed imbibition

It should be noted that a number of treatments upon imbibition could trigger germination of dormant seeds. How these treatments trigger seed germination is poorly understood. They could in principle affect the depth of dormancy present in the dry seed or the germination arrest program, or both. These treatments can be found in Table 1, and references reviewing their putative effects are provided.

Quantitative trait locus (QTL) approaches to study dormancy

In recent years, an increasing number of Arabidopsis mutants with alterations in seed dormancy have been uncovered. As discussed above, genetic analysis of seed dormancy relies on a germination assay and therefore is an intrinsically challenging task due to the inherent difficulty in establishing which of the seed's developmental stages is affected in the mutant, namely: (i) the build up of dormancy during seed maturation; (ii) the release of dormancy during after-ripening; or (iii) the interaction of dry seed dormancy with the germination arrest program (Figs 3, 4).

Different Arabidopsis accessions can produce seeds with vastly different depths of dormancy. Researchers have used QTL or genome-wide association study (GWAS) mapping approaches to identify genetic loci regulating seed dormancy (van Der Schaar *et al.*, 1997; Alonso-Blanco *et al.*, 2003; Laserna *et al.*, 2008; Meng *et al.*, 2008; Bentsink *et al.*, 2010; Yano *et al.*, 2013; Morrison and Linder, 2014; Postma and Agren, 2016).

Table 1. *Methods used to alleviate dormancy of Arabidopsis seeds*

Methods	Examples	Putative effects	References
Stratification	4 d at 4 °C in the dark	Enhanced GA biosynthesis and signaling	Debeaujon *et al.* (2000); Yamauchi *et al.* (2004)
Scarification	Removing the seed coat (testa+endosperm)	Removing ABA release from the endosperm	Bethke *et al.* (2007); Lee *et al.* (2010)
ABA inhibitor	Norflurazon; fluridone	Inhibition of ABA biosynthesis	Ali-Rachedi *et al.* (2004); Lee *et al.* (2010)
Nitrate	KNO_3	Decreased ABA content	Ali-Rachedi *et al.* (2004); Matakiadis *et al.* (2009)
Nitrite	$NaNO_2$	Unknown	Bethke *et al.* (2006a, b)
NO	SNP vapor	Decreased ABA content	Liu *et al.* (2009)
Cyanide	Fe(II)CN vapor; Fe(III)CN vapor; KCN vapor	Probably decreased ABA content (cyanide is the primary dormancy-breaking compound produced by SNP)	Bethke *et al.* (2006a, b)
Karrikin	KAR_1; KAR_2	Enhanced GA biosynthesis and signaling	Nelson *et al.* (2009, 2011)
Strigolactone	GR-24	Enhanced karrikin signaling and GA signaling	Nelson *et al.* (2009, 2011)
ROS	ROS donor: methylviologen; menadione	Unknown	Leymarie *et al.* (2012)

We describe two genetic loci identified in QTL genetic studies that could provide insight on the regulation of the depth of seed dormancy during seed maturation and after-ripening. Supplementary Table S1 compiles a more exhaustive list of mutants affected in seed dormancy.

DELAY OF GERMINATION1 (DOG1)

The *DOG1* QTL was first identified by Alonso-Blanco *et al.* (2003) who studied the natural dormancy variation between the high dormancy Cvi accession and the low dormancy Landsberg *erecta* (L*er*) accession. *DOG1* promotes seed dormancy.

The *DOG1* gene was subsequently identified by Bentsink *et al.* (2006). Although DOG1 has been the subject of numerous reports, its molecular function remains unclear (reviewed in Nonogaki, 2014). Recent reports have proposed that *DOG1*'s promotion of dormancy involves alternative splicing and DOG1 self-binding capacity and regulation of miRNA156 expression (Nakabayashi *et al.*, 2015; Cyrek *et al.*, 2016; Huo *et al.*, 2016).

DOG1 is mainly expressed during seed development, and the DOG1 protein remains present in mature dry seeds (Supplementary Table S1; Nakabayashi *et al.*, 2012). Nakabayashi *et al*. proposed that DOG1 protein levels highly correlate with depth of dormancy in fresh seeds. *DOG1* mRNA and DOG1 protein remain constant throughout after-ripening, suggesting that DOG1 activity to promote dormancy decreases over time in dry seeds (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012). Indeed, Nakabayashi *et al.* showed alterations in DOG1 isoelectric focusing during after-ripening, suggesting a change in DOG1 structure and activity.

Interestingly, *DOG1* mRNA expression decreases, relative to that of dry seed, upon imbibition in both dormant and after-ripened seeds (Bentsink *et al.*, 2006; Nakabayashi *et al.*, 2012). In contrast, DOG1 protein levels remain unchanged upon imbibition in both dormant and after-ripened seeds.

Dekkers *et al*. (2016) presented evidence that *DOG1* promotes seed maturation during seed development. Indeed, the transcriptome of dry *dog1* seeds presents defects in gene expression relative to normal seeds. Moreover, *DOG1* genetically interacts with the ABA response factor *ABI3*, which promotes maturation during seed development (Delmas *et al.*, 2013; Dekkers *et al.*, 2016). Furthermore, *dog1* mutant dry seeds have a lower ABA content. This led Dekkers *et al.* (2016) to propose that DOG1 and ABI3 act in parallel to promote maturation processes. Altogether, these reports support the notion that DOG1 promotes dormancy during seed development. However, how it does so remains unknown.

Concerning the role of DOG1 upon imbibition, available data provide contrasting results. *cyp707a2* mutants accumulate high ABA levels in dry seeds and upon seed imbibition, and are highly dormant (Okamoto *et al.*, 2006). *dog1/cyp707a2* mutants are less dormant than *cyp707a2* mutants (Nakabayashi *et al.*, 2012). However, ABA levels in *dog1/cyp707a2* dry seeds were similar to those of *cyp707a2* mutants although the levels of ABA upon imbibition were not reported (Nakabayashi *et al.*, 2012). Thus, these data suggest that DOG1 positively regulates ABA responses upon imbibition. On the other hand, Bentsink *et al.* (2006) showed that *dog1* mutants respond normally to exogenous ABA, suggesting that DOG1 does not regulate ABA signaling upon imbibition. Interestingly, Kinoshita *et al.* (2010) showed that *DOG1* expression was up-regulated in *abi5* mutants upon seed imbibition in the presence of ABA, suggesting the presence of a crosstalk between ABA responses and *DOG1* expression upon imbibition. The relationship between DOG1 and ABA metabolism and signaling remains to be further understood.

REDUCED DORMANCY 5 (RDO5)/DOG18/IBO

Bentsink *et al.* (2010) identified the *DOG18* QTL, which is associated with high dormancy. The *DOG18* gene was recently identified and shown to be identical to *RDO5* previously identified in a genetic screen for mutants impaired in dormancy (Xiang *et al.*, 2014, 2016). Furthermore, Amiguet-Vercher *et al.* (2015) identified the *IBO* QTL promoting

dormancy in the Loch Ness-0 (Lc-0) accession relative to the Eilenburg-0 (Eil-0) accession. The *IBO* gene turned out to be identical to *RDO5*/*DOG18*.

rdo5 loss-of-function mutant seeds displayed a non-dormant phenotype, suggesting that RDO5/DOG18/IBO is a positive regulator of seed dormancy (Supplementary Table S1; Xiang *et al.*, 2014). Furthermore, Xiang *et al.* (2014) showed that *rdo5* mutants have normal ABA levels in seeds as well as normal responses to ABA.

RDO5/*DOG18*/*IBO* encodes a previously uncharacterized protein phosphatase 2C (PP2C) (Xiang *et al.*, 2014; Amiguet-Vercher *et al.*, 2015). There are 10 recognized groups of PP2Cs (referred as group A–J) in Arabidopsis (Schweighofer *et al.*, 2004). PP2Cs belonging to group A are negative regulators of ABA signaling and seed dormancy (Finkelstein, 2013; Kim *et al.*, 2013).

RDO5/*DOG18*/*IBO* belongs to none of these PP2C groups (Schweighofer *et al.*, 2004). The RDO5/DOG18/IBO protein lacks amino acid residues crucial for phosphatase activity. Unsurprisingly, no RDO5/DOG18/IBO phosphatase activity could be detected *in vitro* (Amiguet-Vercher *et al.*, 2015; Xiang *et al.*, 2016). Interestingly however, IBO^{LC-0} was able to interfere with the activity of other PP2Cs *in vitro* (Amiguet-Vercher *et al.*, 2015).

Amiguet-Vercher *et al.* (2015) introgressed IBO^{Lc-0} into *pp2c* loss-of-function mutant backgrounds, which enhanced sensitivity to ABA. Furthermore, a decrease in general protein phosphorylation during seed imbibition was enhanced in *rdo5* mutant seeds, suggesting that RDO5/DOG18/IBO can indeed influence phosphorylation *in vivo* (Xiang *et al.*, 2016).

Altogether, these results indicate that RDO5/DOG18/IBO could affect ABA signaling by interfering with PP2C activities involved in ABA signaling. RDO5/DOG18/IBO would do so during seed development rather than upon seed imbibition given the normal responses of *rdo5* mutant seeds to ABA upon imbibition. RDO5/DOG18/IBO would therefore affect the depth of dormancy in dry seeds.

Conclusions

Despite decades of research on seed dormancy, the trait remains poorly understood at the molecular genetic level. Surprisingly little is known about what can be considered the essence of dormancy, namely the molecular nature of the primary dormancy intrinsic to the dry seed and how it diminishes over time during the seed's dry after-ripening. This is clearly an issue that deserves closer attention for future investigations. Understanding the nature of dormancy intrinsic to the dry seed would provide a reference point to understand its genesis during seed maturation, including its regulation by environmental cues, and the nature of its interaction with the germination arrest program.

As discussed in this review, the endosperm appears to be necessary and sufficient to implement seed dormancy in Arabidopsis. Focusing on the role of the endosperm in seed dormancy could be a fruitful approach to understand seed dormancy.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. List of mutants affected in seed dormancy.

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