Control of seed dormancy in *Arabidopsis* by a *cis*-acting noncoding antisense transcript

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Seed dormancy is one of the most crucial process transitions in a plant's life cycle. Its timing is tightly controlled by the expression level of the Delay of Germination 1 gene (*DOG1*). *DOG1* is the major quantitative trait locus for seed dormancy in *Arabidopsis* and has been shown to control dormancy in many other plant species. This is reflected by the evolutionary conservation of the functional short alternatively polyadenylated form of the *DOG1* mRNA. Notably, the 3′ region of *DOG1*, including the last exon that is not included in this transcript isoform, shows a high level of conservation at the DNA level, but the encoded polypeptide is poorly conserved. Here, we demonstrate that this region of *DOG1* contains a promoter for the transcription of a noncoding antisense RNA, *asDOG1*, that is 5′ capped, polyadenylated, and relatively stable. This promoter is autonomous and *asDOG1* has an expression profile that is different from known *DOG1* transcripts. Using several approaches we show that *asDOG1* strongly suppresses *DOG1* expression during seed maturation in *cis*, but is unable to do so in *trans*. Therefore, the negative regulation of seed dormancy by *asDOG1* in *cis* results in allele-specific suppression of *DOG1* expression and promotes germination. Given the evolutionary conservation of the *asDOG1* promoter, we propose that this *cis*-constrained noncoding RNA-mediated mechanism limiting the duration of seed dormancy functions across the Brassicaceae.

seed dormancy | *DOG1* gene | *cis*-acting ncRNA | antisense transcript

Plants have evolved elaborate adaptation mechanisms to cope with unexpected and rapid changes in their natural environment (1). The division of the plant life cycle into consecutive developmental phases can be viewed as one such mechanism. This compartmentalization allows plants to focus their resources on particular tasks. The most pronounced developmental phases in plant development are seed dormancy, the juvenile phase, vegetative growth, flowering, and senescence (2). The transition between each successive phase has to be tightly controlled and aligned with the plant's internal metabolic state and external conditions.

Seeds are characterized by their remarkable ability to withstand harsh environmental conditions (3). This is in part because of a seed dormancy mechanism that imposes a block on the ability of seeds to sense permissive conditions and initiate germination (4, 5). This mechanism allows seeds to temporarily bypass favorable conditions to germinate in an environment that will support the entire plant life cycle. Seed dormancy is under strong evolutionary selection because the improper timing of germination often results in immediate death (6). In addition, from an agronomical point of view, seed dormancy has been a subject of intensive selection, because on the one hand strong dormancy leads to uneven germination, but on the other hand weak dormancy may result in preharvest sprouting because of germination on the mother plant (7).

An analysis of seed dormancy variability among *Arabidopsis thaliana* accessions identified the Delay of Germination 1 (*DOG1*) gene as the major quantitative trait locus (QTL) controlling this phenotype (8). *DOG1* is a member of a small gene family (9). The molecular function of the DOG1 protein in seed dormancy control is currently unclear. However, the DOG1 protein has been shown to self-dimerize and to be a highly stable protein that is posttranslationaly modified during after-ripening and germination (10, 11). Genetically, *DOG1* has been shown to control a gibberellin-dependent endosperm-weakening mechanism in *Arabidopsis* and other Brassicaceae family members (12). *DOG1* is also required for the miRNA156-mediated delay of flowering and germination (13).

The level of *DOG1* mRNA is tightly correlated with the strength of seed dormancy. Its expression is seed-specific and shows a strong peak during seed maturation when dormancy is established (9). The *DOG1* gene is alternatively spliced, leading to the production of four mRNA isoforms. *DOG1* alternative splicing is strongly coupled to the speed of polymerase II (PolII) elongation. Transcription factor (TF)IIS knockout and TFIIS dominant-negative mutation lead to the slowdown of PolII elongation, which enhances proximal splice site selection on *DOG1*. In contrast, mutation of the spliceosome cofactor AtNTR1, which increases the rate of PolII elongation, results in the selection of distal splice sites (14). In addition to changes in *DOG1* splice site selection, *tfIIs* and *atntr1* mutants display low *DOG1* expression and consequently a weak seed dormancy phenotype (14–16). Other known factors required for high *DOG1* expression include the histone H2B ubiquitin transferases HUB1 and HUB2 (17),

Significance

Sequential developmental transitions in plant life cycle are tightly controlled by dynamic regulation of key genes. Seed dormancy release is probably the first developmental transition in a plant's life cycle, and it is regulated by the Delay of Germination 1 (*DOG1*) gene. Here we demonstrate that a non-protein-coding antisense transcript originating from a conserved at DNA—but not protein level—*DOG1* region is a negative regulator of *DOG1* expression and seed dormancy establishment. We show that this antisense transcript negatively regulates *DOG1* expression in *cis*. This mechanism is presumably conserved across the Brassicaceae, given the evolutionary conservation of the antisense *DOG1* promoter.

Author contributions: H.F., M.P., and S.S. conceived the study; M.P. and Z.P. designed the adapter *asDOG1* assay; H.F. and M.P. characterized *asDOG1* in the WT; H.F. analyzed the *dog1-5* expression profile, and performed the F1 *dog1-5* × Col-0 and *dog1-5* × *dog1-3 cis/trans* assay and imbibition tests; L.B. performed the RNA stability assay and absolute quantification assay and did the bioinformatic analysis with the help of S.K.; Z.P. performed the 5′ RACE; K.K. and R.Y. prepared the LUC-tagged DOG1 plants; K.K. did the LUC assays and *pasDOG::LUC cis/trans* experiment; and H.F. and S.S. wrote the paper.

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and histone methyltransferase SDG8 (18). The only known example of a negative regulator of *DOG1* expression is histone H3 lysine 9 methyltransferase KYP/SUVH4 (19).

DOG1 is also subject to alternative polyadenylation. Use of the distal polyadenylation site results in the production of a three-exonic long mRNA *lgDOG1* that is poorly expressed and presumably not translated in vivo (11, 20). Selection of the proximal polyA site leads to the production of a two-exon short mRNA *shDOG1* that is translated and can complement the weak dormancy phenotype of the *dog1* mutant (20). The function of the *lgDOG1*-specific exon 3 region, which encodes a nonconserved polypeptide sequence and is probably not translated, is unclear. We and others have shown that a transfer (T)-DNA insertion in exon 3, rather than diminishing seed dormancy, produces a very strong dormancy phenotype, indicating that this region may negatively regulate seed dormancy strength (13, 20).

Developmental transitions are often controlled by elaborate mechanisms that center on a small set of key regulators allowing the plant to integrate diverse positive and negative cues (18, 21). In *Arabidopsis*, this is probably best exemplified by the regulation of Flowering Locus C (*FLC*), a QTL for the transition from vegetative to generative development (22). The majority of known positive and negative regulators that converge on *FLC* control its expression. These regulators include different noncoding RNAs (ncRNAs), including a small RNA targeting the *FLC* 3′ end, an intron-derived sense ncRNA participating in the cold-sensing mechanism, and a long-noncoding antisense transcript. This antisense transcript, named *COOLAIR*, originates from the 3′ end of *FLC* and has a dual function in both cold perception and autonomous pathway-mediated regulation of *FLC* (23–26).

ncRNAs have emerged as important players in the regulation of gene expression (27, 28). Many of these are antisense transcripts, such as Hidden Treasure 1 (*HID1*) (29), $cis\text{-}NAT_{PHO1;2}$ (30), and *asHSFB2a* (31). In these examples, expression of the antisense transcript from an independently integrated transgene led to phenotypic changes, showing that the ncRNA can act in *trans*. In contrast, *FLC* silencing during vernalization has been shown to be *cis*-controlled because two *FLC* alleles can be silenced independently. Thus, it has been hypothesized that the antisense transcript *COOLAIR* acts in *cis*. Indeed, recent single-molecule RNA FISH showed that *COOLAIR* and *FLC* sense transcription is mutually exclusive and confirmed that *COOLAIR* acts in *cis* to silence *FLC* during cold (32). Comparison of the *A. thaliana* and *Arabidopsis alpina FLC* antisense promoter regions revealed DNA sequence conservation, and an analogous antisense transcript was also detected in the latter species (33).

In the present study, we show that a conserved element in the region of the proximal polyA site of *DOG1* serves as an autonomous ncRNA promoter. Its activity leads to the production of a long antisense transcript that is both 5′ capped and polyadenylated, and displays a developmentally regulated expression pattern that is different from the *DOG1* sense transcript. This antisense transcript (*asDOG1*) acts as a negative regulator of seed dormancy that strongly suppresses *DOG1* expression during dormancy establishment, thereby controlling this important developmental transition. Interestingly, *asDOG1*-mediated control of *DOG1* expression and seed dormancy appears to be *cis*-restricted, suggesting a mechanism that may involve *asDOG1* transcription rather than the resulting RNA.

Results

The *DOG1* Exon 3 Region Shows Conservation at the DNA Level but the Encoded Polypeptide Sequence Is Not Conserved. We recently reported that the *DOG1* transcript displays alternative polyadenylation and that the short two-exonic proximally polyadenylated *DOG1* mRNA is functional in the establishment of seed dormancy (20). In contrast, the long three-exonic *DOG1* transcript is unable to complement the *dog1* mutant and does not appear to be translated in vivo (20). DNA sequence alignment of the exon 3 region from selected members of the Brassicaceae revealed mutations, including single-nucleotide insertions and deletions that introduce stop codons (e.g., in *DOG1* of *Arabidopsis lyrata*) (Fig. 1*A*). Despite the apparent lack of evolutionary pressure for ORF integrity, the DNA sequence of exon 3 showed substantial sequence conservation. In addition, a Vista plot of an alignment of the *DOG1* genes from *A. thaliana* and *A. lyrata* suggested the presence of a conserved DNA sequence element within intron 2 (Fig. 1*A*). This element extends beyond the recently described short DOG1 protein ORF generated by use of the proximal polyA site located in intron 2. A multiple alignment of intron 2 revealed extensive DNA sequence conservation of this region in other plants (Fig. 1*A*). Furthermore, whole *DOG1* locus DNA motif analysis, which does not require sequence colinearity, confirmed the presence of conserved DNA motifs corresponding to the *A. thaliana* intron 2–exon 3 element (Fig. 1*B*).

In contrast, the sequence of the polypeptide encoded by *A. thaliana DOG1* exon 3 is not conserved, as evident by the presence of multiple, independently acquired stop codons in relative species (Fig. 1*A*) (exon 3, alignment). The lack of protein conservation of the *DOG1* exon 3 region is also supported by protein sequence alignment, as published previously (20). However, we now show that, together with the intron 2 region, this exon comprises a larger element that is evolutionarily conserved at the DNA sequence level. This is reminiscent of a conserved nonprotein coding sequence element, and may indicate the presence of a potential regulatory element or ncRNA in this region (34).

The *DOG1* Gene Is Transcribed in the Antisense Orientation. We hypothesized that this conserved 3′ region of the *DOG1* gene may encode a nonprotein-coding RNA or contain the promoter mediating the transcription of such an RNA. Reanalysis of strand-specific direct RNA sequencing (DRS) -based mapping of polyadenylation sites in the *Arabidopsis* genome (35) revealed several polyadenylation sites on the *DOG1* antisense strand (Fig. 1*A*). The most prominent of these is in the *DOG1* promoter, but a signal could also be detected in intron 1 (Fig. 1*A*). Using 5′ RACE, we demonstrated the presence of a 5′ capped antisense transcript originating from the second intron of the *DOG1* gene (Fig. 1*C* and Fig. S1). This antisense transcription start site (TSS) coincides with the proximal polyadenylation site of the short sense *DOG1* transcript and the aforementioned conserved intron 2-exon 3 DNA sequence element (Fig. S1) (20).

Our RACE experiments did not reveal any splicing of *asDOG1* transcripts. Therefore, we developed an adapter-mediated RT-PCR assay that allowed us to specifically detect *asDOG1* in the presence of the complementary *DOG1* sense mRNA (36) (Fig. S2). Altogether, DRS, 5′ RACE, and the adapter-mediated RT-PCR assay showed that *DOG1* is transcribed in the antisense orientation, leading to the production of a 5′ capped and polyadenylated transcript. The observation that this antisense transcript's 3′ end extends to the TSS of the *DOG1* sense mRNA (compare sense and antisense in Fig. 1*A*, *Top*) suggests that it may potentially be able to regulate *DOG1* gene expression by a *cis*-acting mechanism like that described for H3K4me2-depositing transcripts in yeast (37).

To further characterize the *DOG1* antisense transcript, we analyzed its stability using a cordycepin-dependent assay (38). This approach demonstrated that *asDOG1* has a half-life of about 46 min (Fig. 1*D*), compared with 50 min for the *shDOG1* sense transcript and 36 min for the short-lived *At3g45970* mRNA. The relatively long (for ncRNA) half-life of *asDOG1*, which is close to that of short-lived protein-coding transcripts, indicated that it may function at a posttranscriptional level, for example by recruiting specific regulators (28). This comparatively high stability of *DOG1* antisense is therefore more consistent with

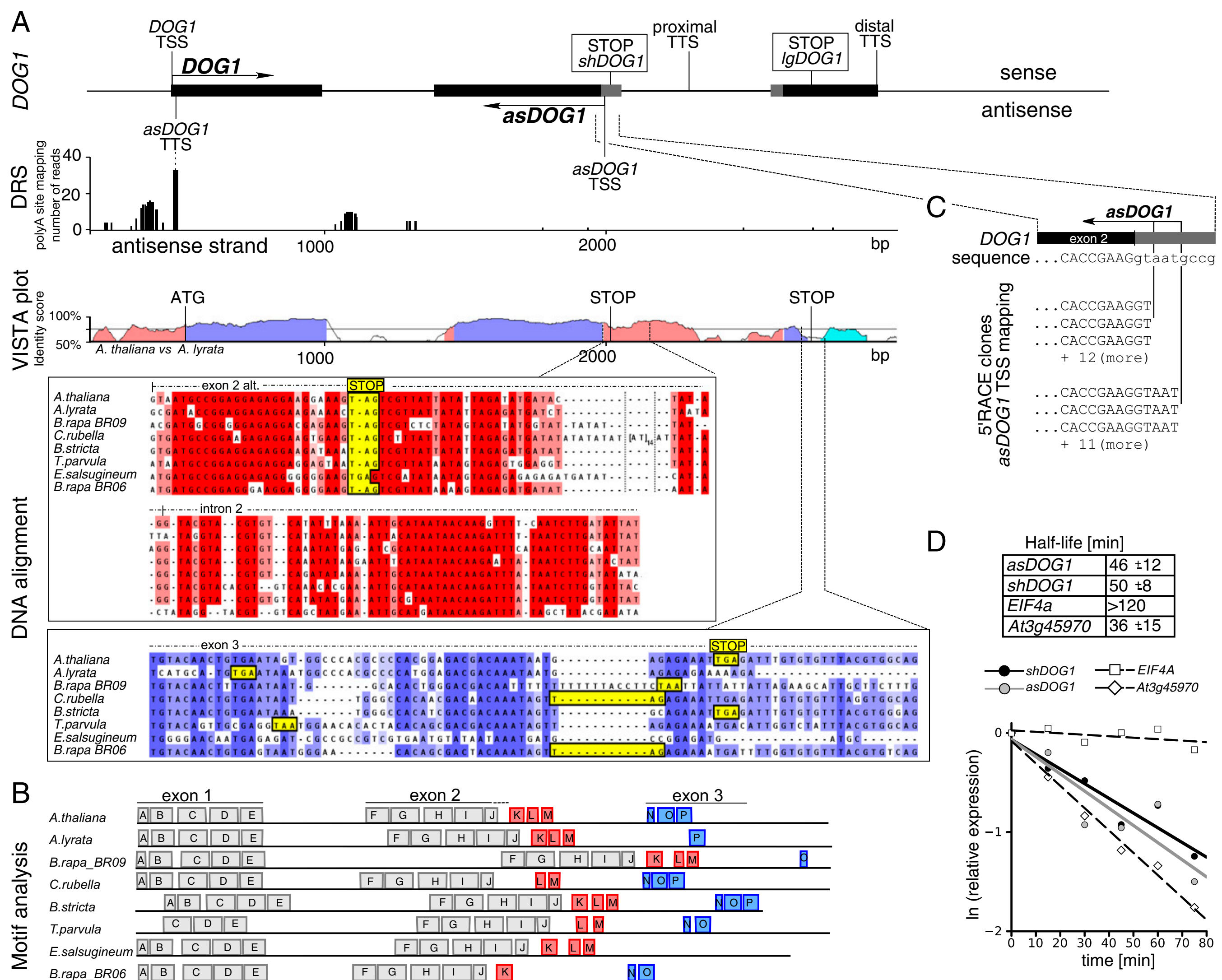

Fig. 1. The *DOG1* 3′ region is conserved at the DNA level (but the encoded polypeptide is not conserved) and colocalizes with an *asDOG1* TSS. (*A*) *DOG1* panel: schematic diagram of *DOG1* gene organization. *lgDOG1*, long three exonic *DOG1* transcript; *shDOG1*, short two exonic *DOG1* transcript; STOP, end of ORF; black boxes, exon sequences; gray boxes, alternative exonic regions, sense and antisense transcripts are marked with arrows. DRS panel: reanalysis of polyA site mapping by Direct RNA sequencing. Reads mapped to the antisense strand represent sites where polyadenylation occurs (TTS antisense). VISTA panel: plot of a pairwise alignment between *A. thaliana* and *A. lyrata DOG1* orthologs. The curve is calculated using default VISTA thresholds based on percentage identity shown on *y* axis and base pair position on the *x* axis (*Materials and Methods*). Regions longer than 100 bp with average conservation score above threshold of 70% were colored (exons in blue, introns and promoter in pink, and UTR in light blue). Alternative splicing is not marked. DNA alignment panel: multiple alignment of *DOG1* orthologs from seven Brassicaceae members, colored according to percentage identity. Exon/intron annotation refers to the *A. thaliana DOG1* gene. Stop codons are marked for each ortholog based on the in silico ORF prediction. (*B*) Motif analysis: DNA motifs in *DOG1* orthologs, identified with MEME software. Only motifs with an e-value $< 1 \times 10^{-14}$ and $P < 1 \times 10^{-14}$ are shown. Exon annotation refers to the *A. thaliana DOG1* gene. Alternative exon 2 is marked with a dotted line. Red boxes, motifs found in intron 2; blue boxes, motifs found in exon 3. (*C*) 5′ RACE sequencing results revealed two TSS of a 5′ capped antisense transcript originating from the end of exon 2. Individual DNA amplicons were cloned and sequenced. (*D*) RNA stability assay performed on Col-0 WT seedlings. Half-life (see table) was calculated based on degradation curves after cordycepin treatment (plot) for *asDOG1*, the sense *shDOG1* transcript, the stable transcript of housekeeping gene *EIF4A* and a short-lived mRNA transcribed from gene *At3g45970*. Presented values are averages with SD from three independent experiments.

a *trans*-acting mode of action, which often requires an RNA molecule to diffuse over a considerable distance in the nucleus (28). In contrast, ncRNAs that act at the level of transcription are often highly unstable (39, 40).

asDOG1 Transcription Is Controlled by an Independent Promoter. The *DOG1* gene is the major QTL for seed dormancy in *Arabidopsis* and, as might be expected, the *DOG1* sense transcript is highly expressed in seeds in comparison with seedlings (9) (Fig. 2*A*). Using the tag-based strand-specific quantitative RT-PCR (RT-qPCR) method, we found that the antisense and sense transcripts of *DOG1* show reciprocal expression profiles (Fig. 2*A*). Therefore, we questioned whether production of the antisense transcript might occur independently of the *DOG1* promoter and sense transcription. Assuming that the conserved noncoding region detected by our bioinformatic analysis contains a promoter for antisense transcription, we cloned the *DOG1* antisense transcript, including its putative promoter region (exon 2–intron 2–exon 3) and fused it to an internal ribosomal entry site–luciferase gene (IRES-LUC) reporter cassette in the antisense orientation (p_{as}*DOG1::LUC*). Separately, we prepared a construct with *LUC* linked to the short *DOG1* sense gene (p_{s}*DOG1::LUC*),

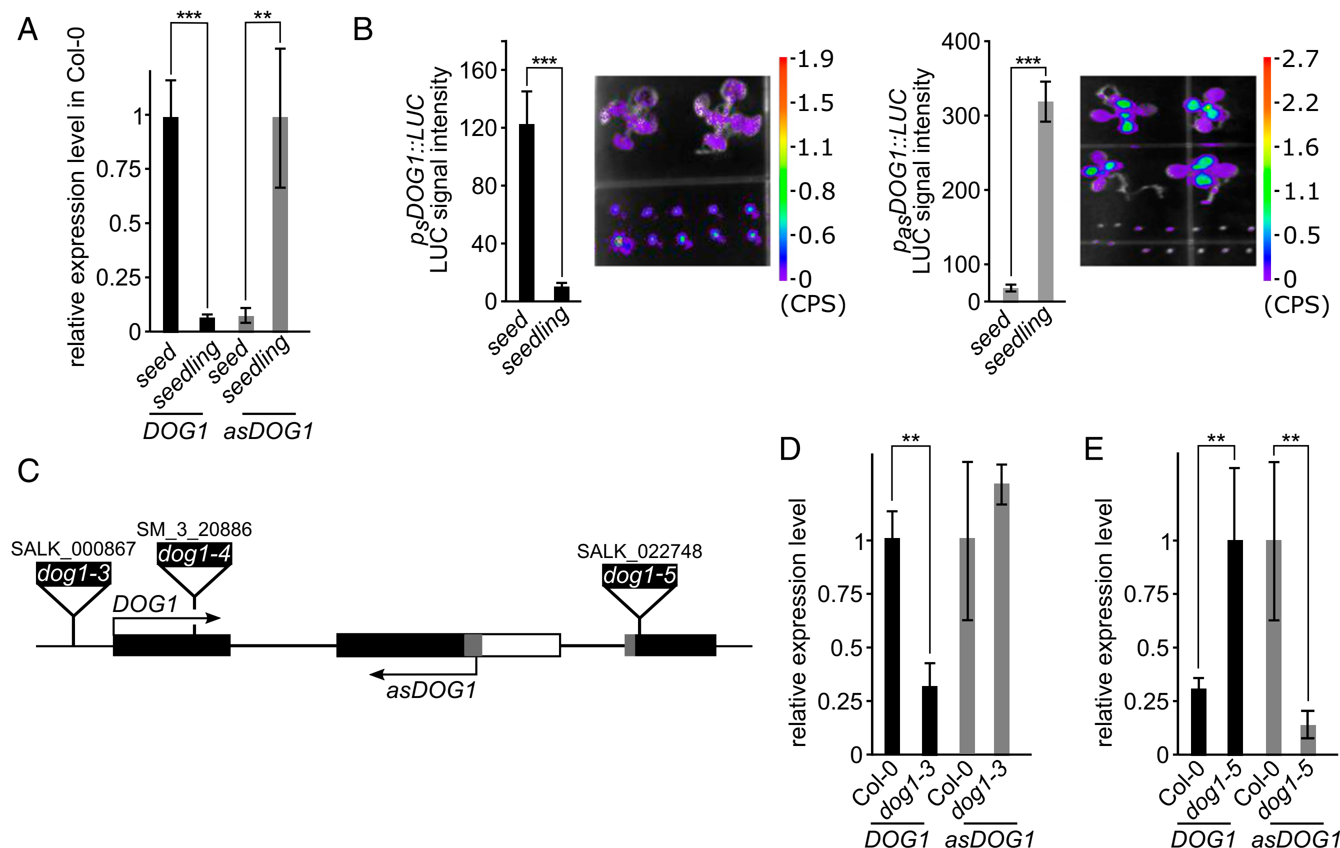

Fig. 2. Tissue-specific expression pattern of *asDOG1*. *DOG1* and *asDOG1* show opposite patterns of expression in freshly harvested seeds and seedlings, when (*A*) assayed in Col-0 (WT) using RT-qPCR or (*B*) by measuring luciferase activity in transgenic plants expressing *DOG1* (*Left*) or *asDOG1* (*Right*), fused with *LUC*. Luciferase activity was quantified and normalized to the signal area. Images of seedlings (*Upper*) and seeds (*Lower*) expressing luciferase were taken with a NightSHADE (Berthold) camera. Colored bar next to image represents light intensity scale in counts per second (CPS). Error bars for *B* represent 95% confidence interval (CI). (*C*) Schematic diagram of *DOG1* gene structure; black boxes, exon sequences; gray boxes, alternative exonic regions; white box region included in alternatively polyadenylated *DOG1* short transcript; arrows show the sense and antisense transcripts. The positions of T-DNA insertions are indicated by black rectangles: *dog1-3* (SALK_000867), *dog1-4* (SM_3_20886) and *dog1-5* (SALK_022748). (*D*) Down-regulation of the *DOG1* sense transcript in *dog1-3* does not affect the expression of *asDOG1*. Relative expression levels in freshly harvested seeds measured by RT-qPCR. (*E*) The *dog1-5* mutant shows up-regulation of short *DOG1* and down-regulation of *asDOG1* expression. Relative expression levels in freshly harvested seeds measured by RT-qPCR. $^{**}P < 0.01$ and $^{***}P < 0.001$. RT-qPCR results were normalized against the *UBC* mRNA level and are the means of three biological replicates with error bars representing SD.

including the sense promoter (Figs. S3 and S4). Stable transgenic lines in the Col-0 (WT) background were produced using both constructs. The native *DOG1* promoter gave an expression pattern similar to that of the endogenous *DOG1* transcript (compare Fig. 2 *A* and *B* and Fig. S3). Interestingly, a LUC signal was also detected in "antisense" lines, indicating that there is indeed a transcriptionally active antisense promoter within the *DOG1* gene. Moreover, the expression pattern produced using the antisense promoter was similar to the endogenous transcript profile determined by adapter-mediated RT-qPCR (compare Fig. 2 *A* and *B* and Fig. S3). These data confirmed that the *DOG1* downstream region is sufficient to activate antisense transcription and may serve as a stand-alone promoter.

To independently corroborate our conclusion, we took advantage of a previously reported loss-of-function mutant *dog1-3*, which has a T-DNA insertion in the *DOG1* sense promoter region (9) (Fig. 2*C*). RT-qPCR analysis confirmed that the *DOG1* sense transcript was clearly reduced in the *dog1-3* mutant, but the level of the *asDOG1* transcript remained unaffected compared with Col-0 WT plants (Fig. 2*D*). This result indicated that sense promoter activity is not required for antisense promoter-driven transcription.

Taken together, our results show that the *DOG1* antisense transcript originates from an independent promoter. The conservation of the DNA sequence in this region (but not the encoded polypeptide sequence) could therefore reflect evolutionary conservation of this promoter.

Disruption of the *asDOG1* Promoter Causes Down-Regulation of Antisense Transcription and Increases the *DOG1* Sense mRNA Level. Although the level of antisense transcript in Col-0 WT plants is low in seeds compared with seedlings, it was further reduced in seeds of the *dog1-5* mutant (Fig. 2 *A* and *E*). The *dog1-5* mutant allele was originally described by our group (20) and, as recently confirmed by others (13), it acts as a gain-of-function mutant showing enhanced seed dormancy (Fig. 3*A* and Fig. S5). In agreement with this phenotype, *dog1-5* showed significant up-regulation of the short proximally polyadenylated *DOG1* transcript (Fig. 2*E*) and raised levels of the DOG1 protein (20). The *dog1-5* mutant carries a T-DNA insertion within *DOG1* exon 3. This exon is included in the long- but absent from the short-*DOG1* transcript. Considering the location of the *asDOG1* TSS, the T-DNA insertion in *dog1-5* was predicted to affect the *asDOG1* promoter (Fig. 2*C*). Indeed, we found a reduction in

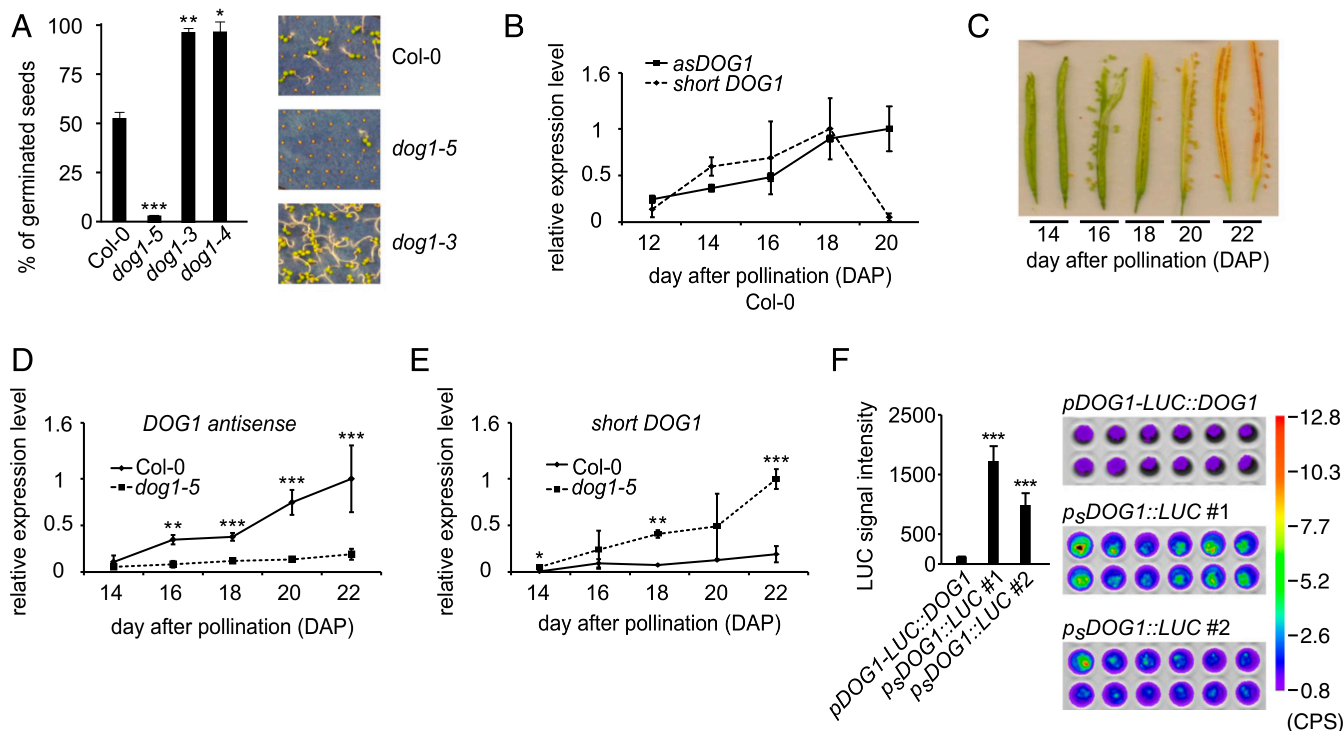

Fig. 3. Seed dormancy is negatively regulated by *asDOG1*. (*A*) The *dog1-5* mutant shows strong seed dormancy. Freshly harvested seeds of Col-0 (WT) and different *DOG1* mutants were scored for germination ability: germination efficiency (%) of tested seeds scored as radical protrusion. *P* value was calculated in comparison with Col-0 (WT) (*Left*); representative seed dormancy test result (*Right*). (Magnification: 0.9×.) (*B*) The expression profiles of the *shDOG1* and *asDOG1* transcripts during silique development in Col-0 (WT) plants show an increase in the antisense transcription in the late stages of seed maturation. (*C*) Siliques from manually pollinated flowers collected at different days after pollination. (*D*) The level of *asDOG1* is decreased in the *dog1-5* mutant during all tested seed developmental stages. (*E*) The *dog1-5* mutant shows strongly increased *shDOG1* expression during silique development. (*F*) Removal of the *asDOG1* promoter results in increased *DOG1* expression. LUC intensities produced by the *psDOG1::LUC* construct, in which *asDOG1* was deleted, and by the *pDOG1-LUC::DOG1* construct, containing the whole *DOG1* gene, were quantified (*Left*) or visualized (*Right*) in freshly harvested seeds from the indicated transgenic lines, error bars for this panel represent 95% CI. *P* value of both p_s*DOG1::LUC* lines was calculated in comparison with *pDOG1-LUC::DOG1*. RT-qPCR data show relative expression levels normalized against *UBC* mRNA and are the means of three biological replicates with error bars representing SD. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

the *asDOG1* transcript level in freshly harvested *dog1-5* seeds (Fig. 2*E*).

This finding led us to speculate that the strong seed dormancy phenotype and underlying up-regulation of the *DOG1* sense mRNA level in the *dog1-5* mutant is actually a secondary effect of the loss of *DOG1* antisense function.

***DOG1* Antisense Is a Suppressor of Seed Dormancy in *Arabidopsis*.** The *DOG1* gene is mainly expressed during seed maturation. So far, all tested *DOG1* mRNA isoforms have displayed parallel expression patterns, consisting of a slow increase phase with a peak that coincides with the seed maturation stage, and a phase of rapid decline at the seed desiccation stage (9–11, 20). Strand-specific RT-qPCR showed that *DOG1* antisense has an expression pattern similar to the *DOG1* sense transcript in the early stages of seed development. However, whereas *DOG1* sense expression reached a peak and began to decline, the level of *asDOG1* continued to increase up to the end of silique maturation (Fig. 3 *B* and *C*).

DOG1 expression has been shown to strongly decrease in response to imbibition (9). In contrast addition of ABA during imbibition diminished the *DOG1* mRNA reduction in *Lepidium sativum* (41). Using RT-qPCR we have confirmed that *DOG1* mRNA is strongly reduced in Col-0 (WT) during imbibition. Our data show that *DOG1* antisense expression exhibits similar strong reduction (Fig. S6). The use of an RNA standard curve, as described in *Materials and Methods*, allowed us to conclude that the sense transcript is ∼400 times more abundant than the antisense transcript in mature freshly harvested seeds (Fig. S6). Our data show that addition of exogenous ABA did not change the observed reduction of *DOG1* sense and *DOG1* antisense during imbibition at the concentration used by us (Fig. S6). Given that *DOG1* antisense and sense transcripts have differential behavior in seed development and similar expression patterns during imbibition, we focused on the seed-dormancy establishment stage.

The *asDOG1* transcript level was strongly reduced in seeds of the *dog1-5* mutant compared with those of Col-0 (WT), showing a fivefold decrease by the late developmental stages (Fig. 3*D*). This finding is in agreement with the low levels of antisense transcript observed in dry seeds of this mutant (Fig. 2*E*). Concurrently, the short *DOG1* transcript was strongly up-regulated (fivefold) in *dog1-5* in comparison with Col-0 (WT) (Fig. 3*E*). Moreover, the average fold-change in *asDOG1* between the WT and the *dog1-5* mutant was similar to that for the short *DOG1* transcript.

DOG1 protein has been reported to be highly stable (10, 12). Therefore, the strong up-regulation of the *DOG1* mRNA in the *dog1-5* mutant during the final stages of seed maturation may account for the substantial overabundance of DOG1 protein in this mutant's mature seeds, providing an explanation for the extremely strong seed-dormancy phenotype of the *dog1-5* mutant (Fig. 3*A*) (20).

To independently confirm the negative influence of *asDOG1* on *DOG1* expression, we compared the expression of *LUC* driven by full-length genomic *DOG1* (*pDOG1-LUC::DOG1*) and

by a truncated version of this gene that lacks the *DOG1* antisense promoter region (p_S*DOG1::LUC*). In agreement with the *dog1-5* mutant analysis, the p_S*DOG1::LUC* construct showed much higher expression than the *pDOG1-LUC::DOG1* control construct (Fig. 3*F*).

In summary, the elimination of *DOG1* antisense expression by T-DNA insertion (*dog1-5* mutant) or deletion of the *asDOG1* promoter (p_S*DOG1::LUC* construct) resulted in significant up-regulation of *DOG1* sense transcription. Therefore, *asDOG1* acts as a negative regulator of *DOG1* expression. Furthermore, based on the enhanced seed-dormancy phenotype of *dog1-5*, we conclude that by controlling *DOG1* expression, *asDOG1* suppresses seed dormancy.

***DOG1* Antisense Acts in *cis*.** Although our data showed that the *DOG1* antisense transcript acts as a suppressor of *DOG1* expression, the mechanism of this regulation remained uncharacterized (42). To determine whether *asDOG1* acts in *cis* or in *trans*, we crossed the *dog1-5* mutant with Col-0 (WT) plants and obtained heterozygous F1 seeds with two copies of the *DOG1* gene: one from Col-0 (WT) transcribed in both sense and antisense orientations, and the other from the *dog1-5* mutant, generating mostly sense transcripts. The resulting *dog1-5* × Col-0 seeds displayed seed dormancy that was only slightly weaker than that of *dog1-5* plants, indicating that the mutant allele can still confer strong seed dormancy in the presence of an antisense transcript originating from the WT *DOG1* gene. This effect was independent of whether the antisense transcript was provided by the maternal or paternal copy of *DOG1* (Fig. 4*A* and Fig. S7).

To define each allele contribution to the *DOG1* mRNA pool in Col-0 × *dog1-5* F1 seeds, we sought to do an allele-specific RT-qPCR in this genetic background. However, it was not possible because of lack of sequence difference between a short *DOG1* mRNA transcript derived from the WT and *dog1-5* copy. We therefore have combined the *dog1-5* antisense-deficient allele with the *dog1-3* allele that produces little sense transcript but a nearly WT level of antisense (Fig. 2*D*). The resulting F1 seeds allowed us to assay the effect of antisense derived from the *dog1-3* on the short *DOG1* sense mRNA level from the *dog1-5* (Fig. S8). We found that in *dog1-3* × *dog1-5* F1 plants, *shDOG1* expression was reduced no more than 50% compared to *dog1-5* × *dog1-5* F1 plants, which is a level predicted by a loss of one sense *DOG1*-producing copy (Fig. S8). One-sample Student's *t* test showed that the null hypothesis about the 50% reduction cannot be rejected ($P > 0.05$). This result is in agreement with previous Col-0 × *dog1-5* F1 analysis and indicates that *asDOG1* is not able to reduce sense *DOG1* mRNA level if expressed in *trans* from a different allele.

However, our observation of the inability of the antisense originating from a Col-0 (WT) or *dog1-3* allele to silence an antisense defective *dog1-5* allele could be also interpreted as a dosage/dilution effect, rather than a lack of *trans* silencing. Therefore, to further confirm the inability of *asDOG1* to act in *trans*, we examined *DOG1* expression in seeds of transgenic plants carrying an additional copy of *DOG1* antisense (Fig. 4 *B* and *C*; see Fig. S4 for construct description). RT-qPCR analysis of three independent lines showed that endogenous sense *DOG1* expression was unaffected by the presence of a cassette expressing *asDOG1* in *trans*.

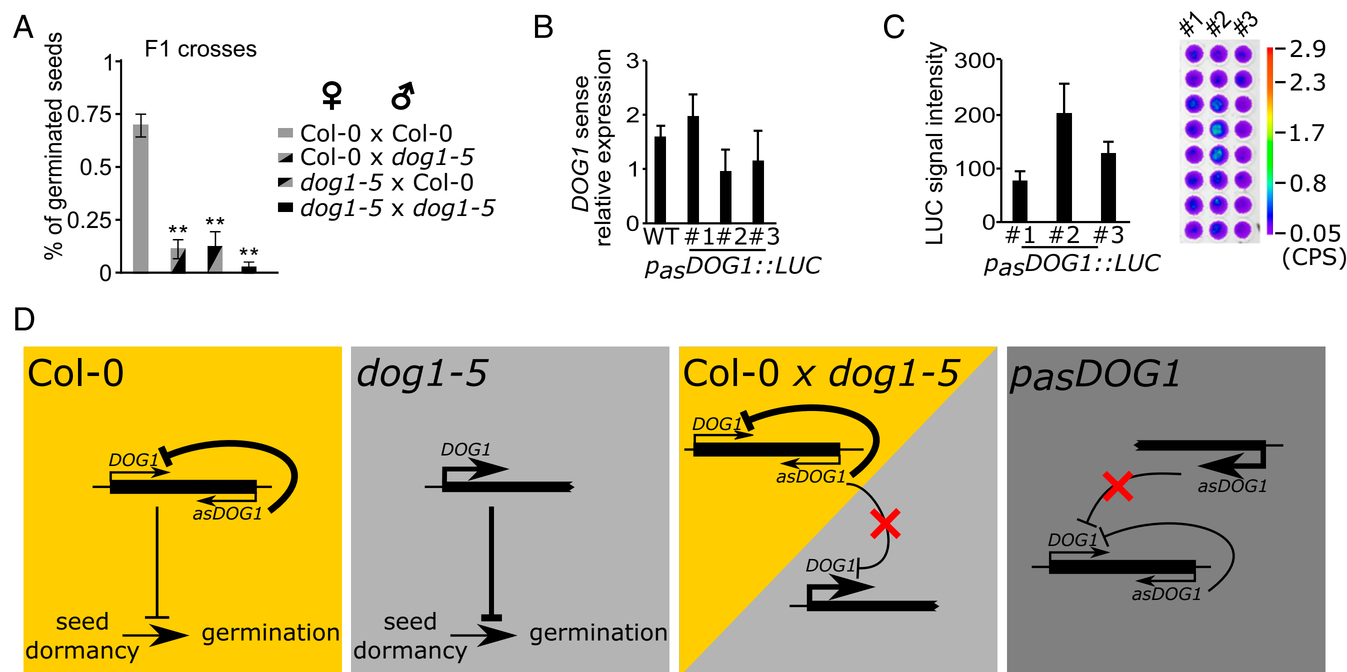

Fig. 4. *DOG1* antisense acts in *cis*. (*A*) Germination efficiency (%) of freshly harvested F1 seeds from a *dog1*-5 × Col-0 cross. *P* values were calculated in comparison with *Col-0 × Col-0*. Mean values from at least three biological replicates with error bars representing SD. $^{**}P < 0.01$. (*B*) An additional copy of *asDOG1* introduced into Col-0 (WT) by transformation does not affect *DOG1* expression. RT-qPCR was normalized against *UBC* mRNA; data are the means of three biological replicates with error bars representing SD. *t* tests show no statistical difference between WT and p_{as}*DOG1::LUC* lines. (*C*) *asDOG1* expressed from construct p_{as}*DOG1::LUC* introduced into Col-0 (WT). The luciferase signal was quantified (*Left*) and visualized (*Right*) in freshly harvested seeds from the indicated transgenic lines. (*D*) Summary of evidence supporting the proposed model of *cis*-restricted *asDOG1* regulation of seed dormancy. Yellow box: *DOG1* antisense activity in Col-0 (WT) plants results in limited *DOG1* expression leading to moderate seed dormancy strength. Gray box: the absence of *asDOG1* in mutant *dog1-5* results in high level expression of *shDOG1*, which strongly inhibits the transition to germination. Yellow/gray box: in Col-0 × *dog1-5* F1 seeds the *asDOG1* originating from Col-0 (WT) *DOG1* is unable to silence the *dog1-5* antisense-less copy of *DOG1* in *trans*. Dark gray box: the addition of a construct expressing additional *asDOG1* does not result in reduced *DOG1* sense expression in *trans*.

Discussion

Seed Dormancy Is Controlled by Antisense ncRNA. The *Arabidopsis* life cycle, like that of many other plants, is composed of a series of developmental transitions (2), starting with the transition from a dormant to a nondormant seed state that allows the embryo to germinate in permissive external conditions. To ensure a plant's survival and reproduction in a changing environment, these transitions are tightly controlled, including regulation by ncRNA. For example, small micro and tasiRNAs control juvenile phase length, and the long antisense transcript *COOLAIR* controls flowering time (24, 43, 44).

Here, we describe ncRNA-mediated control of seed dormancy. The *DOG1* gene is a central positive regulator of seed dormancy strength in *Arabidopsis* (9). We show that seed dormancy and *DOG1* expression are negatively regulated by an antisense transcript. This long ncRNA (lncRNA) is initiated in the region of the proximal transcription termination site (TTS) of the *DOG1* gene and terminates around the *DOG1* TSS (Fig. 1 *A* and *C*). Therefore, we named this transcript *asDOG1* or *1GOD*. The transcript is both 5′ capped and polyadenylated (Fig. 1 *A* and *C*), which suggests that it is transcribed by DNA-dependent RNA polII. This antisense transcript has a tissue-specific expression pattern, indicating that it is not generated by spurious transcriptional noise (45) (Fig. 2*A*). In agreement with this notion, we cloned the antisense promoter that drives its transcription (Fig. 2*B*). Most importantly, the elimination of *DOG1* antisense by T-DNA insertion or deletion resulted in strong *DOG1* sense expression. This finding confirmed that the *asDOG1* transcript acts as a negative regulator of *DOG1* expression and seed dormancy. *DOG1* antisense is strongly induced at the end of seed maturation when levels of the *DOG1* mRNA are reduced (Fig. 3*B*). Thus, the negative effect of *asDOG1* on *DOG1* expression is most prominent at the end of seed maturation. At this time we observed a fivefold increase in *DOG1* mRNA level in the *Arabidopsis dog1-5* mutant, in which antisense production is severely compromised in seeds (Fig. 3*E*).

This finding is corroborated by strong induction of DOG1 protein level in freshly harvested seeds of *dog1-5*, as shown by us previously (20). In agreement, we and others have shown that the *dog1-5* mutant has very strong seed dormancy (13, 20). Given the absence of a long-3 exonic transcript of *DOG1* in *dog1-5* seeds, the presence of a single overabundant DOG1 antibody reactive band in Western blot (20), we previously suggested that the short *DOG1* transcript is the source of the majority of DOG1 protein in vivo, and that it is the short DOG1 protein that controls the seed dormancy (20). Others have shown that DOG1 proteins dimerize and that the multiple splicing/polyadenylation isoforms presented could be required for DOG1 activity in controlling seed dormancy (11). Our published results are partially incompatible with this notion; therefore, more experimental work is needed to resolve those differences (11, 20).

Nevertheless, the *DOG1* antisense has the potential to regulate the *DOG1* alternative processing, including alternative splicing and alternative polyadenylation (9, 11, 20). Unfortunately, the antisense TSS is located in a very close proximity to the alternative splice sites and alternative polyA sites of the *DOG1* gene (Fig. 1*A*), making the study of antisense effect on *DOG1* alternative processing difficult.

Our data clearly show that *DOG1* antisense acts as a negative regulator of the short, two exonic (*shDOG1*) transcript during seed dormancy establishment and, in agreement with this *dog1-5* mutant, show strong seed dormancy (13, 20). Given that the *DOG1* expression in seeds is controlled by seed maturation temperature (46–49), it is possible that *DOG1* antisense may act as a sensor for external and internal stimuli, as shown for other antisense transcripts (26, 30).

DOG1 expression is strongly down-regulated during seed imbibition (9). Our data show a parallel reduction of *DOG1* sense and antisense transcripts during imbibition (Fig. S6), suggesting that *DOG1* antisense may not be involved at this stage but may have functions restricted to seed maturation.

Seed dormancy control by *asDOG1* resembles the regulation of flowering time by *COOLAIR*. Both antisense transcripts act as negative regulators, are 5′ capped, polyadenylated, and originate from the TTS region of the gene they regulate (25). In the case of *FLC*, the *COOLAIR* TSS is located downstream of the *FLC* protein-coding region. In *DOG1*, the antisense transcript is initiated from the proximal TTS of the sense transcript of *DOG1*, and the *asDOG1* promoter overlaps the *DOG1* exon 3 region. Recently, we showed that the short form of the *DOG1* transcript, comprising only exons 1 and 2, is able to complement the *dog1* mutant phenotype and that the amino acid sequence of the encoded protein is evolutionarily conserved. Because we were unable to identify a function for the long form of the DOG1 protein, we focused on *DOG1* exon 3, which encodes its C-terminal region. Previously we reported that the amino acid sequence encoded by exon 3 shows very weak conservation (20). However, despite numerous insertions and deletions resulting in premature stop codons, the exon 3 DNA sequence is relatively well conserved. Using motif analysis we showed that exon 3, together with the intron 2 region, form part of a larger DNA element that could be considered a conserved nonprotein coding-sequence element. The high level of DNA sequence conservation and lack of homology of the encoded polypeptides may be explained by the function of this element as a promoter for *DOG1* antisense transcription (Fig. 1*A*). This finding is reminiscent of the highly conserved *FLC* antisense transcript *COOLAIR* promoter region found in the Brassicaceae family (33).

***DOG1* Antisense Regulates Seed Dormancy in *cis*.** Antisense transcripts represent a substantial proportion of reported lncRNAs. To distinguish them from artificially introduced antisense transcripts used for genetic modification, they have been named natural antisense transcripts (50, 51). In plants, most of the well-characterized natural antisense transcripts act in *trans*, so they are able to regulate their targets when transcribed from a different location in the genome. Examples include: *HID1*, involved in photomorphogenesis (29), *cis-NAT*$_{PHO1;2}$ regulating phosphate homeostasis in rice (30), and *asHSFB2a*, controlling gametophyte development in *Arabidopsis* (31). In all of the above cases, expression of the antisense transcript in *trans* led to phenotypic changes. Similar *trans*-acting lncRNAs have been reported in other kingdoms: for example, human *HOTAIR* (52) and *TY1 CUT* in *Saccharomyces cerevisiae* (53).

Trans-acting antisense transcripts predominantly act as relatively stable RNA molecules that sequester, recruit, or scaffold *trans*-acting factors to regulate their targets (28). Our data show that *asDOG1* is relatively stable compared with the *DOG1* sense transcript and other short-lived protein-coding mRNAs (Fig. 1*D*). This finding indicated that part of the activity of *asDOG1* could be dependent on the RNA molecule itself. Alternatively, the observed stability of *asDOG1* might simply be a consequence of the presence of a 5′ cap and a polyA tail stabilizing this transcript, and does not necessarily indicate that this RNA is functional.

When we expressed the *DOG1* antisense transcript in *trans* (Fig. 4*B*), no changes in *DOG1* sense expression were observed, suggesting that its main mode of activity is in *cis*. However, it is possible that some of the antisense elements missing in our construct are required for its action. To exclude this possibility, we show that *asDOG1* transcribed from its native WT location was also unable to silence the *dog1-5* allele that is deficient in *DOG1* antisense production (Fig. 4*A*). Moreover, a *DOG1* transgene lacking the *asDOG1* promoter was highly overexpressed,

despite the presence of an antisense transcript originating from a WT *DOG1* copy. Thus, the endogenous antisense RNA was unable to silence the transgenic *DOG1* copy. Taken together, these data strongly suggest that *asDOG1* acts in *cis* in seed dormancy control. Examples of *cis*-acting antisense mechanisms include *Xist*-mediated allele-specific X-chromosome inactivation in humans and *PHO* gene regulation in yeast (54, 55). In addition, it has been suggested that in plants the antisense transcript *COOLAIR* acts via the process of its transcription or by formation of an RNA cloud, as seen in single-molecule FISH suppressing FLC transcription in *cis* (22, 32).

In contrast to published examples of *trans*-acting noncoding antisense transcripts in plants (29–31), the function of *asDOG1* is *cis*-restricted: it suggests that in seed dormancy, the act of *asDOG1* transcription, rather than the *asDOG1* RNA molecule, may be important. This suggestion is consistent with the strong requirement for transcription elongation factors in *DOG1* expression (14, 16, 17). However, this does not exclude the possibility that *asDOG1* may regulate other targets in *trans*, or act in *trans* to regulate *DOG1* in other tissues or situations. In agreement with this finding, in fission yeast the same ncRNA trigger has been shown to act in *cis* or *trans* based on its expression level (56) and the local chromatin context of the target (57).

Seed dormancy release represents a key developmental transition in plants and one that is subject to very strong selection. To ensure optimal timing of seed germination, there is a strong counter-selection against inadequate germination (6, 58). For this reason, the expression of *DOG1*, the main QTL for seed dormancy in *Arabidopsis*, is tightly regulated. In the present study we have demonstrated that *DOG1* expression and seed dormancy are controlled by a *cis*-acting antisense transcript. Given the conserved function of *DOG1* in seed dormancy and the evolutionary conservation of the *DOG1* antisense promoter described here, we expect this mechanism to be active in other plants.

Materials and Methods

Plant Materials and Growth Conditions. *A. thaliana* plants were grown in soil in a greenhouse with a long-day (LD) photoperiod (16-h light/8-h dark) at 22 °C/18 °C. For all experiments Col-0 was used as the WT background. The *DOG1* T-DNA insertion mutants, *dog1-3* (SALK_000867), *dog1-4* (SM_3_20886), and *dog1-5* (SALK_022748) were purchased from The European *Arabidopsis* Stock Centre (NASC). The sequences of the examined *DOG1* gene can be found in The *Arabidopsis* Information Resource (TAIR) data library under the accession number *At5g45830*. Material for transcript expression level analysis during seed development was performed as described previously (20). Briefly, flowers were manually emasculated and pollinated, then siliques were collected at the indicated number of days after pollination (DAP).

Seed Dormancy Tests. We used in our study Col-0 and mutants derived from it. Col-0 show a relatively weak seed dormancy phenotype, making the use of standard after-ripening treatment difficult (10). We have therefore taken advantage of a seed dormancy test based on the speed of germination of freshly harvested seeds because it shows a good correlation with *DOG1* transcript and protein levels. Briefly, freshly harvested seeds were sown on wet blue germination paper (Anchor) and germinated in a LD photoperiod (16-h light/8-h dark) at 22 °C/18 °C. Pictures were taken each day using a high-resolution camera and seed radical perfusion was calculated as described previously (59).

Imbibition Experiments. Approximately 150 seeds were imbibed in 300 μL of water with or without 10 μM final ABA concentration. Dry seeds were used as a control. The experiment was performed in a LD photoperiod (16-h light/8-h dark) in 22 °C on a rotating wheel. Samples were collected during the daytime, after 6, 12, and 24 h of imbibition.

RNA Extraction, cDNA Synthesis, and PCR Analysis. RNA extraction and cDNA synthesis were performed as previously described (20). Briefly, RNA was extracted using a phenol-chloroform protocol and treated with DNase (TURBO DNA-free kit, Life Technologies). Reverse transcription of 2.5 μg of RNA was performed using a RevertAid First Strand cDNA Synthesis kit (Fermentas). For cDNA synthesis of sense *DOG1*, oligo(dT) primers were used. The sequences of all primers are given in Dataset S1.

Adapter-Mediated RT-qPCR Assay. RNA extracted as described above was used in cDNA synthesis with a *DOG1* antisense specific primer with tag as shown in Fig. S3. Subsequently, the qPCR was performed as described above with tag-specific primer (AS_SS_RT) and *DOG1* primers (AS_F, AS_R), as shown in Dataset S1. RT-qPCR was performed using a LightCycler 480 real-time system (Roche) with SYBR Green mix (Roche). RT-qPCR results were normalized against the expression level of the *Arabidopsis UBC* gene (60). *P* values presented on graphs indicate $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Absolute Quantification of *DOG1* Sense and Antisense Transcripts. The absolute level of sense and antisense *DOG1* transcripts was calculated based on standard curve method, as described previously (61). First, the PCR fragment produced from a cDNA sample with primers DOG1_total_F and DMD3 was cloned into pJET 1.2 blunt-end cloning vector (all primer sequences are listed in Dataset S1). Resulting plasmids with insert in direct (for sense *DOG1* RNA) and reverse (for antisense *DOG1* RNA) orientation were linearized with a NcoI restriction enzyme and used as templates for in vitro transcription with T7 RNA polymerase. After synthesis, RNA products were recovered using LiCl precipitation and digested with recombinant DNaseI (Roche). Samples were checked for DNA-template contamination by PCR. Subsequently, RNA products were confirmed as nondegraded single-bands on a half-denaturing agarose gel and RNA concentration was measured using Qubit 2.0 fluorimetric assay. Next, dilution series ranging from 10 to 10^9 ng/μL were prepared for both sense and antisense RNAs, each dilution spiked with total yeast RNA to 750 ng/μL. These were used for cDNA synthesis and subsequent qPCR reactions, in triplicate for each dilution. Based on qPCR results, the curve parameters were calculated and used for standardization of data obtained from biological samples. RT-qPCR assays on imbibed seeds were performed using the same primers and procedures as those for standard curve preparation. The obtained Cp values were used to calculate absolute quantities in number of molecules per qPCR reaction. These values were than standarized according to normalized relative levels of the reference transcript *UBC*.

Evolutionary Conservation. *DOG1* ortholog sequences were retrieved from the PLAZA3.0 dicots database (62) and from the National Center for Biotechnology Information GenBank. A pairwise alignment of the *A. thaliana* and *A. lyrata DOG1* orthologs was obtained from the VISTA database of prealigned genomes and visualized using VISTApoint with default settings (63). In brief, for each base pair position, the 100-bp window-averaged identity score was calculated. Regions where the score was higher than 70% were considered as conserved and colored in blue (exons), pink (introns), or light-blue (UTRs). Multiple alignments were prepared using the procoffee algorithm available at the T-coffee web server (tcoffee.crg.cat). This algorithm is specifically designed for the alignment of promoter sequences (64). DNA motifs were identified using the MEME Suite (65). The original MEME output is presented in Fig. S9.

RNA Stability Assay. A cordycepin-dependent RNA stability assay was performed as described previously (20). Col-0 (WT) seedlings were grown on 1/2 Murashige and Skoog Basal Medium with 1% sucrose for 2 weeks in a growth chamber at a LD photoperiod (16-h light/8-h dark) at 22 °C/18 °C. All seeds were stratified at 4 °C for 2 days before sowing. Whole plants were collected and transferred to a flask containing incubation buffer (1 mM Pipes, pH 6.25, 1 mM trisodium citrate, 1 mM KCl, 15 mM sucrose). After 30 min of incubation, cordycepin was added to a final concentration of 150 mg/mL and vacuum-infiltrated (2 × 5 min). At each time-point (0, 15, 30, 45, 60, 75, and 120 min), seedlings representing ∼0.05 g were collected and frozen in liquid nitrogen. Samples were analyzed in triplicate. RNA extraction was performed as described above, and RT-qPCR analysis with primers specific for *EIF4A* and *At3g45970* were used in control reactions for mRNAs showing high and low stability, respectively (38).

5′ RACE. The 5′ RACE was performed using the Invitrogen 5′ RACE System, including dephosphorylation and subsequent TAP treatment. Individual clones were sequenced. Sequencing results are presented in Fig. S1.

Vectors and Plant Transformation. To prepare p_S*DOG1::LUC* and p_{as}*DOG1::LUC* constructs, cloning was performed using the Gateway system (Life Technologies) according to the standard protocol. Plasmid p_S*DOG1::LUC*

was prepared using donor (pDONR201) and destination (pGWB635_LUC) vectors. Plasmid p_{as}*DOG1::LUC* was made using donor (pENTR/D-TOPO) and destination (pGWB635_LUC) vectors. All constructs based on vector pGWB635_LUC were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Col-0 (WT) plants were transformed by the floral-dip method (66).

Luciferase Measurement. Seedlings were sprayed with 0.5 mM luciferin, held in darkness for 1 h, and then emitted light was measured using a NightSHADE LB985 camera, with an exposure time of 10 min. For seeds, about 100 were placed in a well of a white 96-well PCR plate and covered with 10 μL of 1 mM luciferin. After incubating overnight in darkness, emitted light was measured using the NightSHADE camera with an exposure time of 10 min.

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