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Mutations in the Dof Zinc Finger Genes *DAG2* and *DAG1* Influence with Opposite Effects the Germination of Arabidopsis Seeds

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We describe the Arabidopsis gene *DAG2* encoding a Dof zinc finger protein and show that it is involved in the control of seed germination. An Arabidopsis mutant line with a T-DNA insertion in *DAG2* isolated by reverse genetics produces seeds that are substantially more dependent than the wild type on the physical stimuli—light and cold treatment—that promote germination. Mutant *dag2* seeds also are less sensitive to the germination-promotive effect of gibberellins, because a 10-fold higher amount of gibberellins is needed to restore germination when endogenous gibberellin biosynthesis is blocked. The seed germination characteristics of the *dag2* mutant are opposite to those of *dag1*, a knockout mutant of another Dof gene (*DAG1*) that we showed previously to be involved in the control of seed germination, and are similar to those of plants that overexpress *DAG1*. The promoter of the *DAG2* gene is active specifically in the vascular system of the mother plant but not in the embryo, and segregation analysis indicates that the effect of the *dag2* mutation is maternal. Both characteristics are in common with *DAG1*; additionally, the DAG1 and DAG2 proteins share high sequence homology and an identical zinc finger domain. These data suggest, and the germination phenotype of the double mutant is compatible with, a model whereby the zinc finger proteins DAG1 and DAG2 act on a maternal switch that controls seed germination, possibly by regulating the same gene(s).

INTRODUCTION

The Dof proteins are a family of transcription factors specific to plants and not present in yeast and animals. These proteins share a conserved 52–amino acid domain containing a single zinc finger ($CX_2CX_{21}CX_2C$) and a downstream basic region localized toward the protein N terminus (Kisu et al., 1995; Yanagisawa, 1995; Zhang et al., 1995; De Paolis et al., 1996; Vicente-Carbajosa et al., 1997; Mena et al., 1998). Multiple Dof genes have been identified in numerous and evolutionarily distant plants, both monocots and dicots, and they appear to be involved in diverse regulatory circuits. In maize and barley, the Dof proteins identified to date participate in the regulation of genes that encode storage proteins (Vicente-Carbajosa et al., 1997; Mena et al., 1998) and genes involved in carbon metabolism (Yanagisawa and Sheen, 1998; Yanagisawa, 2000). In tobacco, we have shown that a Dof protein is the main *trans*-acting factor regulating the *Agrobacterium rhizogenes* oncogene *rolB* (Baumann et al., 1999). In Arabidopsis, in which the genomic sequence indicates the presence of some 36 members of this family of transcription factors, Dof proteins have been identified that are involved in the regulation of stress response gene(s) (Chen et al., 1996; Kang and Singh, 2000) and in the control of seed germination (Papi et al., 2000).

As a result of the similarity of their DNA binding domains, the Dof proteins all recognize similar target sequences, with a CTTT consensus core (Yanagisawa and Schmidt, 1999). Therefore, their specificity of action presumably rests on their specific interaction with other regulatory proteins and/or their tissue- and cell-specific localization. Outside of the Dof domain, in fact, these proteins usually do not bear significant sequence homology with each other. The maize protein PBF binds to the promoter of the zein genes and interacts with the basic domain/Leu zipper protein Opaque 2 (Vicente-Carbajosa et al., 1997). Its barley counterpart BPBF activates the B-hordein promoter with a similar mechanism that involves the interaction of the Dof transcription factor with another protein (Mena et al., 1998). In Arabidopsis, three Dof proteins, OBP1 (Zhang et al., 1995; Chen et al.,

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1996), OBP2, and OBP3, all bind to the same transcriptional regulator, OBF4, but their genes are expressed in distinctive tissues (Kang and Singh, 2000). Genes that have different expression patterns encode the closely related maize proteins Dof1 and Dof2 (Yanagisawa and Sheen, 1998; Yanagisawa, 2000).

We have shown that the Arabidopsis Dof gene *DAG1* (Dof Affecting Germination) is involved in the maternal control of seed germination. In Arabidopsis, seeds need a red light pulse as a signal to trigger germination. Seeds from the knockout T-DNA insertion mutant *dag1* require much less light to germinate (Papi et al., 2000, 2002). The *DAG1* gene is expressed specifically in the vascular system of the mother plant but not in the embryo or anywhere else in the seed at any stage of development (Papi et al., 2000, 2002). Moreover, the segregation of the mutant phenotype confirms the exclusive role of the mother plant in determining it (Papi et al., 2000).

Here, we describe the identification of another member of the Dof gene family in Arabidopsis, *DAG2*, and we show that it too is involved in the control of seed germination. We isolated a T-DNA insertion mutant in *DAG2* and show that *dag2* mutant seeds have germination properties opposite to those of seeds from the *dag1* knockout compared with the wild type and that the effect of the mutation of *DAG2* is maternal. Moreover, seeds from plants that overexpress *DAG1* under the control of the 35S promoter show germination characteristics analogous to those of *dag2* mutant seeds, whereas seeds from double mutant *dag2 dag1* plants behave like *dag1* seeds. Finally, we show that the promoters of the *DAG2* and *DAG1* genes show very similar tissue and developmental specificities. Based on these data, and in accordance with the phenotype of the double mutant, we propose a model in which *DAG2* and *DAG1* play opposite regulatory roles, possibly on the same maternal gene(s), in committing the seed to germination.

RESULTS

Isolation of the *dag2* Mutant and of the *DAG2* Gene

In a previous work, we isolated the knockout insertion mutant *dag1* in the Dof gene *DAG1* by PCR screening of the Versailles T-DNA–mutagenized population of Wassilewskija (Ws) ecotype Arabidopsis plants (Papi et al., 2000). To isolate mutants of other members of the Dof gene family, the same reverse-genetics approach was used, and the Versailles collection was screened with T-DNA–specific primers in combination with Dof domain–specific primers. Sequencing of the fragment amplified from one of the Arabidopsis lines revealed a new Dof gene with the T-DNA inserted in the coding sequence 138 bp downstream of the Dof domain.

The 5′ region was obtained by 5′ rapid amplification of cDNA ends and was used to isolate the complete cDNA and subsequently the corresponding genomic clone from an Arabidopsis genomic library. Analysis of the putative protein sequence encoded by this Dof gene revealed 77% overall amino acid identity (100% identity in the Dof domain) with the protein encoded by the gene *DAG1* (Papi et al., 2000), as shown in Figure 1. The newly identified Dof gene and its T-DNA insertion mutant allele, therefore, were designated *DAG2* and *dag2*, respectively. The *DAG2* gene was mapped on chromosome 2 by screening of a yeast artificial chromosome library (Camilleri et al., 1998) and was localized between markers Ve019 and AthB7, whereas *DAG1* was mapped previously on chromosome 3 (Papi et al., 2000). DNA gel blot analysis indicated the presence of a single copy of *DAG2* in the Arabidopsis genome (data not shown).

In the DAG2 protein, the Dof domain is positioned between amino acids 82 and 133, at approximately the same position as in DAG1 (amino acids 76 and 127). The 1744-bp coding sequence of the *DAG2* gene is interrupted by a single 634-bp in-

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DAG2    1 MDATKWTQGLQEMMNVKPMEQIMIPNNNTHQPNTTSNARPNTILTSNGVSTAGATVSGVSNNNNNTAVVAERKARPQEKL
DAG1    1 MDATKWTQGFQEMINVKPMEQMISSTNN-NTPQ--Q---QPTFIATNTRPNATASNGGSGGNTNNTATMETRKARPQEKV

DAG2   81 NCPRCNSTNTKFCYYNNYSLTQPRYFCKGCRRYWTEGGSLRNVPVGGSSRKNKRSSSSSSNILQTIPSSLPDLNPPILF
DAG1   75 NCPRCNSTNTKFCYYNNYSLTQPRYFCKGCRRYWTEGGSLRNVPVGGSSRKNKRSSTPLAS---P-SNPKLPDLNPPILF

DAG2  161 SNQIHNKSKGSSQDLNLLSFPVMQDQHHHHVHMSQFLQMPKMEGNGNITHQQQPSSSSSVYGSSSSPVSALELLRTGVNV
DAG1  151 SSQIPNK---SNKDLNLLSFPVMQDHHHH----------------------------------------ALELLRS----

DAG2  241 SSRSGINSSFMPSGSMMDSNTVLYTSSGFPTMVDYKPSNLSFST---DHQGLGHNSNN---RSEALHSDHHQQGRVLFPF
DAG1  184 NGVSSRGMNTFLPGQMMDSNSVLYSSLGFPTMPDYKQSNNNLSFSIDHHQGIGHNTINSNQRAQDNNDDMNGASRVLFPF

DAG2  315 GDQMKELSSSITQEVDHDDNQQQKSHGNNNNNNNSSPNNGYWSGMFSTTGGGSSWZ
DAG1  264 SD-MKELSS--TTQE---------KSHG----------NNTYWNGMFSNTGG-SSWZ
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Figure 1. DAG2 and DAG1 Proteins Are Very Closely Related and Share an Identical DNA Binding Domain.

Alignment of the amino acid sequences of the proteins encoded by the Dof genes *DAG2* and *DAG1*. Green indicates identical amino acid residues, and red indicates conservative substitutions. The Dof domain is underlined.

tron positioned 24 bp downstream of the ATG codon, exactly the same position of the 684-bp intron in the *DAG1* gene (Papi et al., 2000). Thus, the *DAG2* and *DAG1* genes are quite closely related, with the exception of a second small (84-bp) intron present in *DAG1* downstream of the Dof domain where the *DAG2* gene is not interrupted. Figure 2A shows a comparison of the structures of the two genes and the position of the T-DNA insert in the *dag2* mutant. As a result of the 28–amino acid gap caused by the intron in *DAG1* and of other shorter gaps elsewhere in the sequence, the DAG2 protein (369 amino acids) is larger than DAG1 (296 amino acids).

Segregation of the kanamycin resistance marker associated with the T-DNA indicated the presence of a single insertion in the *dag2* mutant line, which was confirmed by DNA gel blot analysis (data not shown). The *dag2* mutant was backcrossed with the wild type, and all subsequent characterizations were performed on the T3 progeny homozygous for the *dag2* allele.

The *DAG2* transcript of the *dag2* mutant was analyzed by reverse transcriptase–mediated PCR. This revealed a *DAG2* mRNA truncated downstream of the Dof domain at a position corresponding to the insertion of T-DNA, in translational fusion with the coding sequence of the β-glucuronidase (*GUS*) reporter gene present in the T-DNA mutagenesis vector (Bouchez et al., 1993), as shown in Figure 2B. Thus, *dag2* can be regarded as a "knockabout" mutant (Krysan et al., 1999), originating a truncated DAG2 protein fused with GUS.

The level of the truncated *DAG2-GUS* chimeric mRNA (Δ-*DAG2*::*GUS*) in the *dag2* mutant was assessed by RNA gel blot analysis on different plant organs. In all organs, the level of Δ-*DAG2*::*GUS* mRNA in *dag2* mutant plants was much lower than the level of the *DAG2* mRNA in Arabidopsis Ws plants, as shown in Figure 2B for RNA extracted from leaves.

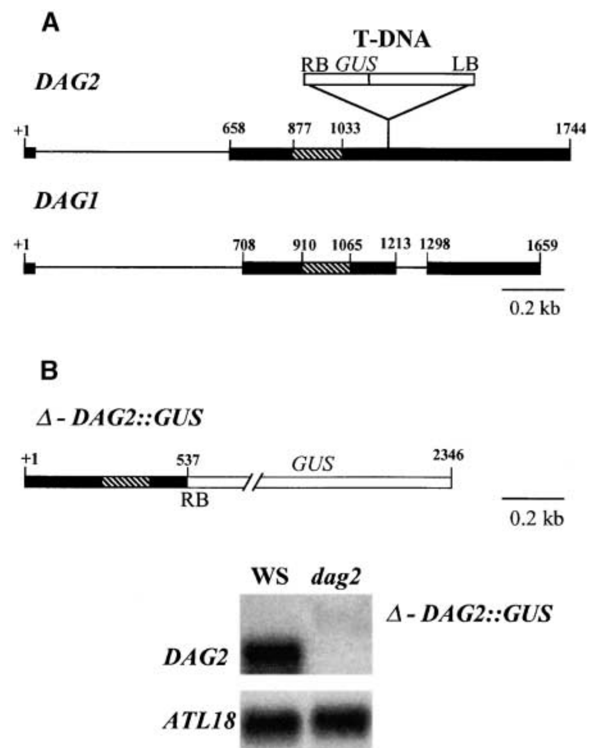

Figure 2. *DAG1* and *DAG2* Genes Have Similar Structures.

Insertion of T-DNA results in a truncated *DAG2-GUS* fusion (Δ-*DAG2*::*GUS*) with a very low level of expression in the *dag2* mutant.

(A) Structure of the *DAG2* and *DAG1* genes and position of the T-DNA insert in the *dag2* mutant. The T-DNA is not drawn to scale. Black boxes indicate exons, and dashed boxes indicate Dof domains. RB and LB, T-DNA right and left border, respectively. Numbers indicate nucleotide positions.

(B) Structure of the *DAG2* transcript in the *dag2* mutant. RNA gel blot hybridization of the *DAG2* 5′ region upstream of the Dof domain (nucleotides 1 to 220) with total RNA extracted from leaves of Ws and *dag2* plants. ATL18 is the mRNA of the Arabidopsis ribosomal protein L18 used to normalize the blots.

Phenotypic Analysis

In view of the close relationship between the *DAG2* and *DAG1* genes, the phenotype of the *dag2* and *dag1* mutants was analyzed comparatively. Until the rosette stage, no morphological differences were observed between *dag2* and Ws plants, as described previously for the *dag1* mutant (Papi et al., 2000). However, as shown in Figure 3, adult *dag2* plants were reproducibly ∼15% taller (41.0 ± 0.7 cm) than Ws plants (35.4 ± 0.6 cm), as opposed to *dag1* adult plants, which were consistently 15% shorter (30.3 ± 1.0 cm) than Ws plants. Flowering time and flower morphology of the *dag2* and *dag1* mutants and of Ws plants did not show appreciable differences. In contrast with the twisted appearance of *dag1* siliques (Papi et al., 2000), the morphology and size of *dag2* siliques were normal (data not shown).

***dag2* and *dag1* Mutant Seeds Have Opposite Germination Properties**

Mutation in the *DAG1* gene affects seed germination most conspicuously (Papi et al., 2000). Therefore, seeds of Ws wild-type and of *dag2* and *dag1* mutant plants were compared for their germination properties under different conditions, and the effects of different factors that influence seed germination—light, cold treatment (stratification), and hormones—were assessed.

Light

Seeds were kept in the dark for 2 days at 4°C before scoring for germination under normal illumination conditions for up to 5 days. As shown in Figure 4A, no substantial difference was observed between Ws and *dag2* and *dag1* mutant seeds, and in all cases, >90% germination was attained

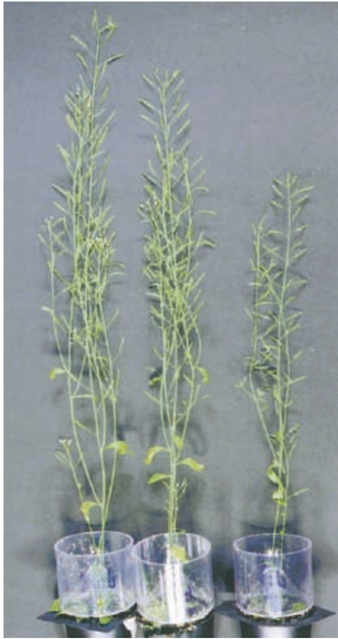

Figure 3. Mutant *dag2* and *dag1* Plants Are Taller and Shorter, Respectively, Than Arabidopsis Ws Plants.

The *dag2* plant is at left, the Ws plant is in the center, and the *dag1* plant is at right.

after 5 days. In contrast, when seeds were kept in the dark after stratification, only ~5% of the *dag2* seeds attained germination after 5 days, as opposed to close to 25% of Ws seeds and close to 50% of *dag1* seeds (Figure 4B). Thus, mutation of *DAG2* substantially reduced the capability of Arabidopsis Ws seeds to germinate in the absence of a light pulse, whereas mutation of *DAG1* enhanced the dark germination of seeds.

Stratification

Omission of the cold treatment before the germination assays under normal illumination conditions resulted in substantial differences in the germination rates of the three seed types. As shown in Figure 4C, *dag2* seeds were slower in germinating and reached a lower germination percentage than Ws seeds, whereas *dag1* seeds were faster and reached a higher germination percentage than Ws seeds. *dag2*, Ws, and *dag1* seeds attained 50% germination after 5, 3, and 2 days, respectively.

In the absence of both light and cold treatment, germination of *dag2* mutant seeds at 5 days was reduced to almost nil, whereas Ws seeds reached almost 10% and *dag1* seeds reached >30% (Figure 4D). Thus, mutation of the *DAG2* gene resulted in substantially higher than normal sensitivity to the lack of the physical factors (light and cold treatment) that stimulate seed germination, an effect opposite to that observed for mutation of the *DAG1* gene (Papi et al., 2000, 2002; this work).

Hormones

It is well established that gibberellins (GAs) promote seed germination. Therefore, we tested seeds from *dag2* and *dag1* mutant plants for the effects of GAs on germination. Seeds from *dag2*, Ws, and *dag1* plants were germinated in the presence of different concentrations of paclobutrazol, a GA biosynthesis inhibitor. As shown in Figure 5A, the different types of seeds were equally sensitive to the inhibitor, and in all cases, a concentration of 5 μg/mL was sufficient to block germination completely, in agreement with previous results from *dag1* seeds (Papi et al., 2000).

To assess the capability of exogenous GAs to rescue germination in the absence of endogenous biosynthesis, different concentrations of GAs were supplied to the seeds in the presence of an inhibitory amount of paclobutrazol (100 μM). As shown in Figure 5B, the sensitivity of the different types of seeds to exogenous GAs differed markedly. To attain 50% germination, a concentration of hormones ~10-fold higher and 10-fold lower than for Ws seeds was needed for *dag2* and *dag1* seeds, respectively. Therefore, the sensitivity of seeds to GAs was affected in opposite ways by mutation of the *DAG2* and *DAG1* genes.

Abscisic acid plays an inhibitory role in seed germination. Thus, we compared the germination rates of *dag2*, Ws, and *dag1* seeds in the presence of different concentrations of abscisic acid. As shown in Figure 5C, 3 μM abscisic acid was sufficient to abate the germination of all types of seeds to <10%. This finding indicates that, as in the case of *DAG1* (Papi et al., 2000; this work), disruption of the *DAG2* gene did not alter the sensitivity of Arabidopsis seeds to the inhibitory effect of abscisic acid.

Overexpression of *DAG1* Causes Phenotypes Similar to Mutation of *DAG2*

Because mutations of *DAG2* and *DAG1* cause opposite phenotypes, the effects of overexpression of one of these genes could be analogous to the effects of mutation of the other. To assess this possibility, we analyzed the germination characteristics of seeds from Arabidopsis plants overexpressing *DAG1* under the control of the 35S promoter. Germination tests were performed under standard illumination but omitting stratification, that is, under conditions that allow substantial germination of wild-type seeds while maximizing differences with the lower germination rate of *dag2* seeds. As shown in Figure 6, seeds from 35S::*DAG1* plants had a substantially lower germination rate than seeds of the corresponding Columbia ecotype used for transformation.

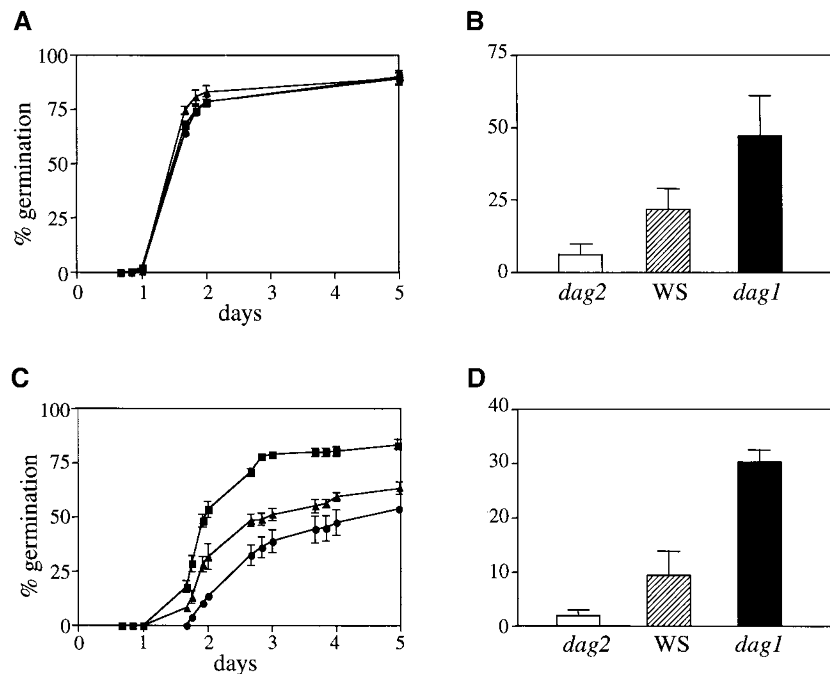

Figure 4. Germination of Mutant *dag2* Seeds Is More Dependent on Light and Stratification Than Germination of Wild-Type Seeds, Whereas Germination of Mutant *dag1* Seeds Is Less Dependent on These Factors.

(A) Germination rates under standard illumination conditions with seeds pretreated at 4°C. Closed circles, *dag2* seeds; closed triangles, Ws seeds; closed squares, *dag1* seeds.

(B) Germination in the dark with seeds pretreated at 4°C. The histogram reports germination percentages scored on day 5.

(C) Germination rates under standard illumination conditions with seeds not pretreated at 4°C. Closed circles, *dag2* seeds; closed triangles, Ws seeds; closed squares, *dag1* seeds.

(D) Germination in the dark with seeds not pretreated at 4°C. The histogram reports germination percentages scored on day 5.

In analogy with the effects of mutation of *DAG2*, germination of 35S::*DAG1* seeds started later than in the wild type and after 5 days reached <40%, compared with 100% of Columbia seeds. In addition, T3 35S::*DAG1* plants were somewhat taller (45.1 ± 0.9 cm) than Columbia untransformed plants (40.8 ± 1.5 cm).

The Promoters of *DAG2* and *DAG1* Have Very Similar Tissue and Developmental Specificities

The pattern of expression of *DAG2* in Arabidopsis plants was analyzed by means of a *DAG2* promoter fusion with the *GUS* reporter gene and compared with the pattern of an analogous construct of *DAG1*. As shown in Figure 7, the two promoters had very similar if not indistinguishable tissue specificities. GUS-specific staining was localized in both cases to the vascular system of the mother plant but was not present in the seed and embryo, in agreement with previous results of *DAG1* mRNA in situ hybridizations (Papi et al., 2000, 2002). Figures 7A and 7B show the strong reporter gene activity of *DAG2*::*GUS* and *DAG1*::*GUS*, respectively, in the vasculature of stage 16 (Smyth et al., 1990) maturing siliques, all through the funiculus connecting the placenta to the ovule, but not in the ovule. The activity of both *DAG2* and *DAG1* promoters was turned off in siliques when they reached full maturation (stage 17; data not shown). No GUS-specific staining was observed in developing or mature embryos. Mature embryos as extracted from imbibed seeds are shown in Figures 7C and 7D.

The activity of the two Dof promoters begins with the development of the plantlet. Figures 7E and 7F show *DAG2*::*GUS* and *DAG1*::*GUS* plantlets, respectively, immediately after germination. In later stages of development and throughout the Arabidopsis plant life span, the activity of the two Dof promoters was restricted to the vascular system in all organs: cotyledons (Figures 7G and 7H), leaves (Figures 7I and 7J), and roots (Figures 7K and 7L).

The Effect of the Mutation of *DAG2* Is Maternal

Because the activity of the *DAG2* promoter is confined to the mother plant, we attempted to determine whether the

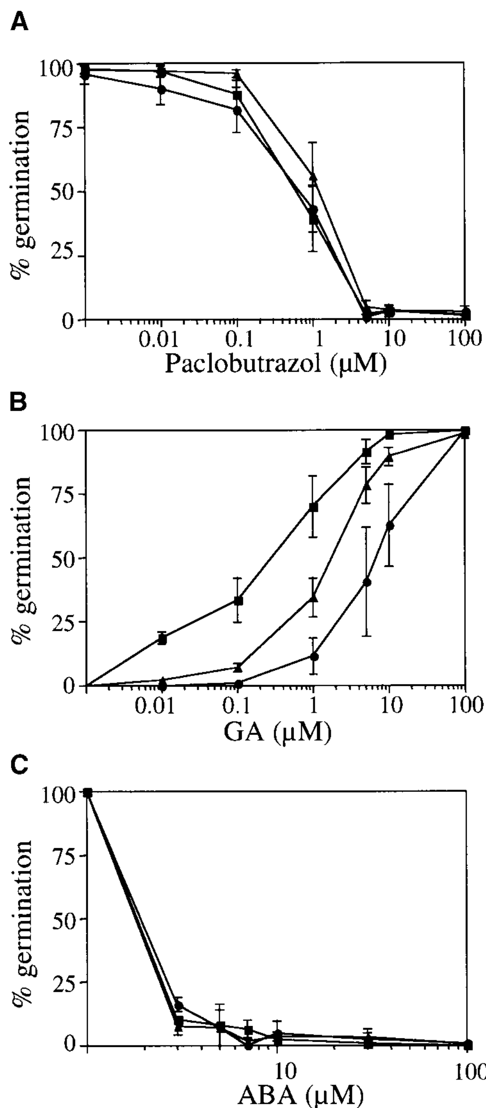

Figure 5. Mutant *dag2* and *dag1* Seeds Require Much Higher and Much Lower Concentrations of GAs, Respectively, Than Wild-Type Seeds to Germinate.

Both mutant seeds were as sensitive to abscisic acid as wild-type seeds. Germination of seeds pretreated at 4°C was under standard illumination conditions. Closed circles, *dag2* seeds; closed triangles, Ws seeds; closed squares, *dag1* seeds.

(A) Germination percentages scored on day 5 in the presence of increasing concentrations of paclobutrazol.

(B) Germination percentages scored on day 5 in the presence of 100 μM paclobutrazol and increasing concentrations of GAs (GA_{4+7}).

(C) Germination percentages scored on day 5 in the presence of increasing concentrations of abscisic acid (ABA).

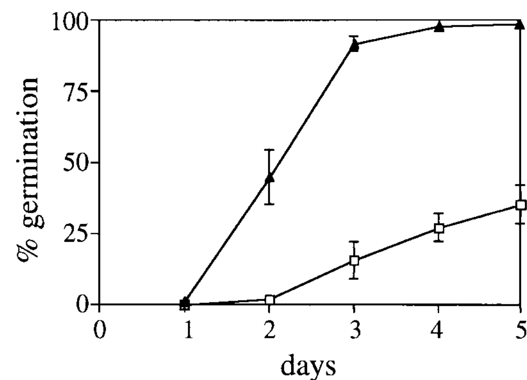

Figure 6. Seeds from Plants Overexpressing *DAG1* (35S::*DAG1*) Have a Lower Germination Rate Than Wild-Type Seeds.

Germination of seeds not pretreated at 4°C was under standard illumination conditions. Closed triangles, Columbia ecotype seeds; open squares, 35S::*DAG1* seeds.

phenotype of *dag2* seeds is attributable to the mother plant or to the developing embryo. Reciprocal *dag2* × Ws and Ws × *dag2* crosses were performed, and seeds from individual F1 siliques were assayed for germination. As discussed above, to maximize differences, germination tests were run under standard illumination conditions on seeds not pretreated at 4°C. The results of assays on seeds from five siliques were averaged for each cross and are presented in Figure 8 in comparison with the germination of Ws seeds. As can be seen, the germination rate of seeds from Ws × *dag2* siliques was virtually indistinguishable from that of Ws seeds, whereas the germination rate of *dag2* × Ws seeds was substantially lower. These results indicate that, consistent with its pattern of expression and analogous to the effect of the mutation of *DAG1* (Papi et al., 2000), the effect of the mutation of *DAG2* was maternal.

Phenotype of the *dag2 dag1* Double Mutant

Because the *DAG2* and *DAG1* genes and the activity of their promoters are very similar, and because the effect of their mutation on seed germination is maternal in both cases, we investigated the genetic interactions between the two genes by constructing *dag2 dag1* double mutant plants. Interestingly, the siliques from *dag2 dag1* plants had the same twisted appearance (Papi et al., 2000) as *dag1* siliques, whereas no statistically significant difference in plant height between double mutant and Ws plants was observed (data not shown). The germination properties of the *dag2 dag1* seeds were scored under standard illumination but omitting stratification, as for the assays reported in Figures 4C, 4D, 6, and 8. As shown in Figure 9, the germination rate of

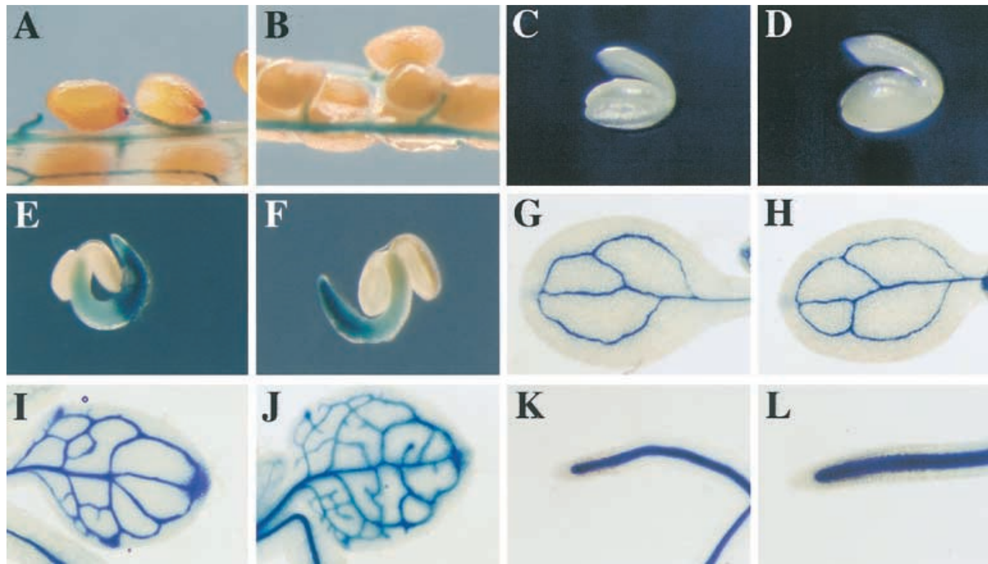

Figure 7. Promoters of the *DAG2* and *DAG1* Genes Show Very Similar Tissue and Developmental Specificities.

Histochemical analysis of T3 Arabidopsis Ws plants harboring either the *DAG2*::*GUS* or the *DAG1*::*GUS* construct.
(A), **(C)**, **(E)**, **(G)**, **(I)**, and **(K)** Activity of the *DAG2*::*GUS* construct in siliques, mature embryo, 7-day-old plantlet, cotyledon, leaf, and primary root, respectively.
(B), **(D)**, **(F)**, **(H)**, **(J)**, and **(L)** Activity of the *DAG1*::*GUS* construct in siliques, mature embryo, 7-day-old plantlet, cotyledon, leaf, and primary root, respectively.

seeds from double mutant plants was substantially higher than that of Ws seeds. These results indicate that at least in terms of silique morphology and seed germination, the effects of the mutation in *DAG1* override the effects of the mutation in *DAG2* (i.e., *DAG1* is epistatic over *DAG2*).

DISCUSSION

Despite the identification of numerous exogenous and endogenous factors and of a number of genes that affect seed germination, the genetic control of this crucial developmental event remains poorly understood. In this article, we report the identification of a new regulatory gene, *DAG2*, that is involved in the control of seed germination. Most interestingly, based on a comparative analysis of mutants isolated by reverse genetics, the regulatory role of *DAG2* appears to be opposite to that of *DAG1*, which was identified previously in our laboratory. The two genes belong to the same gene family encoding Dof zinc finger transcription factors. Neither of the two genes is expressed in the seed itself, and they have very similar patterns of promoter activity limited to the mother plant, suggesting a critical role of a maternal regulatory switch in determining the developmental fate of the mature seed.

In Arabidopsis, breaking of seed dormancy and subsequent germination require triggering by red light that converts the inactive Pr form of phytochrome into the active Pfr form. Germination also is promoted by GAs and can be inhibited by abscisic acid (which promotes seed dormancy). The roles of light and GAs in promoting germination are not clear, but the Pfr-induced pathway apparently involves an increase in seed sensitivity to GAs (Karssen and Laçka, 1986) and/or biosynthesis of GAs (Yamaguchi et al., 1998). GAs, in turn, stimulate the growth potential of the embryo and/or induce enzymes that degrade the seed coat (Groot and Karssen, 1987; Debeaujon and Koornneef, 2000), allowing protrusion of the radicle and completion of the germination process. Exposure of the seeds to low temperatures also promotes germination by breaking seed dormancy. The interplay between light and stratification in controlling seed germination has not been clarified.

We had shown previously that mutation of the *DAG1* gene drastically reduces the requirement of light for Arabidopsis seeds to germinate (Papi et al., 2000, 2002). Here, we show that, in contrast, mutation in the *DAG2* gene results in seeds with a diminished capability to germinate in the absence of light. Accordingly, we show that the requirement of GAs for seeds to germinate also is much increased in the *dag2* mutant, whereas *dag1* seeds require substantially lower levels of GAs than Ws wild-type seeds.

We also show that the promotive effect of stratification is more essential to the germination of *dag2* seeds than that of

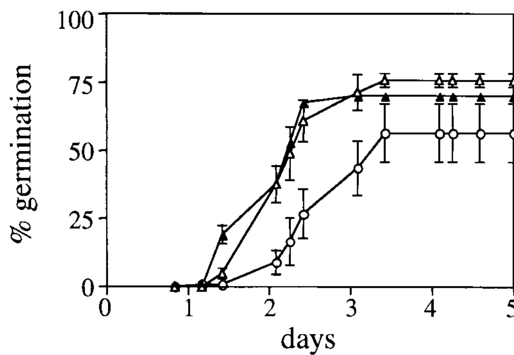

Figure 8. The Effect of the Mutation of *DAG2* Is Maternal.

Germination rates under standard illumination conditions of seeds not pretreated at 4°C. Closed triangles, Ws seeds; open triangles, Ws × *dag2* seeds; open circles, *dag2* × Ws seeds.

Ws seeds, whereas cold treatment is almost superfluous for *dag1* seeds. Ultimately, mutation of *DAG1* increases the germination capability of Arabidopsis seeds considerably, whereas mutation of *DAG2* results in seeds with a substantially lower germination potential than the wild type. Accordingly, overexpression of *DAG1* results in a phenotype analogous to that of mutation in *DAG2* (i.e., reduced germination potential of the seeds).

Judging from the activity of their promoters, neither *DAG1* nor *DAG2* is expressed in the developing seed or in the mature embryo. Based on the segregation of the mutant phenotype, we had demonstrated previously that, in agreement with its pattern of expression, the effect of mutation of *DAG1* is maternal (Papi et al., 2000). Here, we show that the effect of mutation of *DAG2* is maternal as well. In contrast, most of the mutants that control seed germination identified to date act in the developing embryo. The *lec* (*leafy cotyledon*) mutants *lec1* (Meinke, 1992) and *fus3* (Bäumlein et al., 1994; Keith et al., 1994) have altered seed germination characteristics as an effect of mutations in genes that encode transcription factors that control embryo development (Lotan et al., 1998; Luerssen et al., 1998). The abnormal *lec1* and *fus3* embryos develop into abnormal plantlets (Meinke, 1992; Keith et al., 1994).

Embryos of *dag1* and *dag2* mutants are morphologically normal and develop into normal seedlings. The *COMATOSE* (*CTS*) locus also regulates Arabidopsis seed germination potential without affecting embryo development, but the *cts* phenotype is expressed in the embryo (Russell et al., 2000). Embryo mutants of the *aba* class, which are impaired in abscisic acid biosynthesis (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996), or *abi* mutants with reduced sensitivity to abscisic acid (Koornneef et al., 1984) also show altered seed germination control. Neither the *dag1* nor the *dag2* mutant seeds show any alteration in sensitivity to abscisic acid. The altered germination of *rdo* (*reduced dormancy*) mutants (Léon-Kloosterziel et al., 1996) also is embryo determined. The lack of activity of the *DAG1* and *DAG2* promoters in the embryo and the maternal effect of the mutation of these genes on seed germination indicate that major genetic control of this developmental process is exerted in the mother plant.

By extensive in situ mRNA hybridization, we had shown previously that *DAG1* is expressed specifically in the vascular system (Papi et al., 2000, 2002). In this work, the results from analysis with the *GUS* reporter gene confirm the pattern of expression of *DAG1* and show that the activity of the *DAG2* promoter is restricted to the vasculature of the mother plant as well. Finally, we show that the siliques and the germination characteristics of seeds from the double mutant *dag2 dag1* are analogous to those of the *dag1* mutant, indicating that in terms of these traits, *DAG1* acts epistatically to *DAG2*.

A Model for the Role of *DAG1* and *DAG2* in Seed Germination

The results presented in this work suggest that the two regulatory genes are involved in the biosynthesis and/or transport to the seed of some component(s) important for the control of dormancy versus germination. These could be either signal or structural molecules. *DAG1* and *DAG2*, for example, may control the level in the seed of a component needed for light signal transduction or may control the level of structural components (e.g., of the seed coat cell wall) that affect the mechanical aspects of germination. It should be noted that other maternal mutants with altered germination properties are mutants affected in seed coat (testa) integrity (Koornneef and Karssen, 1994; Léon-Kloosterziel, 1997; Debeaujon and Koornneef, 2000; Debeaujon et al., 2000).

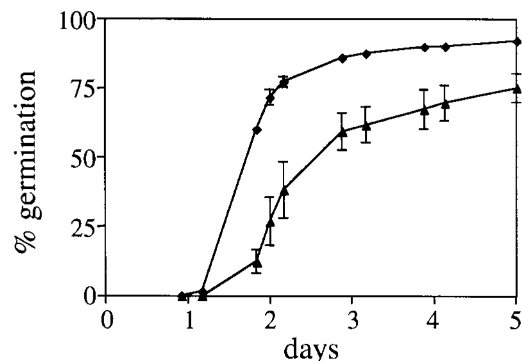

Figure 9. *DAG1* Is Epistatic over *DAG2*.

Germination rates under standard illumination conditions of seeds not pretreated at 4°C. Closed triangles, Ws seeds; closed diamonds, *dag2 dag1* seeds.

Whatever hypothesis proves true, however, it is tempting to speculate that *DAG1* and *DAG2* act on the same maternal gene(s). The two zinc finger proteins they encode share an identical DNA binding domain (Dof); therefore, they must bind the same DNA sequence, with a CTTT core target (Yanagisawa and Schmidt, 1999). Outside of the Dof domain, the two proteins are quite similar (77% overall amino acid identity), the main difference being a 28–amino acid segment present in *DAG2* corresponding to an additional intron present in the *DAG1* gene.

We propose a model of action of the regulatory switch operated by the two DAG transcription factors that also accounts for the epistasy of *DAG1* over *DAG2* in terms of seed germination. In such a model, DAG1 activates maternal gene(s) that promote dormancy or repress germination, whereas DAG2 is either a lower affinity repressor of the same gene(s) or interacts with DAG1 to decrease its activity. In both cases, DAG2 counteracts and in some way titrates the effect of DAG1 to ensure a correct level of dormancy in the seeds. In Ws wild-type plants (in which both DAG1 and DAG2 are present), the prevailing inductive effect of DAG1 on the maternal gene(s) warrants the synthesis and/or transport to the seed of a component needed for seed dormancy (or to inhibit seed germination). The absence of a functional DAG2 protein in *dag2* plants leads to a higher level of expression of the maternal gene(s) and thus to an abnormally high level of the component in the seed, which in turn causes a decrease of the seed germination potential. In both the *dag1* mutant and the double mutant, lack of the DAG1 protein results in a repressed or uninduced state of the maternal gene(s), causing an abnormally low level of the component in the seed and, consequently, an increase in the seed germination potential.

Analysis of Arabidopsis cDNA microarrays with RNAs from mutant and Ws wild-type RNA will provide clues to the target genes of the DAG1 and DAG2 proteins. The study of the possible interactions of the two proteins with each other or with other regulatory proteins will clarify their roles in the transcriptional control of these maternal genes that affect seed germination.

METHODS

Plant Material

All *Arabidopsis thaliana* lines used in this work were grown in a growth chamber at 24/21°C with 16/8-h day/night cycles and light intensity of 300 $\mu E \cdot m^{-2} \cdot s^{-1}$, as described previously (Papi et al., 2000). Plant heights ($\pm$SE) reported are averages of 70 plants.

Germination Assays

For each comparative germination assay, seeds of the different types of plants were harvested on the same day from plants of the same age and stored for 5 weeks in the dark at 18°C. For the assays, seeds were sown in triplicate in Petri dishes (80 to 100 seeds per dish) in the dark on four layers of filter paper 595 (Schleicher & Schüll, Dassel, Germany) soaked with 2 mL of distilled water and assayed for germination under different conditions as described in Results and in the figure legends. For paclobutrazol and gibberellin (GA) sensitivity assays, filter paper 595 was soaked with 2 mL of aqueous solutions of different concentrations of paclobutrazol or 100 μM paclobutrazol and increasing concentrations of GA_{4+7} (Duchefa, Haarlem, The Netherlands). Paclobutrazol and GA_{4+7} were dissolved in absolute ethanol, and a 1000-fold dilution was used for each condition.

Molecular Techniques

Molecular techniques were performed according to standard protocols. Arabidopsis DNA was extracted according to Bouchez et al. (1993). Total RNAs were extracted from wild-type and mutant plants as described previously (Vittorioso et al., 1998).

Isolation of the *dag2* Homozygous Plant

A collection of T-DNA insertion mutants (Laboratoire de Génetique et Amélioration des Plantes, Versailles, France) containing ~22,000 individual lines was screened by PCR using primers corresponding to both the T-DNA right and left borders and primers corresponding to the Dof domain (forward, 5′-TTATTACAACTACAGTCTCACGCAACC-3′; reverse, 5′-GGACGTTACGAAGAGAGCCACCTTCGGCC-3′). One positive line was identified and isolated. The position of the T-DNA within the *DAG2* gene was determined by sequencing the PCR-amplified fragment. The *DAG2*-tagged line was shown to contain a single T-DNA insert and was checked for linkage of the T-DNA insert to the *DAG2* gene. Homozygous *dag2* plants were isolated by kanamycin segregation and confirmed by PCR analysis.

Isolation of *dag1 dag2* Double Mutant Plants

dag1 dag2 double mutant plants were obtained by PCR screening of 50 plants (F2) derived from reciprocal crosses of the two homozygous mutants (F0). PCR was performed on DNA extracted from leaves with a T-DNA–specific primer on the right border and *DAG1* and *DAG2* gene-specific primers, respectively (*DAG1* forward, 5′-TCACACTAAACCCTAATCTTGGCGAAA-3′; *DAG1* reverse, 5′-GTATTGTTGTTCTCTATCTTGGGCAT-3′; *DAG2* forward, 5′-CCATAATAAATCGAAAGGGTCATCAC-3′; *DAG2* reverse, 5′-GTAAAGCACAGTGTTTGAATCCATCATTGA-3′).

Isolation of the *DAG2* gene

The 5′ end region of the *DAG2* cDNA was isolated by 5′ rapid amplification of cDNA ends using a *DAG2*-specific primer for the reverse transcription reaction (5′-GTGATGACCCTTTCGATTTATTA-3′). This region was used as a probe for the screening of an Arabidopsis genomic library (Clontech, Palo Alto, CA). Several positive clones were isolated. A 6.5-kb SalI insert containing the entire *DAG2* gene was cloned into pBluescript KS+ vector (Stratagene). The full-length sequence of the cDNA was obtained by reverse transcriptase–mediated PCR performed according to the manufacturer's instructions (Gibco BRL). DNA sequencing was performed with an ABI373A

automated DNA sequencer as recommended by the manufacturer (Applied Biosystems, Foster City, CA). The analysis of *DAG2* cDNA and derived protein sequences was performed using GCG (Genetics Computer Group, Madison, WI) and BLAST (www.ch.EMBnet.org) or the National Center for Biotechnology Information (Bethesda, MD).

β-Glucuronidase Constructs and Analysis

A 2.2-kb fragment of the *DAG2* promoter region was amplified by PCR (underlines are the restriction sites of HindIII and BamHI; forward, 5′-AAGCTTTGATATCTAAATGGGAAA-3′; reverse, 5′-AAA-AAGGATCCCTGTGTCCACTTCGTAGCG-3′), digested with HindIII-BamHI, and cloned in the binary vector pBI101 previously digested with HindIII-BamHI. A 2.15-kb fragment of the *DAG1* promoter region was amplified by PCR (forward, 5′-AAAAAAGCTTTAGAAGAT-AGGATGTTGCGAAAA-3′; reverse, 5′-AAAAGGATCCTGAACTTC-AACAAAGAGAAAGGC-3′), digested with HindIII-BamHI, and cloned in the binary vector pBI101.3 previously digested with HindIII-BamHI. Wassilewskija plants were transformed by in planta infiltration (Bechtold et al., 1993), and transformants were selected on 50 μg/mL kanamycin. The T3 progeny from several individual kanamycin-resistant homozygous plants for each construct were analyzed for β-glucuronidase expression.

Histochemical staining and microscopic analysis were performed as described previously (Baumann et al., 1999) without vacuum infiltration. The assays were performed on T3 progeny of at least three independent transformants for each construct. Plantlets, seeds, and organs (after chlorophyll removal from green tissues with 70% ethanol) were examined with a Leitz Laborlux D light microscope (Midland, Ontario, Canada) or a Leica MZ12 stereomicroscope (Wetzlar, Germany).

35S::DAG1 Construct and Transgenic Plants

The full-length *DAG1* cDNA was amplified by PCR (forward, 5′-AAAAGGATCCTCTCTTTGTTGAAGTTCATGG-3′; reverse, 5′-AAA-AGATATCGAAAGCTGATAGTTCATGAGC-3′), digested with BamHI-EcoRV, and cloned into pBI121 digested with BamHI-SstI to remove the β-glucuronidase gene. The SstI site was filled in previously. Columbia ecotype plants were transformed by in planta infiltration (Bechtold et al., 1993), and transformants were selected on 50 μg/mL kanamycin. The T3 progeny from several individual kanamycin-resistant plants were analyzed for germination phenotypes.

Accession Number

The EMBL accession number for the *DAG2* gene is AJ237810.

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