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.

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.

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Note that the dry seed samples (0 h) of the qRT-PCR analysis represent CAP + RAD tissue and not whole seeds.

50% endosperm rupture (ER). RAD comprises the radicle plus ca. 1/3 of the lower hypocotyl (embryo growth zone); CAP is the micropylar endosperm tissue.

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 plus ca. 1/3 of the lower hypocotyl (embryo growth zone); CAP is the micropylar endosperm tissue.

Note that the dry seed samples (0 h) of the qRT-PCR analysis represent CAP + RAD tissue and not whole seeds.

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.

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; 21–23). 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.

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 EcoRI or XbaI 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) cdNA 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 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 plus ca. 1/3 of the lower hypocotyl (embryo growth zone); CAP is the micropylar endosperm tissue.

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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-
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 ADOG1 gene homolog LesaDOG1 (23). Here we show by Southern blot analysis that L. sativum actually possesses two DOG1 genes (Fig. 1B), 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 than 50% identity with 2n=16 (See SI Appendix, Fig. S1, Table S1).

A. thaliana

L. sativum

Endosperm rupture [%]

Germination temperature [°C]

B

A

C

D

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 Lesa-DOG1/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 1.0. 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.

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. 1D). Within these 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. 1D). 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. 1D), 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...
investigated if LesaDOG1A, as the most abundant one in seeds (Fig. 1D), 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 *Ler* 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. 2A). 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. 2A). 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 fulfill 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. 1D). In dry seeds of most of the independent homozygous transgenic lines we detected high levels of AtDOG1 transcript (Fig. 2B). The transgenic lines Lesa-OxAtDOG1-A2, -A3, and -A4 (Figs. 2C and 5) were selected for the subsequent experiments.

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 of GA 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, represented by arrows. The transgenic lines especially at later times, but did not affect WT or dog1 mutant seeds (Fig. 2A). 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 fulfill similar seed-related functions in *A. thaliana*.

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A10, B13, E7, and E17 showed an extremely delayed germination phenotype compared to WT (Fig. 2C and SI Appendix, Fig. S24). 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 13.4 h (t0.30%) and thus has a GR x% value of 0.075 h-1 (Fig. 2C). In contrast, the transgenic Lresa-OxAtDOG1-E17 line was far slower and reached 30% ER only at 213 h resulting in a ca. 16-fold lower GR x% value (0.005 h-1) (Fig. 2C). The different Lresa-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. 2D). 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 Lresa-OxAtDOG1 seeds is indeed due to the induction of physiological dormancy. Therefore 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 pretreatment 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 reflect their non-dormant state (Fig. 3A). In contrast, we found that the delayed germination of Lresa-OxAtDOG1 seeds (low GR) was drastically accelerated (high GR) by GA treatment, cold-stratification or after-ripening storage, indicating dormancy breaking (Figs. 3A and S2B, 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 Lresa-OxAtDOG1 seeds. Taken together, this demonstrates that AtDOG1 overexpression confers physiological dormancy to non-dormant L. sativum seeds (Fig. 3A), as did LresaDOG1A overexpression to A. thaliana dog1 mutant seeds (Fig. 24).

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 Lresa-OxAtDOG1-E17 embryos at any tested ambient water potential (Fig. 3B). 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 tests 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. 3C). 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. 4A). Germination of E17 is drastically delayed at 24°C (low GR) but more at 18°C (high GR) at 18°C. DOG1 overexpression in E17 therefore generated a shift of germination temperature optimum towards colder temperatures (Figs. 4A 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. 4B). 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. 4B and 3C). 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. 4B). Because of the identical embryo growth potentials at 24°C of E17 and WT (Fig. 3B) 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. 4A). 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. 4B). We investigated if this narrow temperature window also affected the A. thaliana dog1 mutant and the transgenic At-OxLesaDOG1A lines. Figure 4C shows that A. thaliana Ler 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. 4D). 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. 4D).

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 both temperatures whereas 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 18°C but not at 24°C. 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 18°C (See SI Appendix, Fig. S4D). 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. 4B and S4D). 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. 3A).

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. 5A). 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. 5B and SI Appendix, Fig. S5). Surprisingly, bioactive GAs were generally far more abundant in E17 compared to WT. The total bioactive GA content (GA_1, GA_2, GA_3, GA_4, GA_5) at 50% TR was 1.2±0.6 µg/mg in WT and 40.3±16.1 µg/mg in E17 seeds at their respective optimal temperatures of 18°C and 24°C, respectively. This 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 halved. 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. 4A,B).

To investigate if this observed higher GA accumulation is actually necessary for E17 seeds to germinate we analysed germination responses upon treatment with the GA biosynthesis inhibitor paclobutrazol (PAC, Fig. 5C). 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 inhibition of GA biosynthesis is very effective in delaying germination at 24°C (Fig. 5C). 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 the expression of key genes in 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. 6A). Higher expression of these key enzymes has been shown for the general elevation in the E17 GA metabolite contents. Further early reactions of GA biosynthesis are catalysed by GA$_{20}$-oxidases (GA$_{20}$ox). Most interestingly, we found GA$_{20}$ox expression to be strongly temperature regulated in E17 but not in WT (Figs. 6A and SI Appendix, Fig. S6). During E17 seed germination GA$_{20}$ox is up-regulated at 18°C but not at 24°C, whereas it is down-regulated at both temperatures in WT (Fig. 6A). This specific expression pattern is thus highly associated with the accumulation patterns of the initial metabolites synthesised by GA$_{20}$ox (GA$_{15}$, GA$_{24}$) which are high at 18°C but low at 24°C in E17 (Fig. 5B). Bioactive GAs are synthesised by GA$_{3}$-oxidases (GA$_{3}$ox). Interestingly, we found that, in contrast to GA$_{20}$ox, the GA3ox1 gene is similarly up-regulated during early germination of WT and E17 at both temperatures (Fig. 6A). 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. 4B).

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. 6B). 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 (EXP2A, EXP49, EXLA1, XTH19) increased steadily in WT seeds during germination at both 18°C and 24°C (Fig. 6B). In contrast, in E17 seeds EXP2A, EXP49 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. 6B).

Therefore, the temperature and AtDOG1-regulated transcript expression patterns of EXP2A, EXP49 and XTH19 in WT and E17 were highly associated with accumulation patterns of bioactive GAs (Fig. 5B) as well as with the alteration of endosperm CAP weakening and the resulting germination phenotype (Fig. 4B). 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 necessary 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 GA$_{20}$ox expression only at the colder temperature. DOG1 therefore controls GA$_{20}$ox 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 (maturity) 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 dependent-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 24°C to 18°C). In agreement with this the *A. thaliana* dog1 mutant germinated faster at warmer temperature and overexpression of Lesa-DOG1A 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 warmer temperatures, 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
of seed dormancy. In A. thaliana, but not GA12-encoding, we propose
temperature window and restrict germination at higher tempera-
tures, whereas lower DOG1 levels generate a wide tempera-
ture 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 example, the embryonic layer of the egg
is known to be regulated via the GID1-type GA signalling path-
way (17). When the temperature window is reached, the egg
expression of Ga2ox genes is down-regulated by the high GA contents in E17 seeds for GA2ox 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 GA2ox in a temperature-dependent manner,
potentially through interaction with transcription factors regu-
lating GA2ox expression such as RSG. In contrast to GA2ox,
expression of GAs1, 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 GA2ox 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 the GA-induced weakening of seed
permeability. The elevated GA contents of E17 seeds at 18°C caused
by the GA2ox induction seems to overcome the DOG1-imposed
repression of weakening gene expression for EXP2A, EXP49
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 inhibition of
the GA2ox expression in E17 seeds. The elevated GA contents in imbibed dormant Lesa-OxAtDOG1 seeds might actually be a feedback reaction of the seed to over-
come the block to germination imposed by the AtDOG1 over-
expression. 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 biosyn-
thesis inhibitor of L. sativum seeds overexpressing AtDOG1 is in
greement 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 when GA levels are high in E17 (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 underlined 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 path-
ways in L. sativum and A. thaliana; both species possess the GA re-
cieptors 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-

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dependent repression of CWRP expression inhibiting germina-
tion.  

Taken together, DOG1 regulates Brassicaceae coat dor-
mancy by repressing GA-induced CWRP expression required 
for endosperm CAP weakening of imbibed seeds. DOG1 acts 
by modifying GA-metabolism in a temperature-dependent man-
er 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 mod-
ified custom-made biomechanics machine (load cell range 0-1N). Intact 
endosperm CAP tissue was dissected from the imbibed seeds and glued to a 
manometric tube (0.5 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 0.3 mm diameter probe (hemispherically 
tipped) and a speed of 0.7 mm min⁻¹ was used while force and displacement 
were measured and the puncture force investigated. The displacement-force 
curves were determined from the displacement-force curves as the maximal force. 

Molecular Methods. LesdoG18 genomic and cDNA sequences (Gen-
Bank: KF501341) were used as queries to search the Brassicaceae plant 
databases to determine the closest homologues of A. thaliana DOG1 
overexpression construct used for transformation of L. sativum FR14 was a 
PELLE vector containing a double 35S CaMV promoter and the genomic 
orf of A. thaliana Cvi DOG1 which was provided by Wim Soppe. L. sativum 
LesdoG18 overexpression construct used for transformation of A. thaliana 
dog1-1 was prepared by cloning the LesdoG18 cDNA (23) ORF into the 35S 
CaMV promoter containing pbGW7 vector by using Gateway technology 
(recovery clones). Sense and anti-sense primers were used for generating 
a digoxigenin labeled probe covering 353 bp of exon 1 of LesdoG18 as described (24). 
Western blot analysis was performed as described (24) using a primary polyclonal 
antibody raised against Arabidopsis. 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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