RESEARCH COMMUNICATION

Identification and disruption of an *Arabidopsis* zinc finger gene controlling seed germination

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We describe here the *Arabidopsis* gene *DAG1*, encoding a zinc finger transcription factor of the Dof family, and show that it is involved in the control of seed germination. By a reverse genetics approach, we isolated an *Arabidopsis* mutant line with one T-DNA insertion in *DAG1*. Seeds from homozygous knockout *dag1-1* plants do not develop dormancy and germinate also in the absence of light. Segregation analysis indicates that the effect of the mutation is maternal. Accordingly, in situ mRNA hybridizations reveal expression of *DAG1* in the vascular tissue of the flower and maturing fruit but not in the seed.

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The seeds of several plant species do not germinate immediately after detachment from the mother plant, because the embryos that they contain are dormant. Dormancy, which is initiated during the final stages of seed development, is a trait of considerable adaptive significance—it prevents seeds from germinating under unfavorable conditions—as well as of great agricultural and biotechnological interest. A considerable body of evidence indicates that seed dormancy is induced by the plant hormone abscisic acid (ABA), which is also capable of inhibiting seed germination; mutants impaired in either ABA biosynthesis (*aba*) or sensitivity (*abi*) show reduced dormancy (Koornneef and Karssen 1994; Bewley 1997). Another class of plant growth factors, gibberellins (GA), are necessary for germination, and severe mutant alleles of the *ga1* locus, which are required for GA biosynthesis, fail to germinate (Koornneef and Karssen 1994). Dormancy can be relieved by a period of dry storage of the seeds, and in some plant species, including *Arabidopsis*, germination requires light (Koornneef and Karssen 1994; Bewley 1997). However, the molecular mechanisms controlling seed dormancy and germination are, as yet, very poorly understood. Here, we report the

identification of the *Arabidopsis* gene *DAG1* (Dof affecting germination), encoding a Dof zinc finger transcription factor, and show that it is involved in the control of seed dormancy.

The Dof proteins are a recently discovered family of plant transcription factors, ubiquitously present in the plant kingdom but absent from yeast and animals. These proteins are characterized by a strikingly conserved 52-amino-acid segment, denominated Dof domain, encompassing a single CX₂CX₂CX₂C zinc finger (Yanagisawa 1995; Zhang et al. 1995; De Paolis et al. 1996; Vicente-Carvajosa et al. 1997; Mena et al. 1998); in contrast, their sequences diverge widely outside the Dof domain. These features suggested that the Dof proteins are involved in the regulation of different functions typical of and of general relevance for plants. The few Dof proteins studied so far have been shown to be involved in gene regulation by light in maize (Yanagisawa and Sheen 1998), in the activation of storage protein genes in maize and barley (Vicente-Carvajosa et al. 1997; Mena et al. 1998), and in the regulation of stress-related genes in *Arabidopsis* (Chen et al. 1996). Very recently, we have shown that the tobacco Dof protein NtBBF1 controls expression of the oncogene *rolB* in plants (Baumann et al. 1999).

We identified the *Arabidopsis* Dof gene *DAG1* by sequence homology with the Dof domain of NtBBF1, and we isolated a *dag1* knockout mutant by reverse genetics. The seeds of this mutant are nondormant and capable of germinating in the absence of light. This phenotype and its segregation pattern, as well as the pattern of expression of the gene, point to *DAG1* as a gene that plays a novel and unexpected maternal role in controlling seed germination.

Results

Isolation and structure of *DAG1*

An incomplete *Arabidopsis* *DAG1* cDNA isolated previously by sequence homology with the Dof domain of the tobacco gene NtBBF1 (De Paolis et al. 1996) was utilized to isolate the corresponding genomic clone from an *Arabidopsis* library. Southern blot analysis indicated that the *DAG1* protein is encoded by a single gene (not shown). The *DAG1* gene was then mapped on chromosome 3 by screening of a YAC library (Camilleri et al. 1998) and localized between markers m424 and nga6. Analysis of the genomic sequence (EMBL accession no. AJ224122) revealed that *DAG1* has two introns and encodes a 295-amino-acid putative protein with the single Dof zinc finger domain between amino acid positions 76 and 104.

Isolation of a knockout mutant of *DAG1*

To assess the function of the *DAG1* gene, we utilized a reverse genetics approach based on PCR screening of a transferred-DNA (T-DNA) mutagenized population of *Arabidopsis* plants (Bechtold et al. 1993). Screening of

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~22,000 independent tagged *Arabidopsis* lines of the Wassilewskija (WS) ecotype, led to the identification of one line with a T-DNA insertion in the *DAG1* gene and subsequently to the isolation of the homozygous mutant *dag1-1*. The mutant was backcrossed with the wild type, and all subsequent characterizations were carried out on the F₂ homozygous progeny. Segregation of the kanamycin resistance marker associated with the T-DNA indicated the presence of a single insertion in *DAG1*, which was confirmed by Southern blot analysis (not shown). Sequencing revealed that the T-DNA is inserted close to the 3' splice site of the first intron of the *DAG1* gene, as schematized in Figure 1. No *DAG1* mRNA is detectable by either Northern or RT-PCR analysis in homozygous *dag1-1* plants (not shown).

Phenotypic analysis

Development and growth of the *dag1-1* knockout plants appear normal, and throughout the rosette (vegetative) stage mutants are indistinguishable from wild-type WS plants. Flowering *dag1-1* plants are less branched than WS, but the number of inflorescences per branch is substantially the same and the length of the floral stalk is reduced only modestly (in average, 28.5 ± 0.7 cm for *dag1-1* as compared to 32.8 ± 0.7 cm for WS plants). Flowering time and flower morphology of mutant and WS plants do not show appreciable differences.

Remarkably, however, all of the siliques (fruits) from *dag1-1* plants have a characteristically twisted appearance, as shown in Figure 2, and are shorter than normal WS siliques (10.8 ± 0.2 mm vs. 13.1 ± 0.3 mm). Kanamycin-resistant heterozygous plants segregated the twisted silique phenotype as a recessive trait with a 3:1 ratio and plants with aberrant siliques proved to be homozygous for the T-DNA insertion, indicating that the mutant phenotype is linked to the insertion.

dag1-1 seeds are nondormant and do not need light to germinate

The morphological modification of the siliques observed in the *dag1-1* mutant prompted us to check whether disruption of the *DAG1* gene also affects seed development and/or viability. The twisted siliques were shown to carry a somewhat reduced number of seeds—on average 47.7 ± 4.5 seeds each, as compared to 58.0 ± 5.0 seeds in WS siliques—of normal appearance and containing morphologically normal embryos.

Figure 1. Structure of the *DAG1* gene and position of the T-DNA insertion, which is not drawn to scale. (Solid rectangles) exons; (arrows) primers utilized for the isolation of the mutant line. The *DAG1* cDNA fragment was utilized as a probe for Northern analysis and to derive the sense and antisense riboprobes for in situ mRNA hybridizations.

Figure 2. Siliques from *dag1-1* mutant and WS plants.

Disruption of the *DAG1* gene has, in contrast, strong consequences on the germination of the seeds. As shown in Figure 3A, due to the effect of dormancy the rate of germination of freshly harvested WS seeds is rather low. In contrast, germination of *dag1-1* mutant seeds reaches >95%, indicating that they are substantially nondormant. Because storage gradually releases *Arabidopsis* seeds from dormancy, germination tests were repeated 4 weeks after seed harvest. The capability to germinate normal seeds is, as expected, strongly enhanced by storage, whereas that of *dag1-1* mutant seeds, being almost maximal upon harvest, is only fractionally affected (Fig. 3B).

Seed dormancy is induced by ABA, which also inhibits seed germination. Disruption of the *DAG1* gene does not appear to affect sensitivity of the seeds to the hormone, as germination of both WS and *dag1-1* knockout seeds is inhibited by the same concentration of ABA, as shown in Figure 3C.

Figure 3. Germination rates of WS and *dag1-1* mutant seeds. (A) Freshly harvested seeds; (B) seeds dry-stored for 4 weeks after harvest; (C) stored seeds, germination in the presence of ABA, scored at day 7; (D) stored seeds, germination in the presence of paclobutrazol, scored at day 7; (E) stored seeds, germination in total darkness [(□) WS seeds; (●) *dag1-1* seeds]; (F) stored seeds, germination in total darkness after a red (R) and a far-red (FR) light pulse [(open bars) WS seeds; (solid bars) *dag1-1* seeds].

Like WS seeds, *dag1-1* seeds require GA to germinate: Figure 3D shows that germination of *dag1-1* seeds is as sensitive as that of WS seeds to 10 μ M paclobutrazol, an inhibitor of GA biosynthesis.

Most strikingly, although stored seeds from WS plants are absolutely incapable of germinating in the absence of light, those from *dag1-1* mutant plants germinate in total darkness almost as well as in the presence of light (Fig. 3E). For normal *Arabidopsis* seeds to germinate, a pulse of red light is necessary to convert the (inactive) Pr form of the light receptor phytochrome to the active Pfr form (which is inactivated by far red light). We assessed that this is indeed the case for the WS ecotype seeds utilized in this work, as their negligible dark germination rate increased to 80% when WS seeds were exposed to a pulse of red light before performing the dark germination tests (Fig. 3F). The high germination rate of *dag1-1* seeds in the dark is stimulated further by this initial pulse of red light and reaches 100%. We then tested whether the capability of mutant *dag1-1* seeds to germinate in the absence of light is mediated by active phytochrome, by exposing the seeds to a Pfr-inactivating pulse of far red light prior to incubation in the dark. Figure 3F shows that Pfr is required for both the WS and the mutant seeds to germinate in the dark, as no germination of either was observed under these conditions.

The dag1-1 phenotype is due to disruption of DAG1

Conclusive evidence that the phenotype observed in *dag1-1* plants is due to disruption of the *DAG1* gene was obtained by transforming mutant plants with a genomic clone containing a wild-type copy of *DAG1*. F₃ transformants homozygous for the *DAG1* transgene—identified through segregation of hygromycin-resistance trait conferred by the cloning vector—all showed normal-appearing siliques, indicating that *DAG1* complements the twisted phenotype. The seeds from these siliques germinated in total darkness at the same low level of freshly harvested wild-type WS seeds, indicating that the exogenous *DAG1* complements also the phenotype of *dag1-1* seeds.

The effect of the disruption of DAG1 is maternal

To determine whether the phenotype of *dag1-1* seeds is due to the mother plant or to the developing embryo, we analyzed the germination of stored seeds from heterozygous *DAG1/dag1-1* plants. Two hundred seeds from *DAG1/dag1-1* plants were sown in triplicate and scored for germination after 7 days in total darkness. The results are consistent with a maternal effect of this mutation, as none of the seeds from heterozygous *DAG1/dag1-1* plants is capable of germinating in the absence of light. Instead, up to one-fourth (*dag1-1* homozygous) of the total seeds from heterozygous mothers would be expected to germinate in the dark, if the mutant phenotype were embryo-determined.

DAG1 is expressed in the mother plant but not in the embryo

In view of the maternal effect of the mutation and because seed dormancy initiates during embryo development, we analyzed expression of the *DAG1* gene at different stages of development of *Arabidopsis* flowers, fruits, and embryos. The RT-PCR analysis shown in Figure 4A revealed that the *DAG1* gene is expressed in flowers, as indicated by the amplification bands from flowers at stages 12 (lane F12) and 13 (lane F13), that is, before and, respectively, after flower opening (Smyth et al. 1990). After fertilization, *DAG1* is expressed in developing siliques (stage 16, lane Si16) but not in fully mature ones (stage 17, lane Si17). As also shown in Figure 4A, in *dag1-1* mutant plants no expression of the *DAG1* gene is detectable in the corresponding developmental stages and organs. Control amplifications of the cDNA of ATL18 ribosomal protein from the same samples were all positive (not shown).

A more detailed view of the pattern of expression of *DAG1* was obtained by in situ mRNA hybridizations. As shown in the longitudinal section of a stage 12 flower reported in Figure 4B, panel F12, a clear signal of *DAG1* mRNA is visible in the gynoeceum, specifically localized in the vascular tissue. Transverse sections of the gynoeceum (panel Gy12) confirmed that the *DAG1* mRNA signal is localized in the vasculature. After fertilization, expression of *DAG1* in stage 16 developing siliques (panel Si16) also is clearly localized in the vascular bundles and extends in the funiculus that connects the placenta to the ovule, but no *DAG1* mRNA signal can be detected in the ovule. In agreement with the RT-PCR data, no *DAG1* mRNA signal is detectable in any tissue of stage 17 mature siliques (panel Si17). No *DAG1* mRNA signal is visible at any stage of development in the embryo, as also shown in Figure 4, where sections of globular (panel GE) and torpedo (panel TE) embryos are reported. No *DAG1*-specific signal was detectable in any of the above sections with the *DAG1* sense riboprobe (not shown). Thus, the fact that the pattern of expression of the *DAG1* gene is limited to the mother plant is fully consistent with the maternal effect of the *dag1-1* mutation.

Discussion

Relatively few *Arabidopsis* mutants with reduced or suppressed seed dormancy have been identified so far. In the seed maturation mutants of *lec* (leafy cotyledon) class *lec1* (Meinke 1992) and *fus3* (Bäumlein et al. 1994; Keith et al. 1994), dormancy is affected as a pleiotropic effect of mutations in (as yet unidentified) genes controlling embryo development. Nondormant *lec1* and *fus3* seeds contain morphologically abnormal embryos that develop into seedlings whose anatomical characteristics suggest a homeotic (Meinke 1992) and, respectively, heterochronic (Keith et al. 1994) interpretation of these two mutations. In contrast, embryos of the *dag1-1* mutant appear normal and develop into normal seedlings.

Other seed dormancy mutants are affected in either

Figure 4. Analysis of *DAG1* expression in different organs and developmental stages. (A) RT-PCR with *DAG1*-specific primers on RNA from WS and *dag1-1*: (F12, F13) flowers at stages 12 and 13; (S16, S17) siliques at stages 16 and 17. (–) RT-PCR negative control; (+) PCR performed with WS DNA as a template. (B) In situ mRNA hybridizations with tritium-labeled antisense *DAG1* riboprobe on sections of WS flowers at stage 12 (F12, longitudinal section), gynoeceum of F12 flowers (Gy12, transverse section), siliques at stages 16 (Si16, transverse section) and 17 (Si17, transverse section), late globular-stage embryos (GE, longitudinal section), and torpedo-stage embryos (TE, longitudinal section). (Left) Dark-field images of the hybridizations; (right) corresponding fluorescence micrographs. Arrows point to the vasculature (v), gynoeceum (gy), funiculus (fu) and ovule (ov). Bar 250 μ m in F12 panels, 200 μ m in Gy12, 200 μ m in Si16, 400 μ m in Si17, 60 μ m in GE and TE.

ABA biosynthesis (*aba*, Koornneef et al. 1982; Léon-Kloosterziel et al. 1996a) or ABA sensitivity (*abi*, Koornneef et al. 1984)—major evidence supporting the view that ABA is the main plant hormone responsible for the induction of dormancy. Of the *abi* mutants, only *abi3* shows an effect limited to the embryo (Koornneef et al. 1984) and the *ABI3* gene encodes a seed-specific transcription factor (Giraudat et al. 1992). *ABI1* and *ABI2* encode protein phosphatases 2C (Leung et al. 1994,

1997; Meyer et al. 1994), and their mutation alters several responses of the plant to ABA (Koornneef et al. 1984), as is the case for *aba* mutants (Koornneef et al. 1982; Léon-Kloosterziel et al. 1996a). In contrast, disruption of *DAG1* does not affect sensitivity of the seeds to ABA—germination of *dag1-1* seeds is as sensitive to the hormone as that of WS seeds—and does not lead to the pleiotropic effects on water relations in adult plants characteristic of ABA-deficient mutants. In addition, although *aba* seeds do not require de novo synthesis of GA to germinate, *dag1-1* seeds do: Like WS seeds, *dag1-1* seeds are incapable of germinating in the presence of a concentration (10 μ M) of paclobutrazol that inhibits GA biosynthesis (Léon-Kloosterziel et al. 1996a).

The reduced dormancy mutants *rdo1* and *rdo2*, whose genes have not been isolated yet, are not affected in their response to ABA. In this respect, they are similar to the *dag1-1* mutant, but their reduced dormancy trait is embryo-determined (Léon-Kloosterziel et al. 1996b). In contrast, both the segregation pattern of the mutant phenotype in seeds from heterozygous plants and the lack of expression of the gene in the seeds indicate that *DAG1* exerts its role from the mother plant.

To our knowledge, *DAG1* is the first gene encoding a transcription factor that is specifically involved in the maternal control of seed germination. As yet, the only example of maternally determined loss of seed dormancy is in the mutants of the testa—the seed coat, which is of maternal origin. However, the enhanced germination of most of the testa mutants is due to altered physicochemical and/or mechanical properties of their seed coat—evidence that the seed coat acts in preventing the embryonic radicle from protruding from the seed prematurely (Koornneef 1981, 1990; Léon-Kloosterziel 1997). Although testa mutations result in altered coloration, shape, or composition of the seed coat, *dag1-1* seeds appear normal, suggesting that the maternal effect of the disruption of *DAG1* does not involve the seed testa.

A most interesting trait of the nondormant *dag1-1* mutant seeds is their capability to germinate in total darkness. In contrast, nondormant WS seeds (i.e., after storage-induced release from dormancy) still require (red) light to germinate. Red light is needed to turn the inactive Pr form of the phytochrome into the active Pfr form, which then triggers germination via a pathway, as yet totally unknown. The lack of light requirement of *dag1-1* seeds, however, does not imply that they bypass the phytochrome-mediated pathway to initiate germination. When Pfr is inactivated by a pulse of far-red light, *dag1-1* seeds fail to germinate in the dark.

On the other hand, *DAG1* is expressed in the vascular tissue of the mother plant, suggesting that its mode of action involves transport to the seed of some component necessary for the control of dormancy. This evidence can be rationalized by a model whereupon *DAG1* normally would ensure the inactivity (for dormancy initiation) of a (protein) component—transported from the mother plant to the seed—that would be activated (to trigger germination) as a consequence of red light perception. In plants where *DAG1* is disrupted, this component would be in

the active form independent of red light: dormancy would not be established and seeds would germinate in the absence of light but germination could still be repressed by far-red light.

Further work is needed to verify this model or test alternatives. However, the identification of the first regulatory protein involved in the maternal control of seed germination opens a new avenue of research in this important and still largely obscure aspect of plant adaptation.

Materials and methods

Plant material and seed germination

Seeds from *Arabidopsis thaliana* Heyhn, ecotype WS, were surface-sterilized as described by Santoni et al. (1994), sown into petri dishes on a solid medium containing Murashige and Skoog salts (Sigma), vitamins (Sigma), 0.8% agar, and 1% sucrose, and kept in the dark at 4°C for 2 days. The dishes were then transferred into the growth chamber (24°C/21°C, 16/8 hr day/night, 300 μ Einstein/m²). Seedlings were transferred into individual pots 8 days after germination, and maintained in the growth chamber. Thirty WS and *dag1-1* flowering plants were analyzed for morphometric parameters, and 30 siliques from both types of plants were measured and scored for the number of seeds contained.

For germination experiments, surface-sterilized seeds were sown in triplicate in Petri dishes (30–60 per dish) over one layer of Whatmann paper saturated with 2.5 ml of sterile distilled water and kept in the dark at 4°C for 2 days. Germination tests were subsequently carried out under the illumination conditions utilized for plant growth or in total darkness. In the text, stored seeds are seeds stored for 4 weeks after harvest in the dark under dry conditions at room temperature. For ABA and paclobutrazol sensitivity tests, seeds were sown on 0.8% agar in the presence of the ABA and paclobutrazol concentrations indicated in Figure 3, C, and D, respectively; germination was scored after 7 days of incubation under standard illumination conditions.

For light-pulse experiments, light sources were from Phylips (bulb), and fluence rates were as follows: red light, bulb TL (40 W), fluence, 316 μ W/cm²; far-red light, bulb Phylinea (60 W), fluence, 350 μ W/cm². Seeds were exposed to red or far-red light for 5 min and kept in the dark for 7 days before scoring for germination.

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 in Vittorioso et al. (1998). The RT-PCR analysis was performed with *DAG1*-specific primers, according to the manufacturer's instructions (Perkin-Elmer).

Isolation of DAG1

The Dof domain of the previously isolated partial cDNA of *DAG1* (De Paolis et al. 1996), was utilized for the screening of an *Arabidopsis* genomic library (Clontech). Several positive clones were isolated. An 8-kb *SalI* insert containing the entire *DAG1* gene was cloned into pBluescript KS+ vector (Stratagene) for restriction analysis. The 1.5- and 3.0-kb *EcoRI* fragments containing the promoter (1 kb) and the *DAG1* gene, respectively, were subcloned and sequenced. Full-length sequence of the cDNA was obtained by 5'-RACE, 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). The analysis of *DAG1* cDNA and derived protein sequence was performed using GCG and BLAST, or the National Center for Biotechnology Information (NCBI, Bethesda, MD).

Isolation of the dag1-1 knockout plant

A collection of T-DNA insertion mutants (Laboratoire de Génétique et Amélioration des Plantes, INRA, Versailles, France) containing ~22,000 individual lines, was screened by PCR utilizing pairs of primers corresponding to both the T-DNA right and left borders and the *DAG1* 3'-specific region. One positive line was identified and isolated. The position of the T-DNA within the *DAG1* gene was determined by sequencing the PCR-amplified fragment. The *DAG1*-tagged line was shown to contain a single T-DNA insert and checked for linkage of the T-DNA insert

to the *DAG1* gene. More than 100 putative heterozygous plants (kanamycin resistant with normal siliques) segregated 25% mutant progeny (twisted siliques). Plants heterozygous for the mutation were self-fertilized, and the transmission of the phenotype was confirmed in the F₃ generation.

Complementation of the dag1-1 mutant

A 2.7-kb genomic fragment corresponding to the *DAG1* gene was cloned in the pCambia binary vector (Canberra, Australia). *dag1-1* homozygous plants were transformed by in planta infiltration (Bechtold et al. 1993) and transformants selected on 40 μ g/ml hygromycin. The F₃ progeny from two individual hygromycin-resistant plants was analyzed for complementation of the *dag1-1* phenotypes.

In situ mRNA hybridizations

A 400-bp PCR fragment containing the carboxy-terminus of the *DAG1*-coding sequence up to the 3' UTR, was cloned in the pCR2.1 vector (Stratagene). For the antisense riboprobe, the construct was linearized and in vitro transcribed with T7 RNA polymerase in the presence of ³H-labeled UTP. The sense probe was derived from a construct containing the same PCR-amplified fragment cloned in the opposite orientation. Conditions for tissue fixation, paraffin embedding, hybridization (on 8-mm sections), and washings were as described by Drews et al. (1991).

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