DELAY OF GERMINATION 1 mediates a conserved coat dormancy mechanism for temperature- and gibberellin-dependent control of germination

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Seed germination is an important life-cycle transition as it determines subsequent plant survival and reproductive success. To detect optimal spatio-temporal conditions for germination, seeds act as sophisticated environmental sensors integrating information such as ambient temperature. Here we show that the DELAY OF GERMINATION 1 (DOG1) gene, known for providing dormancy adaptation to distinct environments, determines the temperature optimum for seed germination. We show by reciprocal gene swapping experiments between Brassicaceae species that the DOG1-mediated dormancy mechanism is conserved. Biomechanical analyses show that this mechanism regulates the material properties of the endosperm, a seed tissue layer acting as germination barrier to control coat dormancy. We found that DOG1 inhibits the expression of gibberellin (GA)-regulated genes encoding cell-wall remodelling proteins in a temperature-dependent manner. Furthermore we demonstrate that DOG1 causes temperature-dependent alterations in the seed GA metabolism. This is brought about by temperature-dependent differential expression regulation of genes encoding key enzymes of the GA biosynthetic pathway. These effects of DOG1 lead to a temperature-dependent control of endosperm weakening and determine the temperature optimum for germination. The conserved DOG1 mediated coat dormancy mechanism provides a highly adaptable temperature sensing mechanism to control germination timing.

dormancy gene DOG1 | endosperm weakening | coat dormancy | gibberellin metabolism | germination temperature

Introduction

Seed dormancy is an important adaptive early-life history trait as it controls the distribution of germination in space (e.g. habitat selection) and time (e.g. seasonal temperature changes). Ecophysiological work has shown that seed dormancy is a crucial fitness component with far-reaching consequences for the evolution of entire life histories (1-3). As an innate seed property it defines the environmental conditions in which a seed is able to germinate and ensures that the most vulnerable later phases of the plant life cycle occur during favourable seasonal and environmental conditions. Temperature during seed maturation defines the depth of primary dormancy established upon seed dispersal (4, 5). Furthermore, soil temperature is the major environmental factor for seasonal dormancy cycling of the soil seed bank in temperate regions (6, 7). Especially in regard of a changing climate, it is therefore important to understand the molecular mechanisms of temperature-related traits including dormancy and their role in the adaptation of populations to changing temperatures. The substantial influence of the environment on genetically controlled seed dormancy is mediated, at least in part, by the plant hormones abscisic acid (ABA) and gibberellins (GA) (8-10). Seed contents of and sensitivities to ABA and GA, as well as the properties of the embryo-encasing covering layers are the physiological basis for the germination responses to distinct environments. The genetic basis for the observed natural variation in seed dormancy of *Arabidopsis thaliana* ecotypes are Quantitative Trait Genes (QTGs) including *DELAY OF GERMINATION1 (DOG1)* (3, 10-12). *AtDOG1* is a major dormancy QTGs required for *A. thaliana* seed dormancy and is a decisive component for the environmental adaptation of populations (1, 2, 13-15). Despite its central role neither its (biochemical) function nor its participation in a phylogenetically conserved dormancy mechanism have been elucidated.

Embryo-related developmental processes are mediated by tissue forces in animals and plants. They are determined by the interaction of the embryo and the encasing tissue layers with animal extracellular matrices or plant cell-walls (16, 17). The elongation and straightening of the notochord of vertebrate em-

Significance

Mechanisms of plant seed dormancy evolved to delay germination to a favourable seedling growth season. Germination timing is an important adaptive early-life history trait which determines plant fitness in natural and agricultural ecosystems. The DOG1 gene provides natural genetic variation in dormancy, was the first dormancy-specific gene cloned and encodes a protein of unknown function. We show here that DOG1 controls dormancy of different species by setting the optimal ambient temperature window for germination. This is achieved by temperature-dependent alteration of the gibberellin hormone metabolism which in turn leads to altered expression of genes required for the biomechanical weakening of the coat encasing the embryo. The conserved DOG1-mediated coat dormancy mechanism controls seed germination timing in a temperature-dependent manner.

Reserved for Publication Footnotes

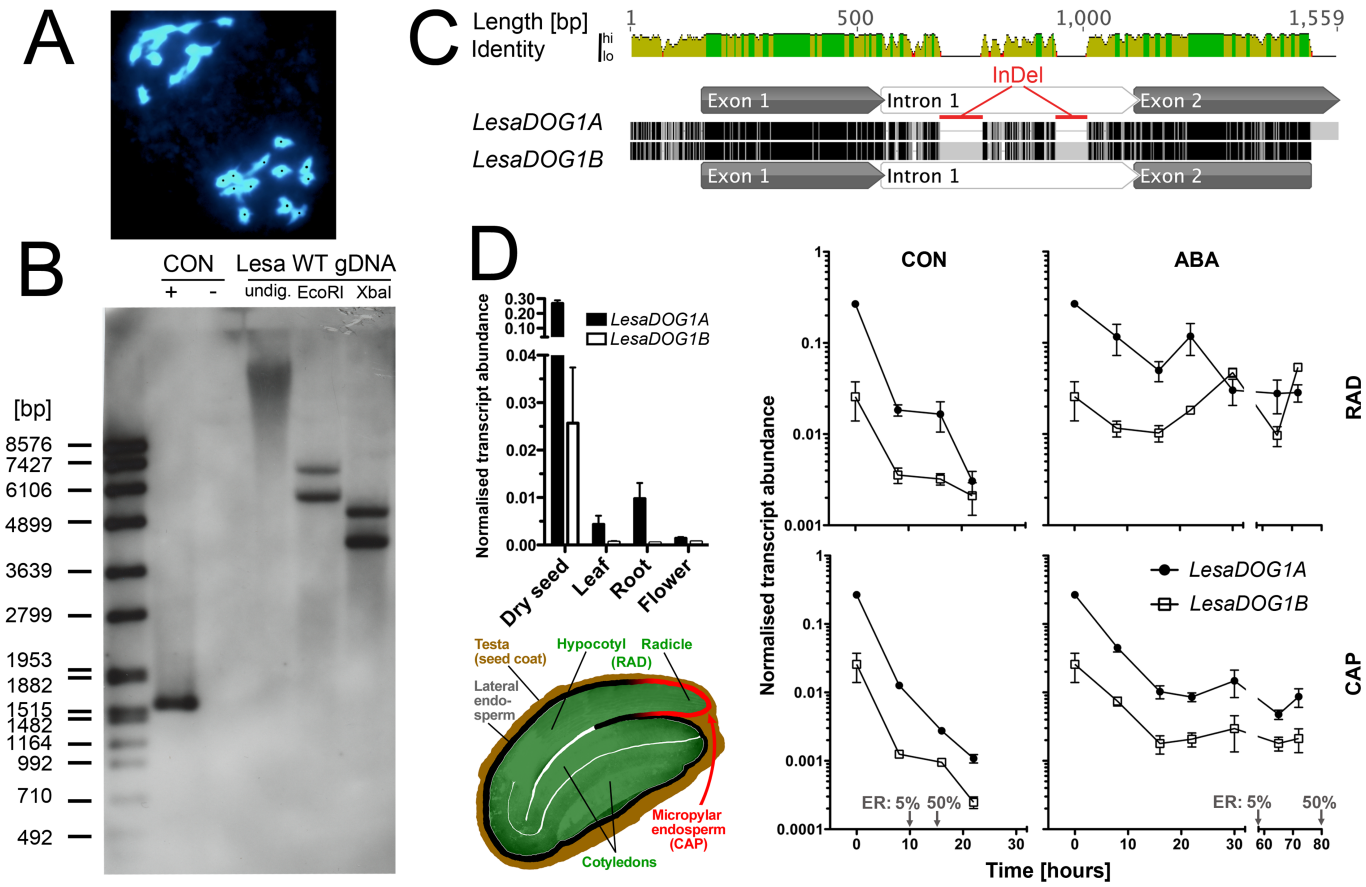

Fig. 1. Two seed-expressed *DOG1* genes, *LesaDOG1A* and *LesaDOG1B*, in the diploid (2n=24) species *Lepidium sativum*. [A] DAPI-stained meiotic (metaphase II) chromosome spreads from flower bud tissue of *L. sativum* exhibiting n=12 chromosomes, thus demonstrating regular meiosis. For better counting, chromosomes are labelled with black dots. [B] Southern blot analysis of the *L. sativum* FR14 genome indicates the presence of two *DOG1* genes. Genomic DNA (Lesa WT gDNA) undigested (undig.) or digested with *Eco*RI or *Xba*I was hybridized with a *LesaDOG1A* probe. Hybridization controls were plasmids with (+) or without (-) *LesaDOG1A* full-length gDNA inserts; *left lane:* DNA molecular mass ladder. [C] Pairwise alignment of *LesaDOG1A* and *LesaDOG1B* (near full-length)gDNA sequences. Identical residues are colored black; mismatches light gray; gaps are indicated as horizontal lines. Exon-Intron annotations were derived by comparison to the respective cDNAs. Two large intronic InDels are marked in red. [D] Transcript abundances of *LesaDOG1A* and *LesaDOG1B* in different plant tissues and during germination in the seed RAD and CAP (as indicated by the schematic *L. sativum* seed drawing) determined by qRT-PCR analysis (N=3, mean ±SEM). Seeds were imbibed without (CON) or with the addition of 10 µM abscisic acid (ABA). Gray arrows indicate time points of 5% or 50% endosperm rupture (ER). RAD comprises the radicle pus ca. 1/3 of the lower hypocotyl (embryo growth zone); CAP is the micropylar endosperm tissue. Note that the dry seed samples (0h) of the qRT-PCR analysis represent CAP+RAD tissue and not whole seeds.

bryos for example depends on the interaction with the surrounding extracellular matrix sheath and its dynamic biomechanical properties (18). In the mature seed of most angiosperms the embryo is encased by two covering layers ('coats'): the living endosperm tissue and the dead testa (seed coat). Whether a plant undergoes a life-cycle transition by completing seed germination or not is controlled by the balance of opposing forces: germination is promoted by the growth potential of the embryo RAD (embryonic radicle-lower hypocotyl axis, embryo growth zone) and inhibited by the restraint weakening of the tissue layers ('coats') covering the RAD (17, 19, 20). Seed germination of many angiosperms including the Brassicaceae *Lepidium sativum* (garden cress) and *A. thaliana* consists of two sequential steps: shortly after imbibition testa rupture (TR) takes place and is subsequently followed by endosperm rupture (ER) and radicle emergence, which is the visible completion of germination. Weakening of the micropylar endosperm (CAP) covering the RAD is a required concurrent process preceding ER. Hormonal signaling and interaction between the key seed compartments RAD and CAP controls the expression of down-stream genes encoding cell wall-remodeling proteins (CWRPs; (21, 22)). These alter the biomechanical properties of cell-walls in RAD (growth) and CAP (weakening) tissues to control germination timing. Little is known about the mechanisms by which QTGs such as *DOG1* mediate the environmental and hormonal control of these processes.

AtDOG1 is a key regulator of seed dormancy because the *A. thaliana dog1* mutant is completely non-dormant and does not exhibit any obvious pleiotropic phenotypes, apart from reduced seed longevity (11, 12). The time required for seed dormancy release during after-ripening storage is determined by AtDOG1 protein levels in dry seeds (5). These accumulate during seed maturation and their accumulation is controlled by temperature. In contrast to the situation during seed maturation, very little is known about the roles of *AtDOG1* during seed germination. The *AtDOG1* gene has been described to belong to a small gene family together with four *AtDOG1-Like* genes in *A. thaliana* and encodes a protein of unknown function (11). Putative *AtDOG1* orthologs are present in *Lepidium* species from varying environments of all continents which exhibit considerable variation in dormancy (23, 24). Monocot *DOG1-Like* genes with a low level of similarity to *AtDOG1* have been found in cereals (25-27). Ectopic expression of some of these cereal *DOG1-Like* genes in *A. thaliana* wild-type seeds delayed germination. It is of interest to investigate if *DOG1* genes from different species provide a conserved dormancy mechanism with a common evolutionary origin as most studies thus far focused on *A. thaliana*.

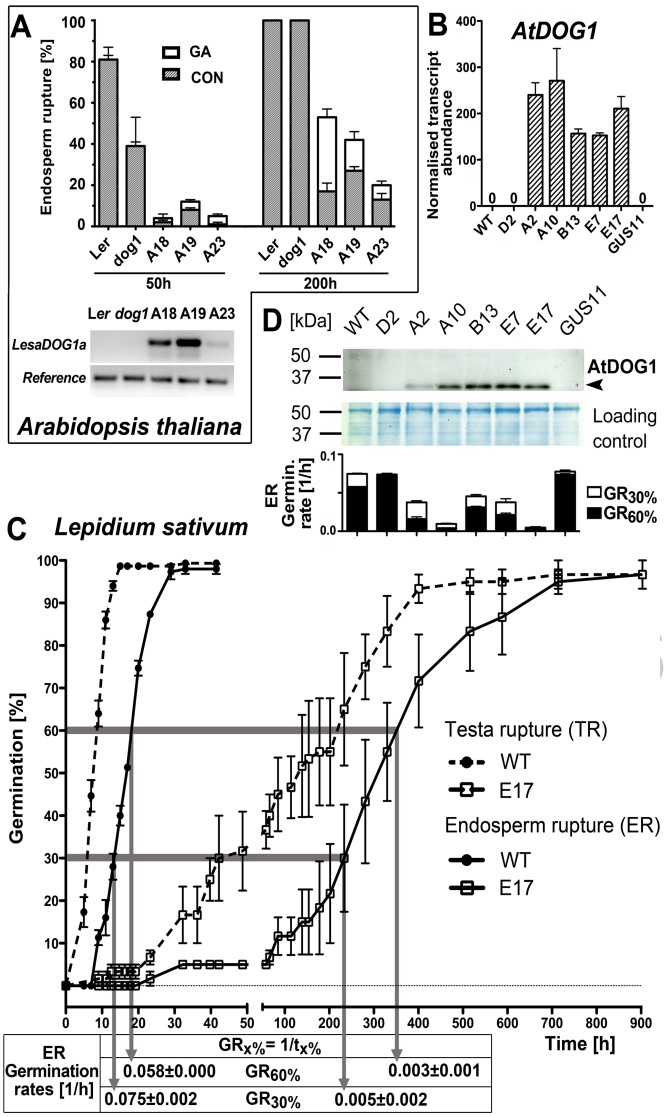

Fig. 2. Reciprocal *DOG1* gene swapping experiments between Brassicaeae reveals a conserved mechanism. Transgenic lines of the *Arabidopsis thaliana dog1* mutant overexpressing LesaDOG1A (*Lepidium sativum)* and transgenic lines of *L. sativum* FR14 overexpressing AtDOG1 (*A. thaliana*) exhibit a delayed germination phenotype. [A] Endosperm rupture of *A. thaliana* Ler wild-type (WT), *dog1* mutant and independent homozygous transgenic *dog1* lines overexpressing LesaDOG1A (At-OxLesaDOG1A-A18, -A19, -A23) during seed imbibition at 24°C. N=3, mean +SEM. The LesaDOG1A-overexpression lines showed a delayed-germination phenotype and a decreased germinability which did not increase beyond 200h. Treatment with 10 µM GA_{4+7} (GA) partially released the dormancy of At-OxLesaDOG1A seeds. *Lower panel:* Semiquantitative RT-PCR indicating expression of the *LesaDOG1A* transgene in dry *A. thaliana* seeds. At2G20000 was used as stable seed-expressed reference gene (45). [B] Independent homozygous transgenic *L. sativum* lines (A2, A10, B13, E7, E17) harboring a chimeric transgene with the CaMV 35S-promoter driving an *A. thaliana* Cvi *DOG1* genomic fragment (Lesa-OxAtDOG1) strongly express *AtDOG1* transcripts (qRT-PCR) in dry seeds. N=4, mean ±SEM.[C] Lesa-OxAtDOG1 lines show a strongly delayed germination phenotype evident from comparative testa and endosperm rupture kinetics of WT and E17. The graph indicates the calculation of the germination rate (GR). N=3, mean ±SEM. [D] Lesa-OxAtDOG1 lines (A2, A10, B13, E7, E17) accumulate AtDOG1 protein in dry seeds and show lower ER GRs compared to WT and GUS11 transformation control.

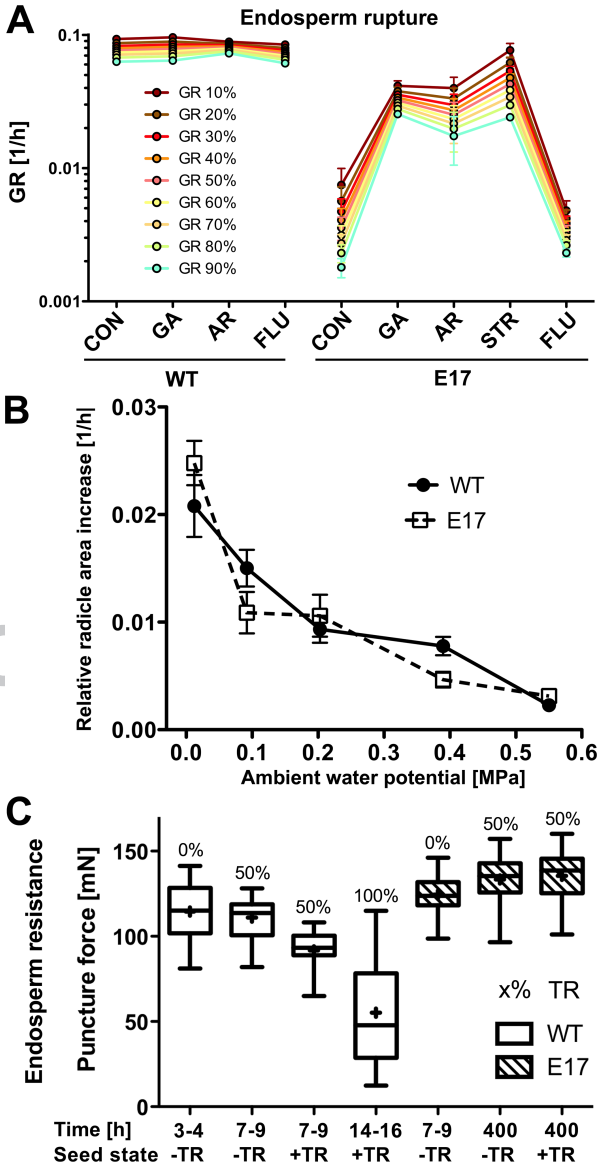

Fig. 3. The delayed germination phenotype of transgenic *Lepidium sativum* is caused by inhibited endosperm CAP weakening. Overexpression of *AtDOG1* in *L. sativum* caused delayed germination which can be rescued by dormancy breaking treatments and is not caused by altered embryo growth potential but by inhibited endosperm CAP weakening. [A] Endosperm rupture germination rates (GRs) of WT and Lesa-OxAtDOG1-E17 at 24°C without (CON) or with dormancy breaking treatments: addition of 10 µM GA_{4+7} (GA), seed dry after-ripening storage for 9 month (AR), addition of 10 µM fluridone (FLU) and cold-stratification pre-treatment at 4°C in the dark for 3 days (STR). N=3, mean ±SEM; for TR data see Fig. S2*B*. [B] Embryo growth potentials of WT and Lesa-OxAtDOG1-E17 as measured by the radicle-hypocotyl axis area increase of excised embryos at different ambient water potentials at 24ºC. Note that there are no significant differences ($p<0.05$) between the embryo growth rates of WT and E17 at any water potential. Each data point represents the relative average radicle area increase (±SEM) of at least 20 imbibed embryos during an incubation period of 27h. [C] Endosperm weakening occurs during germination of *L. sativum* WT and is strongly inhibited in Lesa-OxAtDOG1-E17 seeds. Box plots show endosperm CAP puncture forces of imbibed seeds with (+TR) or without (-TR) testa rupture at 24°C at the times indicated (N=20). TR percentages of the seed population for the respective time points are indicated above box plots.

The Brassicaceae originated as a tropical-subtropical family ca. 37 MYA in a warm and humid climate and subsequently evolved to a dry-adapted family (28). This diversification and radiation upon climate change also required the evolution of mechanisms that adapt seed responses to seasonal temperature cycling. Ancient whole genome duplication (WGD) events lead-

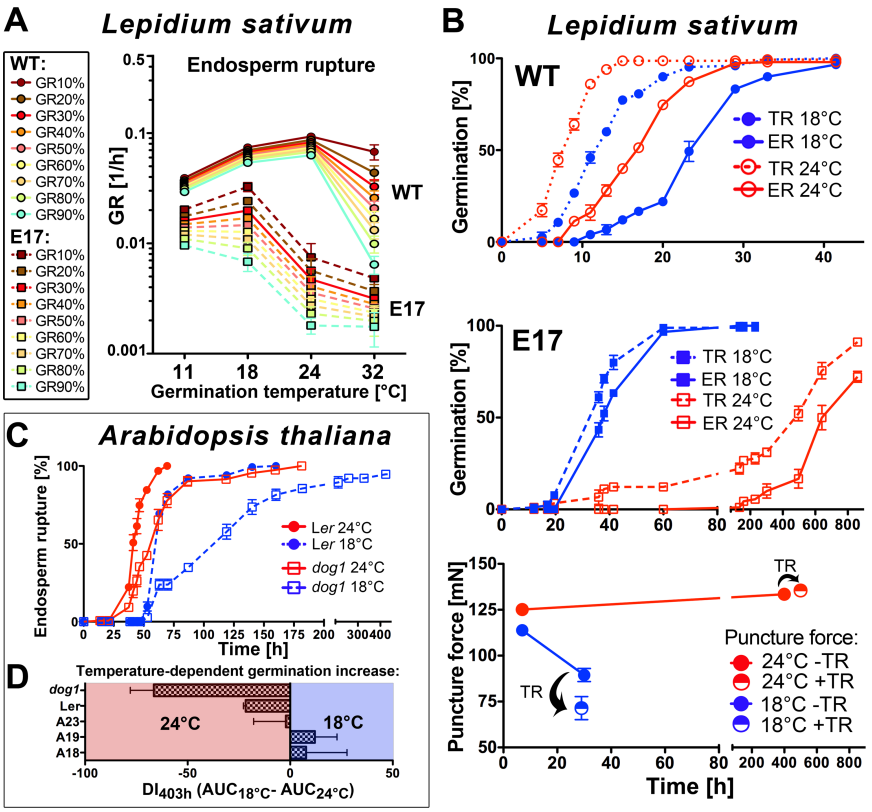

Fig. 4. DOG1 influences delay of germination in a temperature-dependent manner in *Lepidium sativum* and *Arabidopsis thaliana*. [A] Germination rates (GR) for endosperm rupture (ER) at different imbibition temperatures of *L. sativum* WT and transgenic LesaOxAtDOG1-E17 seeds (TR data shown in Fig. S4A). [B] *L. sativum* WT and E17 testa rupture (TR) and ER at 18 and 24°C. Endosperm CAP resistance for E17 quantified by puncture force measurements are shown from seeds either with (+) or without (-) TR. N=20, mean ±SEM. [C] Temperature dependence of *A. thaliana* Ler and *dog1* mutant seed germination. [D] Dormancy index (DI) calculated as the difference of the areas under ER curves at 18°C and 24°C between 0 and 403h. DI is a measure for germination capacity. A positive DI indicates a positive effect of 18°C on germination percentage whereas a negative DI indicates a positive effect of 24°C. N=3, mean +SEM. Note that *dog1* seeds germinate slower compared to *Ler* at 18°C (more negative DI). This effect is reverted in transgenic *dog1* lines overexpressing LesaDOG1A (A18, A19, A23), in that they either germinate faster at 18°C (positive DI) compared to 24°C or no temperature effect is evident (DI=0). All germination kinetics N=3 plates, mean ±SEM.

ing to paleopolyploidy prior to climate changes play a crucial role in the genetic diversification, species radiation and adaptation to new environments (28-30). The monophyletic Brassicaceae genus *Lepidium* (cress) contains a large number of polyploid species suggesting a reticulate evolutionary history and recent allopolyploidization is important for *Lepidium* speciation and range expansion (24, 28, 31). The cultivated spicy sprout crop *L. sativum* is characterized by non-dormant seeds which do not have the after-ripening, cold stratification or light requirements for germination known for the dormant seeds of *A. thaliana*. The larger seeds of *L. sativum* are an established Brassicaceae endosperm CAP weakening model system (21, 23, 32, 33), and thereby provide an interesting choice for studying the potential of dormancy candidate genes from other species: due to the lack of endogenous dormancy, effects of transgenes can be immediately studied on a biomechanical, transcriptional and hormonal level.

We show here by gene-swap experiments between *A. thaliana* and *L. sativum* that DOG1 mediates a conserved GA-related coat dormancy mechanism which determines the seed responses to ambient temperature and has CAP weakening as its major target. Our work provides an integrated view into the underlying molecular mechanisms by which a plant life-cycle transition is controlled in a temperature-dependent manner by alteration of the biomechanical properties of key seed tissues regulating dormancy and germination.

Results

Two Seed-Expressed DOG1 Paralogs Are Present in the Diploid Species (2n=24) *Lepidium sativum*. We have shown previously that *L. sativum* FR14 possesses the seed-expressed *AtDOG1* gene homolog *LesaDOG1* (23). Here we show by Southern blot analysis that *L. sativum* actually possesses two *DOG1* genes (Fig. 1*B*), which we named *LesaDOG1A* (described in (23)) and *LesaDOG1B*. We cloned a near full-length genomic DNA fragment from *LesaDOG1B* and its corresponding cDNA from dry seeds. *LesaDOG1A* and *LesaDOG1B* have conserved intron splice sites and show more than 93% sequence identity in their coding regions. Most differences between the two genes are located in the intronic regions showing less then 50% identity and the presence of two large InDels (Fig. 1*C*). Similar *DOG1* intron sequence variations were also found in other *Lepidium* species (24). The low amount of single nucleotide differences in the coding regions and the occurrence of large intronic InDel blocks point to the young origin of the gene duplication leading to these two paralogs. Such a duplication is likely caused by a recent *Lepidium*-specific polyploidisation event (postulated *Le*-Neo WGD) followed by diploidisation. This is emphasized by our finding that *L. sativum* is diploid (2n=24) with regular meiosis with n=12 (Fig. 1*A*, Table S1) but with a much larger genome size than expected from closely related species. All the investigated 202 *L. sativum* accessions showed a nearly identical relative DNA amount of FC=2.77±0.08 compared to FC=1 for *L. campestre* with 2n=16 (See SI Appendix, Fig. S1, Table S1).

We investigated the expression of the two *LesaDOG1* paralogs and found both predominantly expressed in dry seeds where *LesaDOG1A* shows about 10-times higher transcript abundance compared to *LesaDOG1B* (Fig. 1*D*). Within the seed both genes were expressed in the key compartments RAD (radicle plus lower 1/3 hypocotyl) and CAP (micropylar endosperm), and a rapid decline in the expression levels was evident upon imbibition (Fig. 1*D*). ABA is known to inhibit endosperm CAP weakening required for the completion of germination (21), and ABA also inhibited the decline in transcript abundances for both *LesaDOG1* paralogs (Fig. 1*D*), suggesting a key role for *DOG1* in this process.

The *L. sativum LesaDOG1A* Gene Causes a Delayed-Germination Phenotype upon Overexpression in *A. thaliana dog1* Mutant Seeds. *L. sativum* produces non-dormant seeds although both *LesaDOG1* paralogs are expressed in seeds. We therefore

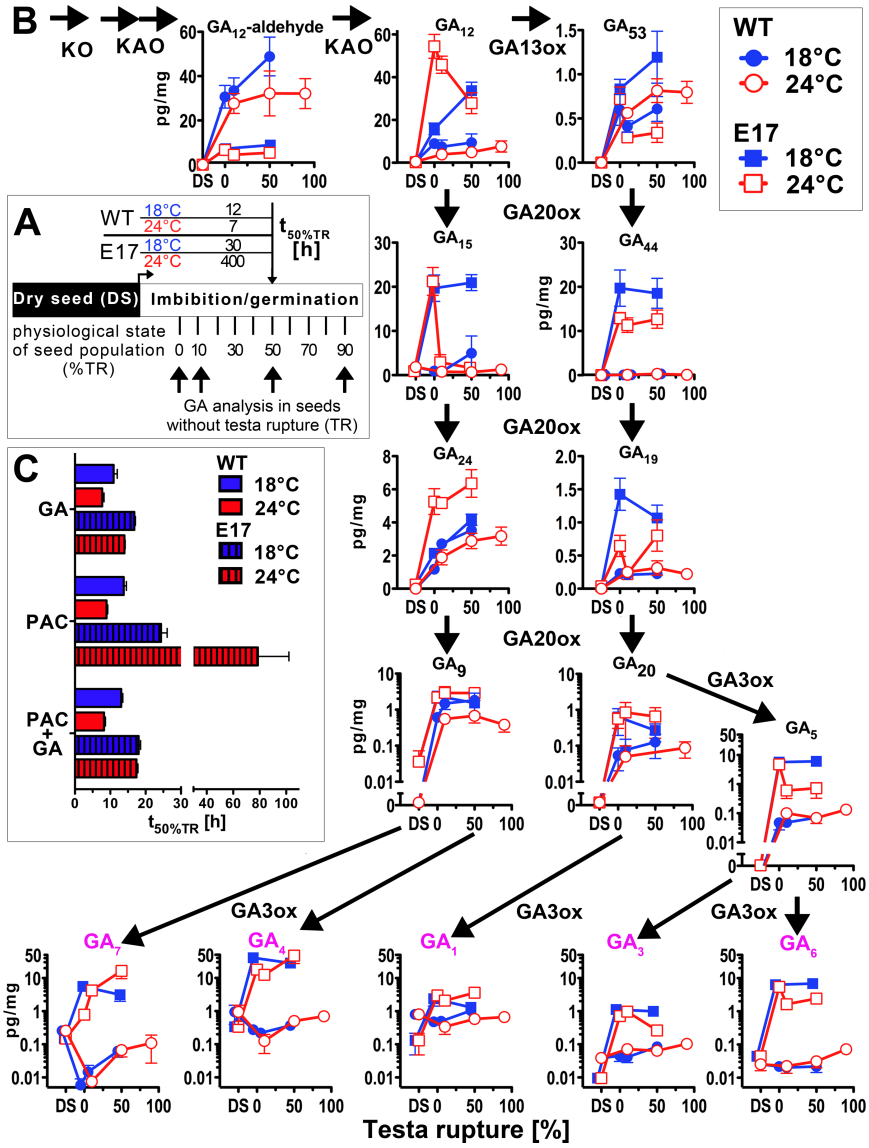

Fig. 5. DOG1 controls the seed gibberellin metabolism in a temperature-dependent manner. Overexpression of AtDOG1 in seeds of *Lepidium sativum* leads to an increase of gibberellin (GA) metabolites and a strongly altered temperature regulation ofGA metabolism during germination. [A] Experimental overview of the GA metabolite analysis shown in B. Physical time to reach a certain physiological state (testa rupture, TR) differs depending on seed imbibition temperature and genotype, representatively shown for the time to reach 50% TR ($t_{50\%TR}$) for *L. sativum* wild-type (WT) and the AtDOG1 overexpression line Lesa-OxAtDOG1-E17 imbibed at 18°C and 24°C. Arrows indicated physiological sampling timepoints for GA analysis. Only seeds without TR within the seed population were sampled. [B] GA metabolite contents of seed populations of *L. sativum* WT and Lesa-OxAtDOG1-E17 reaching different physiological states during imbibition at 18°C and 24°C. Shown are main metabolites of the early 13-non-hydroxylated (left) and 13-hydroxylated (right) pathway. GA biosynthetic enzymes catalysing respective steps are indicated. Bioactive GAs are indicated in purple. Results are presented as amounts per dry weight. N=5, mean ±SEM. DS, dry seed. Results for these and additionally quantified GA metabolites represented on a physical timescale are shown in Fig. S5; and as numeric values in Table S2 . [C] Effect of the GA biosynthesis inhibitor paclobutrazol (PAC, 100µM) on testa rupture ($t_{50\%TR}$) of *L. sativum* WT and Lesa-OxAtDOG1-E17 at 18°C and 24°C compared to germination on GA_{4+7} (GA, 10µM) or on a combination of both (PAC+GA). N=3, +SEM.

investigated if *LesaDOG1A,* as the most abundant one in seeds (Fig. 1*D*), encodes a functional DOG1 protein by analyzing its ability to induce dormancy in *A. thaliana*. A transgene with the *LesaDOG1A* coding sequence driven by a CaMV 35S-promoter was introduced into the completely non-dormant *A. thaliana dog1-1* mutant. We compared the germination behaviour of seeds from three independent homozygous transgenic *A. thaliana dog1* lines overexpressing LesaDOG1A (At-OxLesaDOG1A-A18, -A19, -A23) with the L*er* wild-type (WT) and the *dog1* mutant. All At-OxLesaDOG1A lines showed a delayed germination phenotype compared to WT and *dog1* mutant and markedly reduced germination capacity (Fig. 2*A*). Treatment with GA increased the germination percentages by releasing dormancy of the transgenic lines especially at later times, but did not affect WT or *dog1* mutant seeds (Fig. 2*A*). Taken together, this demonstrates that LesaDOG1A overexpression confers GA-sensitive dormancy and delayed germination to *A. thaliana dog1* seeds. Thus, LesaDOG1A and AtDOG1 seem to fulfil similar seed-related functions in *A. thaliana*.

Transgenic *L. sativum* Seeds Overexpressing AtDOG1 Have a Delayed Germination Phenotype. Proof that *DOG1*-homologous genes cause a delayed germination phenotype has so far only been obtained from work in *A. thaliana*, i.e. by transferring the garden cress *LesaDOG1A* gene into the non-dormant *A. thaliana dog1* mutant (this work) or by ectopically expressing putative cereal *DOG1-Like* genes in the weakly dormant *A. thaliana* accession Columbia (26, 27). It is however unknown if a dormancy mechanism involving *DOG1* genes exists in other species or if the DOG1-signalling pathway is *Arabidopsis*-specific. To address the question about an evolutionary conserved DOG1-mediated pathway we investigated if the function of *A. thaliana and L. sativum DOG1* genes is truly interchangeable by overexpressing the *AtDOG1* gene in non-dormant *L. sativum* seeds.

For this we generated transgenic *L. sativum* lines overexpressing a genomic fragment of the *A. thaliana* Cape Verde Island (Cvi) *DOG1* gene fused to a CaMV 35S-promoter. Overexpression has the advantage of maintaining a high DOG1 protein level allowing functional investigation during germination which is especially important regarding the endogenous regulation of *LesaDOG1A/B* (Fig. 1*D*). In dry seeds of most of the independent homozygous transgenic lines we detected high levels of *AtDOG1* transcript (Fig. 2*B*). The transgenic lines Lesa-OxAtDOG1-A2,

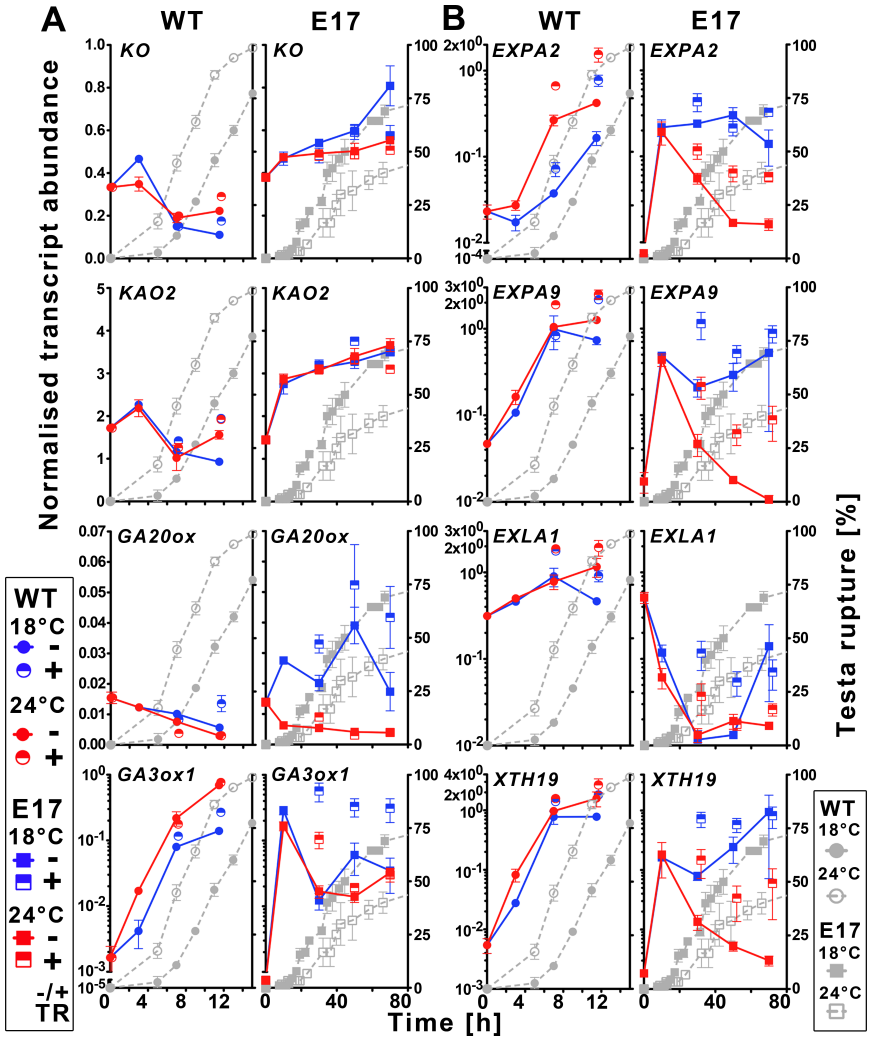

Fig. 6. DOG1 influences the expression of GA biosynthesis and CAP weakening genes in a temperature-dependent manner. Temperature-dependentgene expression profiles of candidate GA biosynthesis and endosperm CAP weakening genes during seed germination of *Lepidium sativum* line E17 overexpressing AtDOG1 compared to wild-type (WT). QRT-PCR transcript abundance analysis of [A] GA biosynthesis genes (*KO*, *KAO1*, *GA20ox*, *GA3ox1*) and [B]candidate cell-wall remodelling genes (*EXPA2*, *EXPA9*, *EXLA1*, *XTH19*) at 18°C and 24°C: WT (*left*) and E17 (*right*) seeds without (-TR) or with (+TR) testa rupture were sampled at the indicated time points which relate to the TR kinetics indicated by the grey curves.N=3, mean ±SEM. Results for other seed batches and lines are shown in Fig. S6. Note that temperature affects transcript abundances and germination differently in WT and E17 seeds but the overexpressed *AtDOG1* transcript and protein amounts themselves in E17 seeds were not temperature-regulated during germination (Fig. S7).

-A10, -B13, -E7, and -E17 showed an extremely delayed germination phenotype compared to WT (Fig. 2*C* and SI Appendix, Fig. S2*A*). Both testa rupture (TR) as well as endosperm rupture (ER) were delayed. For easier comparison of the TR and ER kinetics we determined germination rates (GRs) which are the reciprocal values of the times needed for a seed population to complete a certain percentage of TR or ER ($GR_{x\%} = 1/t_{x\%}$). The *L. sativum* WT seed population reached 30% ER at 13.4h ($t_{30\%}$) and thus has a $GR_{30\%}$ value of $0.075h^{-1}$ (Fig. 2*C*). In contrast, the transgenic Lesa-OxAtDOG1-E17 line was far slower and reached 30% ER only at 213h resulting in a ca. 16-fold lower $GR_{30\%}$ value of $0.005h^{-1}$ (Fig. 2*C*). The different Lesa-OxAtDOG1 lines showed a different degree in the delay of germination reflected by their different GR values and only lines accumulating the transgenic AtDOG1 protein showed a lowered GR (Fig. 2*D*). We conclude that the delayed-germination phenotype is indeed caused by the transgenic overexpression of AtDOG1 in *L. sativum* seeds.

Overexpression of AtDOG1 in *L. sativum* Causes Coat-Imposed Seed Dormancy by Inhibiting Endosperm CAP Weakening Without Affecting the Embryo Growth Potential. We investigated if the delayed germination phenotype of the transgenic *L. sativum* Lesa-OxAtDOG1 seeds is indeed due to the induction of physiological dormancy. Therefor we used several classical dormancy breaking treatments and quantified their effect on germination behaviour. We analysed the influence of GA, the ABA-biosynthesis inhibitor fluridone, cold-stratification pre-treatment and after-ripening storage on germination of freshly harvested mature seeds. The fast germination (high GR) of WT seeds was not appreciably affected by any of these treatments which reflects their non-dormant state (Fig. 3*A*). In contrast, we found that the delayed germination of Lesa-OxAtDOG1 seeds (low GR) was drastically accelerated (high GR) by GA treatment, cold-stratification or after-ripening storage, indicating dormancy breaking (Figs. 3*A* and S2*B*, shown representatively for line E17). Interestingly, fluridone treatment did not affect germination of E17 suggesting that *de novo* ABA synthesis is not involved in the AtDOG1-mediated dormancy of Lesa-OxAtDOG1 seeds. Taken together, this demonstrates that AtDOG1 overexpression confers physiological dormancy to non-dormant *L. sativum* seeds (Fig. 3*A*), as did LesaDOG1A overexpression to *A. thaliana dog1* mutant seeds (Fig. 2*A*).

The balance between the resistance of seed covering layers (testa and endosperm) and the embryo growth potential controls seed dormancy and germination (8, 19). The embryo growth potential determines embryo growth by water uptake and can be quantified using solutions that differ in water potential combined with image analysis (33). Interestingly, we found no significant difference in the growth potential of *L. sativum* WT and Lesa-OxAtDOG1-E17 embryos at any tested ambient water potential (Fig. 3*B*). Thus, overexpression of AtDOG1 does not alter the growth potential of isolated *L. sativum* embryos although intact

seeds germinate much slower. Initial scarification experiments (removal of seed covering layers to release the resistance against the embryo growth potential) suggested that Lesa-OxAtDOG1 lines have coat dormancy as the scarified seeds germinated faster. To test if overexpression of AtDOG1 alters the resistance of the seed covering layers we conducted puncture force measurements (21). Analysis of E17 and WT testa plus endosperm tissues during very early imbibition showed that there is no significant difference in the initial resistance of these seed covering layers indicating absence of general structural differences due to AtDOG1 overexpression (see SI Appendix, Fig. S3). However, puncture force analysis of only the endosperm CAP tissue during the course of germination showed that endosperm CAP weakening was differentially affected in WT and Lesa-OxAtDOG1-E17 seeds (Fig. 3*C*). In WT seeds weakening was initiated by TR and strongly progressed thereafter whereas in E17 it was strongly inhibited and no weaken occurred at all before or after TR, even at very late time points during imbibition. We conclude that the delayed germination phenotype of *L. sativum* seeds overexpressing AtDOG1 is not caused by a decreased embryo growth potential, but is due to an AtDOG1-mediated severe inhibition of endosperm CAP weakening. AtDOG1 has therefore induced endosperm-mediated physiological coat dormancy in *L. sativum* Lesa-OxAtDOG1 seeds.

Germination Temperature Strongly Affects the Delayed Germination Phenotype Caused by DOG1 Overexpression in *L. sativum* and *A. thaliana*. We found that the delay of germination induced by AtDOG1 overexpression in *L. sativum* strongly depended on the seed imbibition temperature. Analysis of four different temperatures showed that 24°C is optimal for *L. sativum* WT germination, whereas 18°C is optimal for Lesa-OxAtDOG1-E17 (Fig. 4*A*). Germination of E17 is drastically delayed at 24°C (low GR) but much faster and more similar to WT (high GR) at 18°C. DOG1 overexpression in E17 therefore generated a shift of germination temperature optimum towards colder temperatures (Figs. 4*A* and *B*).

Puncture force measurements of E17 endosperm CAPs at the two phenotypically very contrasting temperatures 18°C and 24°C showed that endosperm CAP resistance of E17 was differentially affected by the ambient temperature (Fig. 4*B*). No weakening of the E17 endosperm CAPs occurred in seeds at 24°C whereas at 18°C CAP weakening commenced as germination proceeded in a pattern similar to WT (Fig. 4*B* and 3*C*). Interestingly, the E17 endosperm CAPs weakened considerably when the testa ruptured at 18°C whereas at 24°C no endosperm CAP weakening was detected even after TR (Fig. 4*B*). Because of the identical embryo growth potentials at 24°C of E17 and WT (Fig. 3*B*) the observed delayed germination phenotype can thus be explained by temperature-dependent inhibition of endosperm CAP weakening caused by the overexpression of AtDOG1.

Between 18°C and 24°C there is a large shift in the germination response of Lesa-OxAtDOG1-E17 seeds (Fig. 4*A*). Surprisingly, this is a rather narrow temperature window for such an immense difference, i.e. at 18°C 50% of seeds completed germination within 30h whereas at 24°C it took 600h (Fig. 4*B*). We investigated if this narrow temperature window also affected the *A. thaliana dog1* mutant and the transgenic At-OxLesaDOG1A lines. Figure 4*C* shows that *A. thaliana* L*er* WT germinated only slightly slower at 18 compared to 24°C while the *dog1* mutant germinated much slower at 18 compared to 24°C. This delay in germination of the *dog1* mutant at the cooler temperature was also evident from its very negative dormancy index (DI) (Fig. 4*D*). In the transgenic *A. thaliana* lines (At-OxLesaDOG1A-A18, -A19, -A23) this *dog1*-specific temperature-phenotype was completely reverted as evident from their more positive DI (Fig. 4*D*).

In conclusion, these results show that LesaDOG1A and AtDOG1 both affect the temperature responses of *A. thaliana* and *L. sativum* seeds. In both species a high DOG1 level seems to limit germination at warmer temperatures whereas absence or low levels of DOG1 permit germination at warmer temperatures. From the biomechanical analysis of Lesa-OxAtDOG1-E17 seeds we conclude that overexpression of AtDOG1 in *L. sativum* defines the optimal temperature for endosperm CAP weakening which then occurs at 18ºC but not at 24ºC. We propose that temperature-control of seed germination regulated by DOG1 depends on a conserved coat dormancy mechanism within the Brassicaceae with endosperm CAP weakening as target.

Germination Temperature Differentially Affects Gibberellin Contents in Wild-Type and Transgenic *L. sativum* Seeds Overexpressing AtDOG1. ABA is known to maintain coat dormancy and inhibit endosperm weakening, while GA releases coat dormancy and promotes endosperm weakening (8, 10, 32). We found that during imbibition the ABA contents of Lesa-OxAtDOG1-E17 seeds decrease, but there was no difference in this decrease at 24°C compared to 18ºC (See SI Appendix, Fig. S4*D*). Therefore absolute ABA content did not cause the remarkable differences in the temperature responses of E17 seeds, i.e. the strongly inhibited endosperm CAP weakening and delayed germination at the higher temperature (Figs. 4*B* and S4*D*). We conclude that the ABA contents are not causing the delayed and temperature-sensitive germination phenotype induced by AtDOG1 overexpression. This is in agreement with our finding that inhibition of ABA biosynthesis did not increase the delayed E17 germination (Fig. 3*A*).

To investigate the role of gibberellins, we quantified major GA metabolites in dry and imbibed *L. sativum* WT and Lesa-OxAtDOG1-E17 seeds at 18 and 24°C (Fig. 5). We analysed seed populations at physiologically and physically comparable times during germination which had not yet undergone TR (Fig. 5*A*). This allowed identification of gradual changes occurring in seeds which are increasingly preparing to undergo the first visible committed step to the completion of germination. We found that GA metabolite contents in the imbibed state were strongly altered by AtDOG1 overexpression in combination with the ambient imbibition temperature (Figs. 5*B* and and SI Appendix, Fig. S5). Suprisingly, bioactive GAs were generally far more abundant in E17 compared to WT. The total bioactive GA content (GA_1, GA_3, GA_4, GA_6, GA_7) at 50% TR was 1.2±0.6 in WT and 40.3±16.1 pg/mg in E17 seeds at their respective optimal temperatures (24°C for WT, 18°C for E17). This app. 40-fold increase indicates that E17 seeds have a far higher GA requirement for reaching the same germination progression under optimal conditions. Furthermore, the ambient temperature had opposing effects on the GA contents of WT and E17 seeds at the same physiological time point (50% TR). At 18°C the bioactive GA contents in E17 were roughly doubled compared to 24°C whereas in WT they were almost halfed. These temperature-dependent changes were in accordance with the observed germination phenotype i.e. temperatures for optimal germination were associated with higher bioactive GA contents in both genotypes. However, the absolute bioactive GA content in E17 is far higher than in WT although E17 seeds germinate slower at any temperature (Fig. 4*A*, *B*).

To investigate if this observed higher GA accumulation is actually neccessary for E17 seeds to germinate we analysed germination responses upon treatment with the GA biosynthesis inhibitor paclobutrazol (PAC, Fig. 5*C*). Treatment with PAC strongly inhibited E17 testa rupture and subsequent completion of germination at 24°C, and this inhibition was rescued by combined application with GA. Neither PAC nor GA affected the germination responses of WT at any temperature. Interestingly,

the PAC-induced inhibition of E17 seed germination was much weaker at 18°C compared to 24°C.

These results show that DOG1 overexpression established a high GA requirement for germination. E17 seeds seem to react to this DOG1-mediated high GA threshold by producing larger amounts of GA and the ability to produce these amounts is strongly temperature dependent. In agreement with the observed temperature-dependence of the PAC inhibition, the delayed germination phenotype at 24°C thus seems to be caused by a lower GA biosynthesis at this temperature which does not compensate for the AtDOG1 induced GA requirement. This is in agreement with the findings for E17 that addition of GA stimulated germination at 24°C (Fig. 3A), the observed endogenous GA levels are in most cases lower at 24°C (Fig. 5B) and that the inhibtion of GA biosynthesis is very effective in delaying germination at 24°C (Fig. 5*C*). DOG1 therefore seems to define the optimal germination temperature by mediating a germination block based on a high GA threshold for germination which can be reached only at temperatures that allow high GA biosynthesis.

Germination Temperature Combined with DOG1 Presence Differentially Affects GA Biosynthetic and Cell-Wall Remodelling Gene Expression. To elucidate the molecular basis of the drastically enhanced GA levels and their temperature regulation we investigated gene expression of key enzymes of the GA biosynthetic pathway in the *L. sativum* WT and AtDOG1 overexpression line E17. Biosynthesis of GA_{12}, the common precursor for all GAs in plants, is catalysed by ent-kaurene oxidase (KO) and ent-kaurenoic acid oxidase (KAO) (34). The GA_{12} content is strongly increased in E17 seeds compared to WT (Fig. 5B). In agreement with this, we found increased expression of *KO* and *KAO2* during germination of E17 (Fig. 6*A*). Higher expression of these key enzymes may thus be the cause for the general elevation in the E17 GA metabolite contents. Further early reactions of GA biosynthesis are catalysed by GA20-oxidases (GA20ox). Most interestingly, we found GA20ox expression to be strongly temperature regulated in E17 but not in WT (Figs. 6*A* and SI Appendix, Fig. S6). During E17 seed germination GA20ox is up-regulated at 18°C but not at 24°C, whereas it is down-regulated at both temperatures in WT (Fig. 6*A*). This specific expression pattern is thus highly associated with the accumulation patterns of the initial metabolites synthesised by GA20ox (GA_{15}, GA_{44}) which are high at 18°C but low at 24°C in E17 (Fig. 5*B*). Bioactive GAs are synthesised by GA3-oxidases (GA3ox). Interestingly, we found that, in contrast to GA20ox, the GA3ox1 gene is similarly up-regulated during early germination of WT and E17 at both temperatures (Fig. 6*A*). However, after this initial up-regulation, the transcript contents were down-regulated in E17 at both temperatures whereas they continued to be up-regulated in WT. Surprisingly, later during germination the expression of this gene is strongly up-regulated during TR of E17 seeds at 18°C but not at 24°C. This is an intriguing pattern regarding the fact that endosperm weakening of E17 occurs during TR only at 18°C but not at 24°C (Fig. 4*B*).

To gain insight into the underlying molecular downstream mechanisms of the strongly AtDOG1- and temperature-dependent CAP weakening we analysed the expression of GA-regulated candidate endosperm CAP weakening genes (Fig. 6*B*). We investigated known genes encoding cell-wall remodelling proteins (CWRPs) of the expansin and xyloglucan endo-transglycosylases/hydrolase families (35). Transcript abundances of these genes (*EXPA2*, *EXPA9*, *EXLA1*, *XTH19*) increased steadily in WT seeds during germination at both 18°C and 24°C (Fig. 6*B*). In contrast, in E17 seeds *EXPA2*, *EXPA9* and *XTH19* were strongly temperature-regulated. After an initial increase in transcript abundance at both temperatures during the first hours of E17 seed imbibition transcripts declined dramatically at 24°C but not at 18°C (Fig. 6*B*). Therefore, the temperature and AtDOG1-regulated transcript expression patterns of *EXPA2*, *EXPA9* and *XTH19* in WT and E17 were highly associated with accumulation patterns of bioactive GAs (Fig. 5*B*) as well as with the alteration of endosperm CAP weakening and the resulting germination phenotype (Fig. 4*B*). In contrast, *EXLA1* was temperature independently down-regulated in E17 but up-regulated in WT seeds.

We conclude that DOG1 established a high GA requirement for E17 seed germination by repressing the GA-induced expression of CWRP genes needed for endosperm weakening. A constantly high GA content is neccessary to overcome this DOG1 induced repression of CWRP expression. In the presence of DOG1 this constantly high GA content is only maintained at colder but not at warmer temperatures. This seems to be due to the specific up-regulation of GA20ox expression only at the colder temperature. DOG1 therefore controls GA20ox expression and germination in a temperature-dependent manner.

Discussion

DOG1 Mediates a Conserved Physiological Coat Dormancy Mechanism in the Brassicaceae *Lepidium sativum* and *Arabidopsis thaliana*. We establish here that *DOG1* genes mediate a common dormancy mechanism, i.e. that the function and role(s) of DOG1 are conserved. Environmentally and hormonally regulated *DOG1* gene expression prior to seed dispersal (maturation) is important for the control of Brassicaceae seed germination (2, 4, 5, 11, 24), and for *A. thaliana DOG1* it is known that it provides adaptation to local environments (1, 14, 36). After seed dispersal, *AtDOG1* transcript levels in the soil seed bank are central to sensing seasonal temperature patterns and differed characteristically during dormancy cycling in summer and winter annual *A. thaliana* ecotypes (6, 7). We show here by reciprocal overexpression that *AtDOG1* and *LesaDOG1A* confer dormancy to non-dormant *L. sativum* WT and *A. thaliana dog1* mutant seeds, respectively. We found that *DOG1* genes induce primary physiological seed dormancy that can be released by after-ripening storage of dry seeds, by cold stratification of imbibed seeds, and by treatment of imbibed seeds with bioactive GAs.

From a mechanistic point of view dormancy and germination are regulated by two opposing forces, the growth potential of the embryo counteracting the restraint of the seed covering layers (17, 19, 37). By using embryo growth imaging (33) at different ambient water potentials, we demonstrate here that the growth potentials of Lesa-OxAtDOG1 and WT embryos did not differ. However, we found that the overexpression of AtDOG1 severely inhibits endosperm CAP weakening of imbibed Lesa-OxAtDOG1 seeds. Therefore AtDOG1 confers endosperm-mediated coat dormancy to *L. sativum* seeds. Thus, we demonstrate that the target of DOG1 to induce dormancy and delay germination is not the embryo growth potential but the seed covering layers.

We conclude that an evolutionary conserved role of DOG1 confers physiological coat dormancy and delayed germination to Brassicaceae seeds. This DOG1-mediated dormancy pathway does not alter the embryo growth potential, but has endosperm CAP weakening as its major target and enables temperature depended-control of dormancy during imbibition through regulation of CWRP gene expression and GA metabolism as outlined below.

DOG1 Determines the Temperature Window for Germination by Regulating Endosperm CAP Weakening Gene Expression Through Temperature Control of the Gibberellin Metabolism. We found for *L. sativum* that overexpression of AtDOG1 leads to a shift of the optimal germination temperature towards colder temperatures (from 24ºC to 18ºC). In agreement with this the *A. thaliana dog1* mutant germinated faster at warmer temperature and overexpression of LesaDOG1A abolished its preference for the warmer temperature as germination optimum. This points to

the fact that the amount of functional DOG1 protein determines the germination temperature optimum of a seed. We conclude that more DOG1 shifts the germination temperature optimum to colder and less DOG1 to warmer temperatures. Endogenous *AtDOG1* expression actually cycles in *A. thaliana* seeds in the soil seed bank through the year in association with soil temperature and dormancy state and it was proposed that AtDOG1 acts as a seed thermal sensor determining the depth of dormancy (6). In our *L. sativum* Lesa-OxAtDOG1 lines overexpressed AtDOG1 does not cycle during imbibition (See SI Appendix, Fig. S7), and the constantly high expression maintains the inhibition of germination at warmer temperatures. In agreement with this, germination of *A. thaliana* Cvi seeds known for high *AtDOG1* expression is also inhibited at warmer temperatures (7). Thus, on the one hand, endogenous DOG1 expression is influenced by the environment, i.e. up-regulated at low temperature during maturation as well as in imbibed seeds of *A. thaliana* (2, 4-7) and *L. sativum* (See SI Appendix, Fig. S8). On the other hand, the amount of DOG1 seems to determine the temperature sensitivity window for germination. Higher DOG1 levels generate a narrow temperature window and restrict germination at higher temperatures, whereas lower DOG1 levels generate a wide temperature window and allow germination at higher temperatures. This regulation seems to be conserved in *L. sativum* and *A. thaliana* and points to a general underlying mechanism by which DOG1 regulates coat dormancy to define the temperature window for germination. As for embryo-related developmental processes in animals (16, 18), the embryo-encasing tissue layers of the seed are of key importance: for *L. sativum* we directly showed that the temperature-dependent inhibition of germination is caused by the DOG1-mediated temperature-specific inhibition of endosperm CAP weakening.

Our findings show that DOG1 is involved in the temperature-dependent control of GA metabolism. Strikingly, analysis of the GA metabolism demonstrated that imbibed dormant Lesa-OxAtDOG1 seeds have a strongly increased GA metabolite content, including the bioactive GAs. Furthermore, GA metabolites show a strong temperature-dependent accumulation in the presence of DOG1. In *L. sativum* WT seeds the *KO* and *KAO2* genes, encoding the enzymes for GA_{12} biosynthesis, are expressed during early germination and their expression decreases during late germination; this expression pattern is very similar to *A. thaliana* seeds (38). In contrast, these genes are constantly up-regulated when DOG1 is overexpressed in *L. sativum* E17 seeds. Interestingly, only *KAO2* but not *KAO1* expression is affected by *DOG1* overexpression (See SI Appendix, Fig. S9), which points to different mechanism in the regulation of these two genes, as has been shown for duplicated *KAO* genes in pea and sunflower (34). *KO* expression is directly regulated by the bZIP transcription factor REPRESSION OF SHOOT GROWTH (RSG, (39)) which was also suggested to regulate *KAO* expression (40). Thus, it is tempting to speculate that DOG1 interacts with certain bZIP transcription factors to regulate GA biosynthesis. Since the drastic DOG1-mediated up-regulation of GA metabolite contents is evident for GA_{12}, but not GA_{12}-aldehyde, we propose that the DOG1-mediated induction of *KAO2* plays a major role in the general increase in the GA metabolite contents. While up-regulated DOG1-mediated *KAO2* expression can explain the generally enhanced GA biosynthesis pathway, it is however not temperature-dependent and therefore does not explain the observed temperature-dependent differences in the GA metabolite accumulation.

Most intriguingly, we found that GA20ox gene expression is temperature-regulated by DOG1 overexpression, and this regulation is in agreement with the temperature-dependent accumulation of GA metabolites catalysed by GA20ox enzymes. Gene expression of *GA20ox*, but not of *KO* or *KAO,* has been shown to be regulated by a GA negative feedback mechanism controlled by the RSG transcription factor in tobacco (34, 39). In agreement with this, neither *KO* nor *KAO2* expression is down-regulated by the high GA contents in E17 seeds. *GA20ox* expression is however down-regulated in E17 seeds imbibed at 24°C, but not at 18°C (Fig. 6A). This suggests that DOG1 interferes with the negative feedback regulation of *GA20ox* in a temperature-dependent manner, potentially through interaction with transcription factors regulating *GA20ox* expression such as RSG. In contrast to *GA20ox*, expression of *GA3ox1*, which is also feedback regulated (41) but not by RSG (39), seems to be strongly down-regulated during the course of germination when GA levels are high in E17. We therefore propose that DOG1 specifically interferes on the level of *GA20ox* expression to mediate the temperature-dependent regulation of bioactive GA accumulation.

The downstream-mechanism for endosperm CAP weakening depend on the GA-induced expression of CWRP genes in the CAP such as expansins and XTHs (9, 35, 42-44). Although E17 seeds contain elevated GA contents compared to WT at any imbibition temperature, expression of candidate CWRP genes is reduced, especially at 24°C. DOG1 therefore seems to repress GA-induced CWRP expression especially at higher temperatures, and this subsequently inhibits endosperm weakening and germination. The elevated GA contents of E17 seeds at 18°C caused by the GA20ox induction seem to overcome the DOG1-imposed repression of weakening gene expression for *EXPA2*, *EXPA9* and *XTH11*. The fact that DOG1 repressed *EXLA1* at both temperatures in E17 but not in WT point to the fact that DOG1 might regulate the expression of diverse CWRP genes differently. Constantly high GA contents seem to be required to overcome this repression and to release the DOG1-imposed coat dormancy. The elevated GA contents in imbibed dormant Lesa-OxAtDOG1 seeds might actually be a feedback reaction of the seed to overcome the block to germination imposed by the AtDOG1 overexpression. Non-dormant *L. sativum* WT seeds do not require *de novo* GA biosynthesis for germination. In contrast, dormant Lesa-OxAtDOG1 seeds require *de novo* GA biosynthesis as they are highly responsive to GA biosynthesis inhibition. They are not GA-insensitive per se, but have a high GA requirement which at 24°C is not saturated by the elevated endogenous bioactive GA. This decreased GA sensitivity and increased sensitivity to GA biosynthesis inhibitor of *L. sativum* seeds overexpressing AtDOG1 is in agreement with the finding that *A. thaliana dog1* mutant seeds have increased GA sensitivity and decreased sensitivity to a GA biosynthesis inhibitor (5, 11). The *A. thaliana dog1* mutant in a GA-deficient background needed 10-fold less added GA to reach the same germination progression as the corresponding control (11). Furthermore, ecophysiological work demonstrated that *A. thaliana* DOG1 alleles causing delayed germination in the field are associated with increased seed GA contents (15). Interestingly, the enhanced contents in GA metabolites were not evident in dry Lesa-OxAtDOG1 seeds, but were induced during seed imbibition and underline a role of DOG1 in coat dormancy maintenance in the imbibed state.

Besides regulating the seed GA contents in a temperature-dependent manner to control CWRP expression, DOG1 may also regulate GA signalling pathways important for the GA-induction of CWRP genes. Expression of the CWRP genes described above is known to be regulated via the GID1-type GA signalling pathways in *L. sativum* and *A. thaliana*; both species possess the GA receptors *GID1A*, *GID1B* and *GID1C* (35). In contrast to WT seeds, down-regulation of *GID1A* expression is evident in E17 seeds during early germination (See SI Appendix, Fig. S9) and may be involved in causing lower CWRP expression in E17 despite the elevated GA contents. Furthermore temperature-dependent up-regulation of *GID1B* in E17 during late germination might provide the GA sensitivity needed to overcome the temperature-

dependent repression of CWRP expression inhibiting germination.

Taken together, DOG1 regulates Brassicaceae coat dormancy by repressing GA-induced CWRP expression required for endosperm CAP weakening of imbibed seeds. DOG1 acts by modifying GA-metabolism in a temperature-dependent manner to overcome the CWRP repression at certain temperatures. This confers temperature-responsive control of endosperm CAP weakening and thereby determines the optimal seed germination temperature.

Materials and Methods

Physiological Assays. Analysis of seed germination (24) and embryo growth potential (33) was as in Suppl. Methods (See SI Appendix). Puncture force measurements were conducted as described previously (32) with a modified custom-made biomechanics machine (load cell range 0-1N). Intact endosperm CAP tissue was dissected from the imbibed seeds and glued to a metal sample holder (0.6 mm hole size) using Loctite 454 glue (Henkel). A rounded metal pin was driven into the sample while force and displacement were recorded simultaneously. A 0.3mm diameter probe (hemisphere shaped tip) and a speed of 0.7mm min^{-1} was used while force and displacement was recorded. The CAP puncture force (tissue resistance) was determined from the displacement-force curves as the maximal force.

Molecular Methods. *LesaDOG1B* genomic and cDNA sequences (Genbank ID KF501341) were cloned as described in (23, 45). *A. thaliana DOG1* overexpression construct used for transformation of *L. sativum* FR14 was a pLEELA vector containing a double 35S CaMV promoter and the genomic ORF of *A. thaliana* Cvi *DOG1* which was provided by Wim Soppe. *L. sativum LesaDOG1A* overexpression construct used for transformation of *A. thaliana dog1-1* was prepared by cloning the *LesaDOG1A* cDNA (23) ORF into the 35S CaMV promoter containing pB2GW7 vector by using Gateway technology (Invitrogen). Southern blot analysis was performed using a digoxigenin-labeled probe covering 353 bp of exon 1 of *LesaDOG1A* as described (24). Western blot analysis was performed as described (24) using a primary polyclonal antibody raised against AtDOG1 (5). RNA extraction, qRT-PCR analysis (45), GA and ABA metabolite quantification, and plant transformation were as in Suppl. Methods (See SI Appendix).

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1. Kronholm I, Picó FX, Alonso-Blanco C, Goudet J, Meaux J de (2012) Genetic basis of adaptation in *Arabidopsis thaliana*: local adaptation at the seed dormancy QTL *DOG1*. *Evolution* 66:2287–2302.
2. Chiang GCK et al. (2011) *DOG1* expression is predicted by the seed-maturation environment and contributes to geographical variation in germination in *Arabidopsis thaliana*. *Mol Ecol* 20:3336–3349.
3. Alonso-Blanco C et al. (2009) What has natural variation taught us about plant development, physiology, and adaptation? *Plant Cell* 21:1877–1896.
4. Kendall SL et al. (2011) Induction of dormancy in *Arabidopsis* summer annuals requires parallel regulation of *DOG1* and hormone metabolism by low temperature and CBF transcription factors. *Plant Cell* 23:2568–2580.
5. Nakabayashi K et al. (2012) The time required for dormancy release in *Arabidopsis* is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. *Plant Cell* 24:2826–2838.
6. Footitt S, Douterelo-Soler I, Clay H, Finch-Savage WE (2011) Dormancy cycling in *Arabidopsis* seeds is controlled by seasonally distinct hormone-signaling pathways. *Proc Natl Acad Sci USA* 108:20236–20241.
7. Footitt S, Huang Z, Clay HA, Mead A, Finch-Savage WE (2013) Temperature, light and nitrate sensing coordinate *Arabidopsis* seed dormancy cycling, resulting in winter and summer annual phenotypes. *Plant J* 74:1003–1015.
8. Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytol* 171:501–523.
9. Holdsworth MJ, Bentsink L, Soppe WJJ (2008) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytol* 179:33–54.
10. Graeber K, Nakabayashi K, Miatton E, Leubner-Metzger G, Soppe WJJ (2012) Molecular mechanisms of seed dormancy. *Plant Cell Environ* 35:1769–1786.
11. Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. *Proc Natl Acad Sci USA* 103:17042–17047.
12. Bentsink L et al. (2010) Natural variation for seed dormancy in Arabidopsis is regulated by additive genetic and molecular pathways. *Proc Natl Acad Sci USA* 107:4264–4269.
13. Horton MW et al. (2012) Genome-wide patterns of genetic variation in worldwide *Arabidopsis thaliana* accessions from the RegMap panel. *Nat Genet* 44:212–216.
14. Silady RA, Effgen S, Koornneef M, Reymond M (2011) Variation in seed dormancy quantitative trait loci in *Arabidopsis thaliana* originating from one site. *PLoS ONE* 6:e20886.
15. Barua D, Butler C, Tisdale TE, Donohue K (2012) Natural variation in germination responses of *Arabidopsis* to seasonal cues and their associated physiological mechanisms. *Ann Bot* 109:209–226.
16. Heisenberg C-P, Bellaïche Y (2013) Forces in tissue morphogenesis and patterning. *Cell* 153:948–962.
17. Linkies A, Leubner-Metzger G (2012) Beyond gibberellins and abscisic acid: how ethylene and jasmonates control seed germination. *Plant Cell Rep* 31:253–270.
18. Keller R (2006) Mechanisms of elongation in embryogenesis. *Development* 133:2291–2302.
19. Nonogaki H (2006) Seed germination - the biochemical and molecular mechanisms. *Breeding Sci* 56:93–105.
20. Barrero JM, Talbot MJ, White RG, Jacobsen JV, Gubler F (2009) Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant Physiol* 150:1006–1021.
21. Linkies A et al. (2009) Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: a comparative approach using *Lepidium sativum* and *Arabidopsis thaliana*. *Plant Cell* 21:3803–3822.
22. Dekkers BJW et al. (2013) Transcriptional dynamics of two seed compartments with opposing roles in *Arabidopsis* seed germination. *Plant Physiol* 163:205–215.
23. Graeber K et al. (2010) Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae *DOG1* genes. *Plant Mol Biol* 73:67–87.
24. Graeber K et al. (2013) Spatiotemporal seed development analysis provides insight into primary dormancy induction and evolution of the *Lepidium DELAY OF GERMINATION1* genes. *Plant Physiol* 161:1903–1917.
25. Sugimoto K et al. (2010) Molecular cloning of *Sdr4*, a regulator involved in seed dormancy and domestication of rice. *Proc Natl Acad Sci USA* 107:5792–5797.
26. Ashikawa I, Abe F, Nakamura S (2010) Ectopic expression of wheat and barley *DOG1*-like genes promotes seed dormancy in *Arabidopsis*. *Plant Sci* 179:536–542.
27. Ashikawa I, Abe F, Nakamura S (2013) *DOG1*-like genes in cereals: Investigation of their function by means of ectopic expression in *Arabidopsis*. *Plant Sci* 208:1–9.
28. Franzke A, Lysak MA, Al-Shehbaz IA, Koch MA, Mummenhoff K (2011) Cabbage family affairs: the evolutionary history of Brassicaceae. *Trends Plant Sci* 16:108–116.
29. Schranz ME, Mohammadin S, Edger PP (2012) Ancient whole genome duplications, novelty and diversification: the WGD Radiation Lag-Time Model. *Curr Opin Plant Biol* 15:147–153.
30. Haudry A et al. (2013) An atlas of over 90,000 conserved noncoding sequences provides insight into crucifer regulatory regions. *Nat Genet* 45:891–898.
31. Dierschke T, Mandakova T, Lysak MA, Mummenhoff K (2009) A bicontinental origin of polyploid Australian/New Zealand *Lepidium* species (Brassicaceae)? Evidence from genomic *in situ* hybridization. *Ann Bot* 104:681–688.
32. Müller K, Tintelnot S, Leubner-Metzger G (2006) Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiol* 47:864–877.
33. Voegele A et al. (2012) Embryo growth, testa permeability, and endosperm weakening are major targets for the environmentally regulated inhibition of *Lepidium sativum* seed germination by myrigalone A. *J Exp Bot* 63:5337–5350.
34. Hedden P, Thomas SG (2012) Gibberellin biosynthesis and its regulation. *Biochem J* 444:11–25.
35. Voegele A, Linkies A, Müller K, Leubner-Metzger G (2011) Members of the gibberellin receptor gene family *GID1* (*GIBBERELLIN INSENSITIVE DWARF1*) play distinct roles during *Lepidium sativum* and *Arabidopsis thaliana* seed germination. *J Exp Bot* 62:5131–5147.
36. Huang X et al. (2010) The earliest stages of adaptation in an experimental plant population: strong selection on QTLS for seed dormancy. *Mol Ecol* 19:1335–1351.
37. Bewley JD (1997) Breaking down the walls - a role for endo-β-mannanase in release from seed dormancy? *Trends Plant Sci* 2:464–469.
38. Ogawa M (2003) Gibberellin biosynthesis and response during *Arabidopsis* seed germination. *Plant Cell* 15:1591–1604.
39. Fukazawa J et al. (2011) bZIP transcription factor RSG controls the feedback regulation of NtGA20ox1 via intracellular localization and epigenetic mechanism. *Plant Signal Behav* 6:26–28.
40. Helliwell CA, Chandler PM, Poole A, Dennis ES, Peacock WJ (2001) The CYP88A cytochrome P450, ent-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. *Proc Natl Acad Sci USA* 98:2065–2070.
41. Toh S et al. (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in *Arabidopsis* seeds. *Plant Physiol* 146:1368–1385.
42. Chen F, Bradford KJ (2000) Expression of an expansin is associated with endosperm weakening during tomato seed germination. *Plant Physiol* 124:1265–1274.
43. Carrera E et al. (2008) Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. *Plant J* 53:214–224.
44. Yamauchi Y et al. (2004) Activation of gibberellin biosynthesis and response pathways by low temperature during imbibition of *Arabidopsis thaliana* seeds. *Plant Cell* 16:367–378.
45. Graeber K, Linkies A, Wood ATA, Leubner-Metzger G (2011) A guideline to family-wide comparative state-of-the-art quantitative RT-PCR analysis exemplified with a Brassicaceae cross-species seed germination case study. *Plant Cell* 23:2045–2063.