

Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*

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Genetic variation for seed dormancy in nature is a typical quantitative trait controlled by multiple loci on which environmental factors have a strong effect. Finding the genes underlying dormancy quantitative trait loci is a major scientific challenge, which also has relevance for agriculture and ecology. In this study we describe the identification of the *DELAY OF GERMINATION 1* (*DOG1*) gene previously identified as a quantitative trait locus involved in the control of seed dormancy. This gene was isolated by a combination of positional cloning and mutant analysis and is absolutely required for the induction of seed dormancy. *DOG1* is a member of a small gene family of unknown molecular function, with five members in *Arabidopsis*. The functional natural allelic variation present in *Arabidopsis* is caused by polymorphisms in the cis-regulatory region of the *DOG1* gene and results in considerable expression differences between the *DOG1* alleles of the accessions analyzed.

natural variation | seed germination | abscisic acid | gibberellin | cis variation

Seed dormancy, defined as the inability of a viable seed to germinate under conditions that allow germination, is assumed to be an important adaptive trait in nature (1). It can prevent germination when environmental conditions are suitable for germination but where the probability of completing the life cycle is low (2, 3). In *Arabidopsis* a large number of mutants affecting seed dormancy have been generated artificially, and the genetic, physiological, and molecular characterization of these mutations is starting to shed light on the complexity of the regulation of this process. For instance, mutants in genes such as *ABA-insensitive 3* (*ABI3*) (4, 5), *FUSCA3* (*FUS3*) (6, 7), and *LEAFY COTYLEDONS* (*LEC1* and *LEC2*) (8–10) with defective seed maturation are nondormant, indicating that dormancy is part of the developmental program established during the later phases of seed development. The analyses of testa mutants originally identified by their altered seed color or shape provided strong evidence for the importance of the testa in the control of seed germination (11). Nongerminating mutants affected in the biosynthesis of the plant hormone gibberellin (GA) (12) and the nondormant mutants deficient in abscisic acid (ABA) (13) have shown the important role of ABA in the induction and maintenance of dormancy and the opposing roles of GA and ABA in the control of dormancy and germination (reviewed in ref. 14). The downstream effects of the hormones are less well known. Light-induced stimulation of seed germination is affected in phytochrome photoreceptor-deficient mutants (15). Furthermore, genes that most likely do not specifically affect hormone or light signaling pathways have been described as dormancy regulators. These include genes encoding transcription regulators such as *DOF* affecting germination (*DAG*) (16, 17) and several genes with unknown functions such as those disrupted in the *reduced dormancy 1–4* mutants (*rdo 1–4*) (18, 19). An additional set of genes that play roles in seed dormancy are those identified by the study of natural variation. The identification of the genes underlying this natural variation for seed dormancy may help to further increase our understanding of the mecha-

nisms involved in this process. At the same time, it will provide insights into the way nature shaped genetic variability for this trait during adaptive evolution. Currently, quantitative trait locus (QTL) mapping is a standard procedure in quantitative genetics. The identity of individual QTL, i.e., the DNA sequences (coding or noncoding) responsible for the QTL, can be established (20).

Natural variation for seed dormancy has been described in many species (21, 22), and several dormancy QTL regions have been validated in introgression or near isogenic lines (NILs) for *Arabidopsis*, barley, and rice (refs. 23–26 and reviewed in ref. 21). However, in none of these cases has the molecular function of the genes underlying dormancy QTL been as yet identified.

Previous research demonstrated that there is considerable genetic variation for seed dormancy between the laboratory strain Landsberg *erecta* (*Ler*) with low seed dormancy and the dormant Cvi accession from the Cape Verde Islands (24). QTL analysis for seed dormancy on a recombinant inbred line population derived from a cross between these two accessions identified *Delay of Germination 1* (*DOG1*) as an important determinant of seed dormancy within this population. *DOG1*, for which the Cvi allele increased the level of seed dormancy, explained 12% of the variance observed in seed dormancy in this recombinant inbred line population. Here we report the positional cloning and analysis of the seed dormancy QTL *DOG1*, which, based on the phenotype of the loss-of-function mutants, seems to specifically control seed dormancy whereas previously described seed dormancy and germination genes often affect multiple plant processes.

Results

Identification of a Mutant at the *DOG1*-Cvi Locus. To study the role of *DOG1* in seed dormancy we used a NIL containing a Cvi introgression at the position of this QTL in a *Ler* genetic background (called NILDOG17-1) (24). This line, which is more dormant than the *Ler* accession (Fig. 1A), confirming the presence of a dormant Cvi allele in that region, was submitted to a mutagenesis treatment. Mutants with no or strongly reduced seed dormancy were selected in this experiment. Among these were mutants at the *ABA1* and *ABA2* loci, indicating that ABA is needed for the functioning of *DOG1*. One line had a mutation

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Abbreviations: QTL, quantitative trait locus; ABA, abscisic acid; GA, gibberellin; NIL, near isogenic line; *Ler*, Landsberg *erecta*.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EF028465–EF028472).

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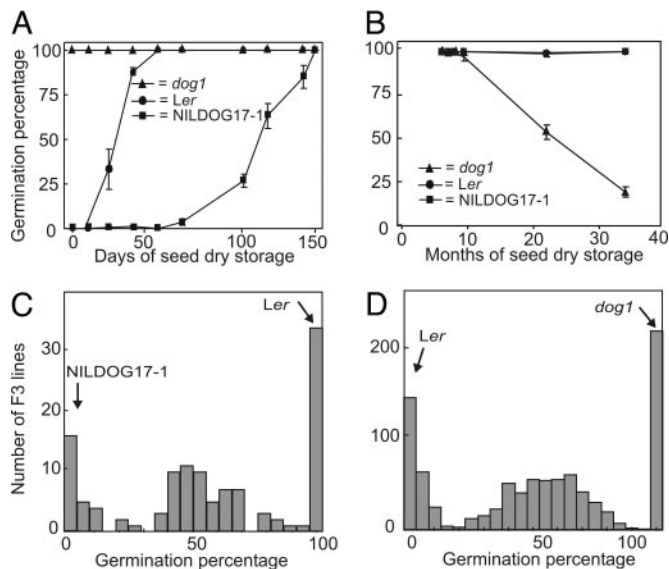


Fig. 1. Phenotypes of the different *DOG1* alleles. (A) Germination behavior of *Ler*, *NILDOG17-1*, and *dog1* at different time points after seed harvest. The means and SE of four replicates are shown. (B) Seed storability phenotypes of the different *DOG1* alleles. Germination behavior of the different *DOG1* alleles, *Ler*, *NILDOG17-1*, and *dog1* at different time points after seed harvest. The means and SE of triplicates are shown. (C and D) Segregation of *DOG1* alleles. Shown are frequency distributions of germination percentages of F_3 seed batches harvested from individual F_2 plants derived from the crosses *Ler* \times *NILDOG17-1* (C) and *Ler* \times *dog1* (D).

in the *DOG1* gene itself (*dog1*), as was concluded from the fact that no recombinants between this mutation and the dormant *Cvi* allele could be detected among 2,061 gametes analyzed in the F_2 progeny of a *Ler* \times *dog1* cross. The *dog1* mutant does not show any obvious pleiotropic effects except that it cannot be stored as long as *Arabidopsis* wild-type seeds at room temperature (Fig. 1B). To study the genetics of the different *DOG1* alleles, the germination behavior of F_3 seeds, obtained from F_2 plants derived from crosses of *Ler* with either *DOG1-Cvi* (*NILDOG17-1*) or *dog1*, were tested. Segregation ratios were in agreement with monogenic inheritance and suggest semidominance of the *DOG1* alleles (Fig. 1 C and D).

Germination Characteristics of the Three *DOG1* Alleles. The germination behavior of the *dog1* mutant resembles ABA-insensitive and ABA biosynthesis mutants (13). The *aba1-1* mutant is characterized by the absence of dormancy but also by germination in darkness and germination without the need of *de novo* GA biosynthesis. This was shown by germination on GA biosynthesis inhibitors and the ability of double mutants with the GA biosynthesis mutant *gal-3* to germinate (13, 27). To determine whether *dog1* has the same germination characteristics we performed similar experiments with *dog1*. This revealed that *dog1* mutant seeds still require light and GA for germination, although less than *Ler* and *NILDOG17-1* (Fig. 2A). Reduced germination in darkness can be partly overcome by cold stratification (Fig. 2B), probably because cold can increase GA sensitivity (27). *DOG1* is not specifically involved in ABA signal transduction, because the *dog1* mutant has a normal sensitivity to applied ABA (Fig. 2C). However, to achieve dormancy conferred by the *DOG1-Cvi* allele, ABA is required, because combining *NILDOG17-1* with the *aba1-1* mutant led to nondormant seeds (Fig. 2D). In conclusion, *dog1* seeds still require light-induced GA biosynthesis to overcome inhibition by ABA present in imbibed nondormant seeds (28–30).

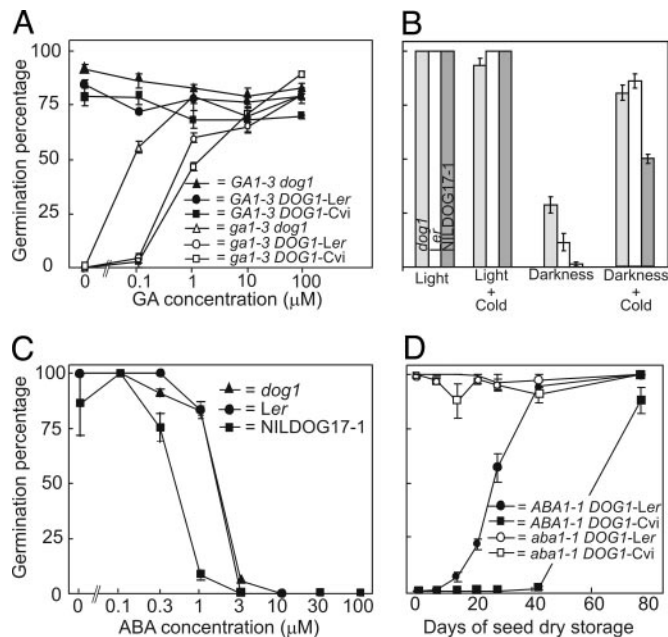


Fig. 2. Germination behavior of *DOG1* under different environmental circumstances and in different genetic backgrounds. (A) GA requirement for germination. Germination on various concentrations of GA_{4+7} of after-ripened seeds of *GA1-3 dog1*, *GA1-3 DOG1-Ler*, and *GA1-3 DOG1-Cvi* and on the GA-deficient seeds *gal-3 dog1*, *gal-3 DOG1-Ler*, and *gal-3 DOG1-Cvi*. Seeds of *GA1-3 dog1* and *gal-3 dog1* were 1 month after-ripened, and seeds of the other combinations were after-ripened for 25 months. Percentages are averages of four measurements \pm SE. (B) Germination in darkness. Shown are germination percentages of after-ripened seeds of *dog1*, *Ler*, and *NILDOG17-1* under different environmental conditions, in light, in light after a cold stratification, in darkness, and in darkness after a cold stratification. The means and SE of triplicates are shown. (C) ABA sensitivity of the three *DOG1* alleles. Shown is germination in different ABA concentrations of after-ripened seeds of *dog1*, *DOG1-Ler*, and *DOG1-Cvi*. Seeds of *dog1* and *DOG1-Ler* were after-ripened for 4 months, and seeds of *DOG1-Cvi* were after-ripened for 25 months. The percentages are means of triplicates \pm SE. (D) ABA dependency of *DOG1*. Shown is germination behavior of *aba1-1* in two different *DOG1* backgrounds (*DOG1-Ler* and *DOG1-Cvi*) at different time points after seed harvest.

Isolation of *DOG1*. To understand the molecular function of *DOG1*, the gene was cloned by using positional information and the *dog1* mutant described above. Recombination mapping, using the progeny of crosses between a *Ler* morphological marker line carrying the *gl3* and *tz* mutation and *NILDOG17-1* and *Ler* \times *dog1*, placed the *DOG1* locus between marker *cMRA19z* and marker *K15I22* in the region where the QTL was mapped on chromosome five. This region contained 22 ORFs. Because the loss-of-function phenotype of *DOG1* was known, we decided to look for the candidate gene in that region by the identification of a gene whose knockout phenotype resembled the phenotype of the *dog1* mutant described above. Among the insertion mutants from the Salk collection (<http://signal.salk.edu>), Salk line 000867 was identified as completely nondormant. This line had a T-DNA insertion in the promoter region of *At5g45830*. Three additional lines (SM_3.20808, SM_3.20873, and SM_3.20886 from J. H. Clarke, John Innes Centre, Norwich, U.K.) with transposons inserted at position 213 of the same gene and therefore probably representing the same transposition event, also have nondormant phenotypes (positions of the insertions are indicated in Fig. 3). Sequence analyses of *At5g45830* in *NILDOG17-1* and *dog1* identified a 1-bp deletion at position 914 (based on *Col* genomic sequence) in the *dog1* mutant. This deletion changes the reading frame and terminates

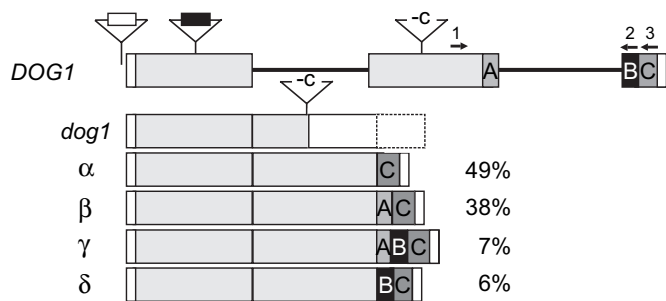


Fig. 3. Schematic illustration and relative abundance of the *DOG1* transcripts. At the top of the figure the *DOG1* genomic DNA is shown. The insertions of SALK 000867 (white box), SM.3.20808, SM.3.20873, and SM.3.20886 (black box) and the position of the mutation in the *dog1* mutant (-c) are indicated on top of this genomic structure. Boxes on the black solid line indicate the exons, white boxes indicate 3' and 5' untranslated regions, and the different greyscales are used to illustrate the different exon compositions of the transcripts α , β , γ , and δ , which are shown below. The percentages indicate the relative abundance of the different transcripts; this is an average of the abundance measured in dry dormant and nondormant seeds of several accessions analyzed. Primers used for PCR amplification are indicated by the black arrows (1, pyro F2; 2, pyro R2; 3, pyro R3).

the ORF 6 aa after the deleted nucleotide (Fig. 3). To prove that At5g45830 caused the dormancy phenotype a 5.6-kb PCR fragment containing the Cvi allele of At5g45830 and both the 5' and 3' region was cloned into the binary vector pBIB-HYG (31) (pBIB-*DOG1*-Cvi) and transformed into *Ler* by using *Agrobacterium tumefaciens*. Two independent transformants homozygous for the construct had a strong dormant phenotype. This confirmed that the Cvi allele of At5g45830 is responsible for the strong seed dormancy in NILDOG17-1 (Fig. 4).

***DOG1* Is a Member of a Novel, Plant-Specific Gene Family.** *DOG1* (At5g45830) belongs to a small gene family. Based on the gene structure of *DOG1* we were able to identify four additional genes of this type in the *Arabidopsis* genome. We have called them *DOG1-Like 1–4* (*DOGL1–4*), which encode At4g18660, At4g18680, At4g18690, and At4g18650, respectively. Four of the *DOG1* family genes are highly conserved; the sequence similarity of *DOGL1* to *DOGL3* with *DOG1* is, respectively, 54.3%, 43.1%,

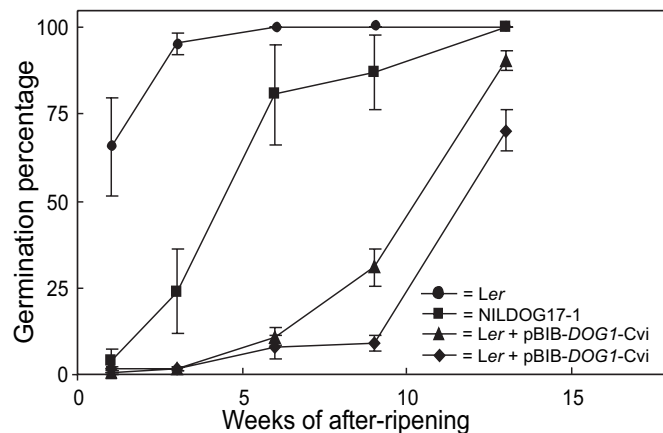


Fig. 4. Introduction of the *DOG1*-Cvi allele into the *Ler* genetic background. The germination of homozygous transformants obtained by *Agrobacterium*-mediated transformation of *Ler* with the genomic fragment of *DOG1*-Cvi is shown. Shown is germination behavior of *Ler*, NILDOG17-1, and two independent transformants at different time points after seed harvest. The means and SE of triplicates for *Ler* and NILDOG17-1 and 11 and 12 replicates for the two transformants, respectively, are shown.

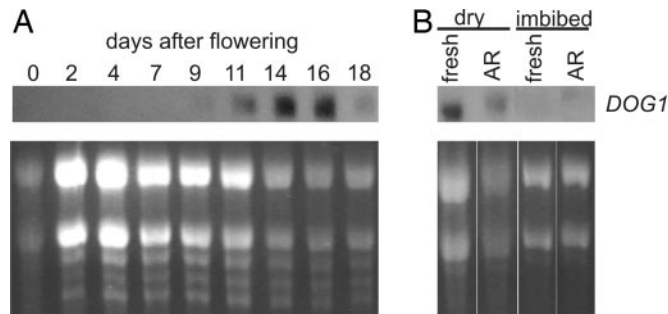


Fig. 5. Northern blot analyses of the expression of *DOG1* in seeds of NILDOG17-1. (A) The expression at different developmental stages, starting at the day of pollination until 18 days after pollination, when the seeds are mature and the silique opens (analyzed in developing siliques). (B) The expression in fresh (dormant) and after-ripened (AR; nondormant) dry and imbibed mature seeds is shown. (Upper) Autoradiograph. (Lower) RNA loading stained with ethidium bromide.

and 39.3% (Fig. 7, which is published as supporting information on the PNAS web site). *DOGL4* is much more distinct, with only a 23.4% sequence similarity with *DOG1*. To test whether these *DOGL* genes affect seed dormancy we obtained presumed null mutants for *DOGL1*, *DOGL2*, and *DOGL3* from the Salk T-DNA insertion project (<http://signal.salk.edu>). However, insertions in these genes did not result in a germination phenotype (data not shown). To assign a possible function for the *Arabidopsis* *DOG1* gene family proteins we compared them with similar genes in other species. The highest percent similarity found is with a *Brassica napus* EST [embryo library; tBLASTx in NCBI EST database (CN827162); global identity 37.8%, local 53.4% in 206 aa]. This gene has not been annotated. The highest percent similarity with a gene that has a known function is that with the wheat transcription factor HBP-1b (CAA40102) (global 13.3%; local 42% in 33 aa). *DOG1* contains three protein domains (PD870616, PD004114, and PD388003) as defined by ProDom (<http://prodom.prabi.fr/prodom/current/html/home.php>) (32). PD870616 is present only in the five related *Arabidopsis* genes mentioned above and has not been annotated. PD004114 is present in the D bZIP transcription factors described in ref. 33. This group of transcription factors contains the bZIP domain and an additional conserved motif (box 1). However, *DOG1* does not show any homology in these two regions but only with the region between the bZIP domain and box 1. Protein domain PD388003 has been annotated as tumor-related protein-like (BAA05470.1) (23.7% global identity) because it was found in the hybrid *Nicotiana glauca* \times *Nicotiana langsdorffii*, which shows tumorous cell growth. Another gene with the same DNA structure as *DOG1* is BAB08196 from *Oryza sativa* (17.9% global identity). The functions of both these genes are unknown. This low percent similarity with genes of unknown function in other species means that a conclusion about the molecular function of *DOG1* cannot be drawn as yet.

***DOG1* Expression Is Seed-Specific.** We analyzed the *DOG1* expression in different plant tissues; seedlings, roots, flowers with closed buds and flowers with open buds, and developing siliques at different time points and in dormant and after-ripened dry and imbibed seeds. *DOG1* transcription is seed-specific; transcription starts during the seed development 9 days after pollination and reaches its highest level during the last phases of seed development (as was detected by analyzing developing siliques) (Fig. 5). This is in agreement with data reported by the Genevestigator online search tool Gene Chronologer (www.genevestigator.ethz.ch) (34). *DOG1* transcripts remain present in after-ripened (nondormant) dry seeds. Upon imbibition the transcripts rapidly disappear in both

dormant and after-ripened seeds (Fig. 5). To determine whether the promoter region of *DOG1* contains motifs that can explain its seed-specific expression we have run 2,349 bp of genomic sequence 5' of the *DOG1* ATG in PLACE (a database of plant cis-acting regulatory DNA elements) (35). We could identify a RY repeat (CATGCA; -500 from the ATG) required for seed-specific expression. In addition, we found ABRE motifs (TACGTGTC; -1671 and -1650 from the ATG) that are known to be required for ABA responsiveness and that have been found as well in 24 genes that are GA-down-regulated during *Arabidopsis* seed germination (36).

Alternative Splicing of the *DOG1* Gene. The At5g45830 gene is described as encoding a predicted ORF of 291 aa (GenBank accession no. NM123951). From sequence analyses performed on cDNA pools we could identify at least four different cDNAs (α - δ) present in *Ler* and in *Cvi*, suggesting that they are the result of alternative splicing. The four different cDNAs are different combinations of three exon fragments, A, B, and C (C only = cDNA α , A+C = β , A+B+C = γ , and B+C = δ) (Fig. 3). To investigate the possibility that the different phenotypes of the *DOG1-Ler* and NILDOG17-1 could be explained by different relative levels of the spliced cDNAs, we developed a pyrosequencing assay to quantify the relative abundance of the different transcripts in mature dry seeds (Fig. 3). This method did not detect a significant difference in amount of the different transcripts between the genotypes analyzed or between dormant (fresh) and nondormant (after-ripened) seeds. CDNA β and γ encode the same protein, which is the result of a stop codon in exon A. We do not know whether all of the cDNAs are translated into peptides or not.

Sequence Diversity of *DOG1* in Different Accessions of *Arabidopsis thaliana*. To understand the basis for the allelic differences at *DOG1*, we compared the nucleotide sequences of the *DOG1-Ler* and *DOG1-Cvi* alleles (in total \approx 5.5 kb based on the *Col* sequence; starting 2,349 before the translational start). Three polymorphisms and one indel have been identified in the coding region, and two of the polymorphisms lead to an amino acid substitution, serine-927 to proline and proline-1694 to glutamine. The indel resulted in an extra glycine in *DOG1-Ler* compared with *DOG1-Cvi*. In addition, we sequenced *DOG1* alleles of six additional accessions (*Col*, *An-1*, *Kond*, *Sha*, *Kas-2*, and *Fei-0*) of which recombinant inbred line populations derived from crosses with *Ler* have been constructed (37–39) (Carlos Alonso-Blanco, personal communication). In four of these populations (*Sha*, *Kond*, *Kas-2*, and *Fei-0*) a QTL at *DOG1* or a closely linked locus has been identified (Table 1, which is published as supporting information on the PNAS web site) (ref. 38 and L.B., unpublished results). The relative dormancy levels of the different accessions are indicated in Table 1. The sequence comparison showed various polymorphisms in the coding regions of *DOG1* alleles; however, we did not observe a correlation between the sequence and the dormancy level of the accessions. Therefore, we assume that these polymorphisms are not responsible for the functional allelic differences found for *DOG1*.

Expression Diversity of *DOG1*. In addition to the sequence diversity in the coding region we observed 19 polymorphisms and 10 indels in the putative cis-regulatory region of the *DOG1* gene. To determine whether these differences could cause the difference in phenotype we analyzed the functional variation of the *DOG1* cis-regulatory region. To quantify this functional variation as a result of the cis-regulation of *DOG1* mRNA levels specifically, we used F₁ seeds in which distinct promoter alleles were compared within a common trans-regulatory background and quantified the relative levels of the two parental mRNA forms in the hybrid by pyrosequencing (as described in refs. 40 and 41). F₁ seeds were analyzed by comparing *Ler* with each of nine other genotypes (the

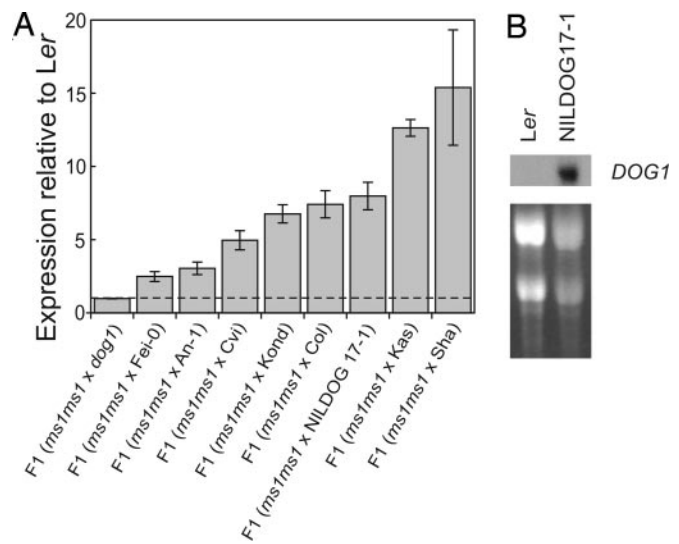


Fig. 6. Expression analyses of *DOG1* in different genetic backgrounds. (A) Relative expression levels were measured by pyrosequencing of cDNAs derived from RNA extracted from F₁ seeds made by pollinating a *male sterile 1* (*ms1*) mutant in a *Ler* background with pollen of each of nine other genotypes (the accessions *Fei-0*, *An-1*, *Cvi*, *Kond*, *Col*, *Kas-2*, and *Sha* and NILDOG17-1 and *dog1*). The relative values are means of triplicates \pm SE. The horizontal dashed line indicates a relative expression level of 1. (B) Northern blot analyses of the expression of *DOG1* in mature seeds of *Ler* and NILDOG17-1. (Upper) Autoradiograph. (Lower) RNA loading stained with ethidium bromide.

accessions *Fei-0*, *An-1*, *Cvi*, *Kond*, *Col*, *Kas-2*, and *Sha* and NILDOG17-1 and *dog1*). The *Ler* coding region harbored a single nucleotide polymorphism allowing us to distinguish this *DOG1* allele (*DOG1-Ler*). The relative amount of each parental allele was quantified by pyrosequencing (Fig. 6A). This procedure did not distinguish between the differentially spliced transcripts (α - δ). Differences in *DOG1* expression found between *Ler* and NILDOG17-1 were confirmed by RT-PCR (data not shown) and Northern blot analyses (Fig. 6B). Cis-regulatory alleles have a 2- to 15-fold difference in activity relative to the *Ler* cis-regulatory allele, which is much larger than what was previously observed in *A. thaliana* at another locus (41). *DOG1-Ler* and the *dog1* mutant have the lowest expression levels. *DOG1-Cvi* in the background of the *Cvi* accession has clearly a lower expression level than *DOG1-Cvi* in the background of NILDOG17-1, which could be because of loci present outside the introgression in *Cvi* that reduce the mRNA level of *DOG1* (24).

Discussion

The genetics of seed dormancy has been studied mainly by analyzing mutants that were selected based on a germination phenotype. This revealed several mutants defective in hormonal pathways.

Natural variation provides another genetic resource to identify genes that are involved in the control of seed dormancy. One of these genes which determines the genetic variation for seed dormancy in nature in *Arabidopsis* is the *DOG1* locus, identified by QTL analysis of the progeny from a cross between the accessions *Ler* and *Cvi* (24). In the present work we have identified the underlying gene of the *DOG1* QTL by fine (high-resolution) mapping of the QTL and by the identification of a loss-of-function allele, which resulted in loss of dormancy. The latter allowed us to screen for T-DNA insertion mutants in the available collections in the low-dormancy *Col* accession. *DOG1* is encoded by At5g45830 and represents a novel gene with unknown function. *DOG1* belongs to a small family of proteins in *Arabidopsis* that contains three conserved domains

(PD870616, PD004114, and PD388003) of which the functions are unknown. *DOG1* by itself has a strong effect on seed dormancy whereas the *DOG1*-like genes, which are highly similar to each other, have no proven influence on seed dormancy.

DOG1, of which the different alleles behave in a codominant manner, is a gene that specifically controls seed dormancy. Apart from a seed longevity phenotype in *dog1* mutant seeds, no other pleiotropic phenotypes have been identified. *DOG1* function is clearly related to ABA, based on the double mutant analysis. Preliminary data suggest that it may affect ABA levels in dry seeds (J. Zeevaert, personal communication) and slightly changes ABA sensitivity (Fig. 2C). The observation that the *DOG1* transcript levels drop during seed imbibition (Fig. 5) and that ABA also has an effect in imbibed seeds where it antagonizes the effect of GA (induced by light) might indicate that *DOG1* functions only in the establishment of dormancy during seed maturation, whereas ABA may function during imbibition also. This might be in agreement with the observation that the *dog1* mutant still requires light and GA to induce germination in the nondormant seeds that need the latter to initiate germination. Another nondormant mutant that still requires light and GA is *fusca 3* (42). This mutant belongs to a group of genes that control the seed maturation program.

The amino acid substitutions that were detected in the structural part of the *DOG1* gene when comparing different accessions most likely are not functional. Therefore, the natural variation, present in *DOG1*, may be controlled by differences in cis-regulatory region(s). Expression analyses performed on F₁ seeds of *Ler* crossed with the different accessions indeed show clearly that the natural allelic variation in the *DOG1* gene between these accessions causes differences in gene expression level. The 5' upstream cis-acting region controlling the expression has also been sequenced, and, although many polymorphisms were shown to be present in this region upstream of the putative translational start codon, it is difficult to define the responsible polymorphism(s) that affect the expression of the *DOG1* gene. To judge whether certain haplotypes are responsible for the effect on the expression, more accessions need to be analyzed.

With the isolation of *DOG1* the first seed dormancy gene accounting for variation occurring in natural populations has been identified at the molecular level. The identification of the first seed dormancy QTL is an important step toward the elucidation of a genetic pathway that controls this important agronomical trait.

Methods

Plant Material. NILDOG17-1 has a 14-cM Cvi introgression around *DOG1* in a *Ler* background as previously described by Alonso-Blanco *et al.* (24). *Ler* (NW20), Cvi (N8580), An-1 (N944), Kond (CS6175), Col (CS907), Kas-2 (N1264), Sha (CS929), and the transgenic lines SALK 000867 (N500867), SM_3.20808 (N105944), SM_3.20873 (N105997), and SM_3.20886 (N106006) were provided by the Nottingham *Arabidopsis* Stock Centre, and Fei-0 (CS22645) was a gift from Carlos Alonso-Blanco (Centro Nacional de Biotecnología, Madrid, Spain). The morphological marker line carrying the *gl3* and *tz* mutation was constructed in our laboratory (43).

Mutant Isolation. Approximately 10,000 M₀ seeds of NILDOG17-1 were soaked in tubes containing 10 mM KNO₃ and 0.2% agar for 20 h at 4°C, mutagenized with 300 Gy of γ -irradiation, and dispersed on soil in an air-conditioned greenhouse. M₂ seeds were harvested from groups of \approx 100 M₁ plants. In total there were 90 bulks, and a few hundred seeds of every bulk were sown on water-saturated filter paper (no. 595; Schleicher & Schuell, Dassel, Germany) in Petri dishes on the day of harvest. Seeds that germinated within 3 days after seed harvest were selected as putative mutants. Freshly harvested NILDOG17-1 seeds did

not germinate under these conditions. The mutant phenotype was confirmed by retesting the germination of the seeds harvested from the plants grown from these selected seedlings. To discard possible nondormant contaminants, the selected mutants were checked for the presence of the Cvi introgression by using Cleaved Amplified Polymorphic Sequence marker DFR (*TT3* gene; the *Arabidopsis* Information Resource, www.arabidopsis.org).

Growth Conditions. The growth conditions used were described in ref. 39. The *gal1* mutants were germinated on 10 μ M GA₄₊₇, and the resulting seedlings were planted in the greenhouse, where they were sprayed once a week with 100 μ M GA₄₊₇ to stimulate growth, anther development, and seed production. Seeds were harvested in cellophane bags and stored in a cardboard box at room temperature.

Seed Dormancy Measurements and Germination Assays. Germination tests in water under white light were performed at each time point by incubating seeds for 1 week as follows. Between 50 and 100 seeds of a genotype were evenly sown on a filter paper soaked with 0.7 ml of demineralized water, 0–100 μ M GA₄₊₇, or 0–100 μ M ABA (mixed isomers; Sigma-Aldrich, St. Louis, MO) in a 6-cm Petri dish. GA₄₊₇ was dissolved in a few drops of 1 M KOH and diluted with a phosphate citrate buffer containing 3.3 mM K₂HPO₄·3H₂O and 1.7 mM citric acid (pH 5.0).

To allow germination the Petri dishes were placed in moisture chambers consisting of plastic trays containing a filter paper saturated with tap water and closed with transparent lids. These moisture chambers were transferred to a climate room (25°C, 16-h light/day, model TL57; Philips, Eindhoven, The Netherlands), and germination was scored after 7 days. The total number and the number of germinating seeds were scored, and the percentage of germinating seeds was calculated.

For germination in the dark, seeds were sown under green-light conditions, and the Petri dishes were wrapped in two layers of aluminum foil and stored in a closed box under the same conditions as the light-germinated seed.

Cold stratification was performed by placing the imbibed seeds on water-soaked filter paper for 7 days at 4°C in darkness.

Germination analyses to study the genetic behavior of the different *DOG1* alleles were performed on F₃ seeds from individual F₂ plants of the crosses *Ler* \times NILDOG17-1 and *Ler* \times *dog1*. Seeds of *Ler* \times NILDOG17-1 were sown 28 days after seed harvest to allow the *Ler* seeds to germinate 100% and seeds of *Ler* \times *dog1* were sown directly after seed harvest.

DNA Extractions. DNA was extracted by using the Wizard magnetic 96 (FF3760; Promega, Madison, WI) DNA isolation kit.

Fine-Mapping of *DOG1*. Details of the molecular markers used for the fine-mapping of *DOG1* can be found in Table 2, which is published as supporting information on the PNAS web site.

Complementation of *DOG1*. A genomic DNA fragment of 5.6 kb of *DOG1*-Cvi was amplified by PCR using primers EcoRI-5791 (CCGAATTCTCGTCTTGGAAATGTGTTTCCCATGG) and KpnI-10944 (CCGGTACCAAATTGTTTGTGCATGCTTCAGC) and AccuPrime Pfx DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). This PCR product was cloned into the pCR-Blunt II-TOPO vector (Invitrogen Life Technologies). From this vector it was moved to pBIBkan-hyg (31) and inserted at the EcoRI/KpnI restriction sites to create pBIB *DOG1*-Cvi. pBIB *DOG1*-Cvi was introduced by electroporation to *A. tumefaciens* (AGLO strain) (44). The transformed AGLO strain was used to transform *Ler* plants by floral dip. T₁ primary transformants selected on the basis of their resistance to hygromycin B

were transferred to the greenhouse to set T_2 seeds that were collected to perform germination experiments.

Sequencing. PCR products were sequenced by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence products were separated by Greenomics (Wageningen, The Netherlands).

RNA Extractions. RNA extractions were performed according to ref. 45, with small modifications. The phenol, phenol/chloroform, and chloroform extractions were performed only once. The RNA pellet was dissolved in 50 μ l of DEPC-treated MQ and subsequently applied to a RNeasy column to clean the RNA and to perform DNA treatment (RNA purification; Qiagen, Valencia, CA).

Northern Blot Analyses. Northern blot analysis was performed following the protocol supplied with the Hybond-N nylon membranes (Amersham Pharmacia, Uppsala, Sweden) by using 10 μ g of RNA isolated as described above. *DOG1* expression was detected by using an 866-bp probe corresponding to the coding region of the *DOG1* gene.

Pyrosequencing. Pyrosequencing was used to quantify allele frequencies and transcript forms (alternative splicing) in RT-PCR products. Sequence primers were developed by using SNP Primer Design software (version 1.01) from Biotage (Uppsala, Sweden).

cDNA synthesis. For the first-strand cDNA synthesis 800 ng total RNA was used in a volume of 20 μ l by using M-MLV reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's protocol. The standard dT₁₂₋₁₈ adapted primer was used.

Alternative splicing. For RT-PCR 10 μ l of 8 \times diluted cDNA was used with 10 pmol of each primer, 100 μ M of each deoxynucle-

otide, 1 unit of Super TaqDNA polymerase, and the Super TAO reaction buffer provide by Sphaero Q (Gorinchem, The Netherlands). For PCR the following primers were used: pyro F2, biotinylated GAGTGGGGAAGTATGAGAGATCGT; pyro R2, CCCACTATTACAGTTGTACATGC. Primer positions are indicated in Fig. 3.

The SNP was analyzed by using sequence primer Seq2 (TG-TACATGCATCGAATATTA). To discriminate between cDNA transcripts γ and δ a second PCR was performed by using the following: pyro F2, biotinylated GAGTGGGGAAGTATGAGAGATCGT; pyro R3, GCAAAATGCCACGACGTGA. The SNP was analyzed by using sequence primer Seq3 (TGC-CACGACGTGAATAAA).

Quantification of allele frequencies. For RT-PCR 2 μ l of 5 \times diluted cDNA was used under the same PCR conditions as mentioned above for the alternative splicing. For PCR the following primers were used: pyro F1, TACAAGAAGACGACGCGGATAT; pyro R1, biotinylated CGGAGATAGAATCCCGAGGA. The SNP was analyzed by using sequence primer Seq1 (GGAGAAT-GTCGGAGAG).

As controls, DNA of F₁ plants was used. The relative expression of *DOG1-Ler* to the other *DOG1* of the other accessions should be 1 in the F₁ genomic DNA.

To control for possible position effects in the thermocycler, cDNA samples together with DNA extracted from heterozygous plants were randomly distributed across 96-well plates before PCR.

Pyrosequencing was carried out according to the manufacturer's standard protocols (Biotage).

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- Bewley JD (1997) *Plant Cell* 9:1055–1066.
- Donohue K (2005) *Seed Sci Res* 15:175–187.
- Fenner M, Thompson K (2005) *The Ecology of Seeds* (Cambridge Univ Press, Cambridge, UK).
- Ooms JJ, Léon-Kloosterziel KM, Bartels D, Koornneef M, Karssen CM (1993) *Plant Physiol* 102:1185–1191.
- Nambara E, Keith K, McCourt P, Naito S (1995) *Development (Cambridge, UK)* 121:629–636.
- Bäumlein H, Miséra S, Luersen H, Kölle K, Horstmann C, Wobus U, Müller AJ (1994) *Plant J* 6:379–387.
- Luersen H, Kirik V, Herrmann P, Misera S (1998) *Plant J* 15:755–764.
- Meinke DW, Franzmann LH, Nickle TC, Yeung EC (1994) *Plant Cell* 6:1049–1064.
- Lotan T, Ohto M, Yee KM, West MA, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) *Cell* 93:1195–1205.
- Stone SL, Kwong LW, Yee KM, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada JJ (2001) *Proc Natl Acad Sci USA* 98:11806–11811.
- Debeaujon I, Léon-Kloosterziel KM, Koornneef M (2000) *Plant Physiol* 122:403–414.
- Koornneef M, Van der Veen JH (1980) *Theor Appl Genet* 58:257–263.
- Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) *Theor Appl Genet* 61:385–393.
- Kucera B, Cohn RA, Leubner-Metzger G (2006) *Seed Sci Res* 15:281–307.
- Casal JJ, Sanchez RA (1998) *Seed Sci Res* 8:317–329.
- Papi M, Sabatini S, Bouchez D, Camilleri C, Costantino P, Vittorioso P (2000) *Genes Dev* 14:28–33.
- Gualberti G, Papi M, Bellucci L, Ricci I, Bouchez D, Camilleri C, Costantino P, Vittorioso P (2002) *Plant Cell* 14:1253–1263.
- Léon-Kloosterziel KM, van de Bunt GA, Zeevaart JAD, Koornneef M (1996) *Plant Physiol* 110:233–240.
- Peeters AJ, Blankestijn-De Vries H, Hanhart CJ, Léon-Kloosterziel KM, Zeevaart JA, Koornneef M (2002) *Physiol Plant* 115:604–612.
- Salvi S, Tuberosa R (2005) *Trends Plant Sci* 10:297–304.
- Bentsink L, Soppe W, Koornneef M (2007) in *Seed Development, Dormancy, and Germination*, eds Bradford KJ, Nonogaki H (Blackwell, Oxford), pp 113–132.
- Koornneef M, Bentsink L, Hilhorst HWM (2002) *Curr Opin Plant Biol* 5:33–36.
- Han F, Ullrich SE, Clancy JA, Romagosa I (1999) *Plant Sci* 143:113–118.
- Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-De Vries H, Koornneef M (2003) *Genetics* 164:711–729.
- Gao W, Clancy JA, Han F, Prada D, Kleinhofs A, Ullrich SE (2003) *Theor Appl Genet* 107:552–559.
- Takeuchi Y, Lin SY, Sasaki T, Yano M (2003) *Theor Appl Genet* 107:1174–1180.
- Debeaujon I, Koornneef M (2000) *Plant Physiol* 122:415–424.
- Yamaguchi S, Sun T, Kawaide H, Kamiya Y (1998) *Plant Physiol* 116:1271–1278.
- Grappin P, Bouinot D, Sotta B, Miginiac E, Jullien M (2000) *Planta* 210:279–285.
- Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, Grappin P, Jullien M (2004) *Planta* 219:479–488.
- Becker D (1990) *Nucleic Acids Res* 18:203.
- Servant F, Bru C, Carrere S, Courcelle E, Gouzy JP, Peyruc D, Kahn D (2002) *Brief Bioinform* 3:246–251.
- Jakoby M, Weisshaar B, Droge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F (2002) *Trends Plant Sci* 7:106–111.
- Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) *Plant Physiol* 136:2621–2632.
- Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) *Nucleic Acids Res* 27:297–300.
- Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S (2003) *Plant Cell* 15:1591–1604.
- Lister C, Dean C (1993) *Plant J* 4:745–750.
- Clerkx EJM, El Lithy ME, Vierling E, Ruys GJ, Blankestijn-De Vries H, Groot SPC, Vreugdenhil D, Koornneef M (2004) *Plant Physiol* 135:432–443.
- El-Lithy ME, Bentsink L, Hanhart CJ, Ruys GJ, Rovito D, Broekhof JL, van der Poel HJ, van Eijk MJ, Vreugdenhil D, Koornneef M (2006) *Genetics* 172:1867–1876.
- Wittkopp PJ, Haerum BK, Clark AG (2004) *Nature* 430:85–88.
- de Meaux J, Goebel U, Pop A, Mitchell-Olds T (2005) *Plant Cell* 17:676–690.
- Raz V, Bergervoet JH, Koornneef M (2001) *Development (Cambridge, UK)* 128:243–252.
- Koornneef M, Van Eden J, Hanhart CJ, Stam P, Braaksm FJ, Feenstra WJ (1983) *J Hered* 74:265–272.
- Lazo GR, Stein PA, Ludwig RA (1991) *Biotechnology* 9:963–967.
- Vicent CM, Delseny M (1999) *Anal Biochem* 268:412–413.