DELAY OF GERMINATION1 requires PP2C phosphatases of the ABA signalling pathway to control seed dormancy

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The time of seed germination is a major decision point in the life of plants determining future growth and development. This timing is controlled by seed dormancy, which prevents germination under favourable conditions. The plant hormone abscisic acid (ABA) and the protein DELAY OF GERMINATION 1 (DOG1) are essential regulators of dormancy. The function of ABA in dormancy is rather well understood, but the role of DOG1 is still unknown. Here, we describe four phosphatases that interact with DOG1 in seeds. Two of them belong to clade A of type 2C protein phosphatases: ABA-HYPERSENSITIVE GERMINATION 1 (AHG1) and AHG3. These phosphatases have redundant but essential roles in the release of seed dormancy epistatic to DOG1. We propose that the ABA and DOG1 dormancy pathways converge at clade A of type 2C protein phosphatases.
Accurate timing of seed germination is important for the adaptation of plants to their environment. Seeds shed from plants with a life cycle adapted to seasonal changes are usually not able to germinate directly due to dormancy, which is defined as the incapacity of an intact viable seed to complete germination under favourable conditions. Seeds will only germinate after dormancy has been released. Seed dormancy impacts on agricultural production and was under negative selection during domestication. Low dormancy ensures fast and uniform germination of crop seeds, but it can also lead to an unwanted early germination on the mother plant (pre-harvest sprouting) and reduced seed quality. A good understanding of dormancy control will benefit both ecological understanding and crop management. The induction and release of dormancy are regulated by developmental and environmental factors. Dormancy is induced during seed maturation. Regulators of seed maturation and environmental conditions during seed development affect the strength of seed dormancy. Dormancy is released by imbibition at low temperatures (stratification) or extended dry storage of seeds (after ripening).

The role of hormones in dormancy and germination has been intensively studied. Abscisic acid (ABA) regulates seed maturation and is required for the induction of dormancy. Gibberellins (GA) are needed for germination. It is in particular the balance between ABA and GA that determines germination potential. ABA regulates dormancy by reducing the activity of protein phosphatase 2C (PP2C) clade A proteins like ABI INSENSITIVE 1 (ABI1) and ABI2. As a consequence these phosphatases lose their ability to inhibit the activity of class II SNF1-related protein kinase 2 (SnRK2) by dephosphorylation. Several of these SnRks positively control dormancy and the triple mutant snrk2.2 snrk2.3 snrk2.6 shows a loss of seed dormancy.

Two major dormancy genes, DELAY OF GERMINATION 1 (DOG1) and REDUCED DORMANCY 5 (RDO5), have been identified that seem to function independent from the plant hormones. RDO5 is a member of the PP2C family of protein phosphatases that does not show phosphatase activity. The molecular function of DOG1 has not been solved and its protein lacks domains with a known function. Both genes control natural variation in seed dormancy between Arabidopsis accessions. Mutations in DOG1 and RDO5 completely abolish or reduce seed dormancy, respectively. DOG1 is highly conserved in the plant kingdom and homologues in various crop species have been shown to control seed dormancy. The amount of DOG1 protein in seeds determines the time they have to be stored to release dormancy and we have shown that the protein loses its function during this after-ripening process. The regulation of DOG1 is complex and involves polyadenylation, alternative splicing and self-binding of its splicing forms. In addition, DOG1 transcription is influenced by environmental factors occurring during maturation and in the seed bank such as low temperature, which is associated with enhanced DOG1 transcript and protein levels. DOG1 is predominantly located in the nucleus suggesting that it might function as a transcriptional regulator. DOG1 has been proposed to act by a temperature-dependent alteration of the GA metabolism, leading to weakening of the endosperm. In addition, it was recently shown that DOG1 influences transcript levels of genes involved in miRNA processing causing altered accumulation of miRNAs that control developmental phase transitions in Arabidopsis and lettuce. These two potential functions of DOG1 are not mutually exclusive, but none of them has been conclusively proven to be part of the primary mechanism by which DOG1 regulates dormancy. Genetic and transcriptomic analyses suggested that DOG1 is likely to function independent from ABA. However, both ABA and DOG1 have to be present to induce seed dormancy as absence of a single one of these two regulators results in complete lack of dormancy even when the other regulator is highly accumulated. DOG1 was also shown to be required for multiple aspects of seed maturation, partially by interfering with ABA signalling components. This suggests that the two pathways converge at downstream steps.

**Results**

DOG1 forms complexes with protein phosphatases in seeds. The DOG1 gene is essential for dormancy in Arabidopsis and seeds lacking functional DOG1 protein are completely non-dormant. The molecular mechanism by which DOG1 controls dormancy is not obvious because the primary amino acid sequence of DOG1 lacks domains with a known function. To obtain an insight into its function, we set out to identify proteins that interact with DOG1 in vivo using pull-down experiments. The antibody that we used for DOG1 detection in previous experiments is not suitable for pull-down experiments.

**Fig. 1** The YFP-DOG1 transgene complements the dog1-1 non-dormant phenotype. **a** Germination of freshly harvested seeds on 0.05% ethanol (control) or on 0.05% ethanol plus the indicated concentrations of fluridone. Radical emergence was scored 7 days after sowing. Shown are averages ± s.d. of three independent batches of seeds for each genotype. **b** YFP-DOG1 protein accumulation in the complementation lines. (top) Ponceau S staining. DOG1 protein was detected using GFP antibody. After stripping, the same membrane was blotted with H3 antibody as loading control. Cdog1#1-4 represent four independent dog1-1 transformants containing the YFP-DOG1 transgene.

**Fig. 2** The DOG1 regulating seed dormancy is shown by the increased seed germination of freshly harvested seeds treated with 5 µM fluridone. **a** Germination of freshly harvested seeds on 0.05% ethanol (control) or 0.05% ethanol plus the indicated concentrations of fluridone. Radical emergence was scored 7 days after sowing. Shown are averages ± s.d. of three independent batches of seeds for each genotype. **b** YFP-DOG1 protein accumulation in the complementation lines. (top) Ponceau S staining. DOG1 protein was detected using GFP antibody. After stripping, the same membrane was blotted with H3 antibody as loading control. Cdog1#1-4 represent four independent dog1-1 transformants containing the YFP-DOG1 transgene.
Therefore, transgenic dog1-1 plants were generated containing a yellow fluorescent protein (YFP) fused with DOG1 at the N-terminus, expressed from a strong DOG1 promoter derived from the Cape Verde Islands (Cvi) accession (pDOG1:YFP-DOG1). Independent homozygous single-insertion lines were selected and analysed for their seed-dormancy level. Seeds from the dog1-1 mutant germinated 100% directly after harvest whereas its wild-type background, NIL DOG1, only showed low germination (Fig. 1a). The transgenic lines (named Cdog1 #1, #2, #3 and #4) complemented the dog1-1 phenotype and their dormancy levels correlated with YFP-DOG1 protein accumulation (Fig. 1). This indicated that our YFP-DOG1 fusion protein is functional. Seeds from the dormant NIL DOG1 and the complemented lines fully germinate on the ABA biosynthesis inhibitor fluridone confirming the essential role of ABA biosynthesis in dormancy even in seeds with high levels of DOG1 protein19. The line with the highest dormancy level and protein accumulation (Cdog1 #4) required 11 weeks of dry storage to fully release dormancy (Supplementary Fig. 1a) and was selected for pull-down experiments together with the dog1-1 mutant as a control.

Dormant and non-dormant seed samples were taken from the same batch after different storage durations (Supplementary Fig. 1a). Proteins were isolated in their native state from dry and 24 h imbibed seeds in three biological replicates to pull-down YFP-DOG1 protein complexes using green fluorescent protein (GFP) -binding agarose beads. Gel analysis of the pull-down procedure revealed a high specificity and efficiency for YFP-DOG1 protein enrichment (Supplementary Fig. 1b). Pulled-down proteins were analysed by quantitative mass spectrometry (Supplementary Data 1), and stringent data processing (see Methods) identified 184 protein groups, which were reproducibly quantified and exclusive for the Cdog1#4 pull-downs in all three YFP-DOG1 replicates of a given condition but in none of the corresponding control pull-downs (Fig. 2). To identify proteins present in DOG1 complexes that can be relevant for its function in seed physiology we selected targets annotated with a gene ontology (GO) biological process related to seeds. We obtained 46 matches for 17 unique proteins (Fig. 2a and Supplementary Table 1) suggesting that DOG1 can interact with proteins implied in diverse seed developmental processes including dormancy. In addition, seven matches of candidates implied in ABA responses were found suggesting that DOG1 might interfere with the ABA hormone signalling pathway by complexing with ABA-related proteins (Fig. 2a and Supplementary Table 1).

Dormant seeds showed the highest number of proteins co-purifying with YFP-DOG1 (138 proteins in dry and 80 proteins in imbibed dormant seeds; Fig. 2b). Loss of seed dormancy induced a drastic decrease in the number of interacting protein (six proteins in dry and two proteins in imbibed non-dormant seeds). The decrease in number of interacting proteins upon dormancy alleviation might be a direct effect of the loss of DOG1 activity during after-ripening19. Two proteins, RDO5 and AHG1, were found to interact under all tested conditions (Fig. 2b). RDO5 belongs to the PP2C clade A phosphatase family and has been described as a positive regulator of dormancy with seed-specific expression14. AHG1 is a PP2C clade A phosphatase27 and members of this clade are negative regulators in the ABA signalling pathway28, 29. AHG1 has a seed-specific expression pattern and shows the highest transcript levels in dry seeds among all PP2C clade A phosphatases27 (Supplementary Fig. 2). Furthermore, amongst the identified interacting proteins, AHG3 and PROTEIN PHOSPHATASE 2A SUBUNIT A2 (PP2AA/PDF1) were identified in dry as well as in imbibed dormant seeds. AHG3 belongs to the same clade of PP2Cs as AHG1, while PDF1 is a scaffolding subunit of PP2A protein phosphatase. We focussed our further studies on AHG1/3, RDO5 and PDF1 because they share relevant characteristics. They are all expressed in seeds14, 27, 30, 31, interact with YFP-DOG1 in dormant seeds and are involved in the same molecular process: phosphoserine/threonine dephosphorylation. In addition, three of them, AHG1, AHG3 and RDO5, have been implicated in either ABA signalling or dormancy14, 27, 30.

To confirm the interaction of DOG1 with the identified PP2C phosphatases and the PP2A phosphatase subunit, we performed a yeast two-hybrid GAL4 assay. Co-transformation of pACT2: DOG1 with pAS2:RDO5/AHG1/AHG3 restored yeast growth on selective medium and β-galactosidase activity. This restoration was also observed for pAS2:PDF1, although at a weaker level (Supplementary Fig. 3). We subsequently analysed the interaction of DOG1 with these proteins in planta using bimolecular fluorescence complementation (BiFC) in epidermis cells of Nicotiana benthamiana leaves. Interaction of DOG1 with AHG1, AHG3 and RDO5 was observed in the nucleus (Fig. 3). The specificity of DOG1 binding to AHG1 and AHG3 within the PP2C clade A phosphatases was analysed by testing its interaction with another phosphatase of this clade, ABI2, which had not been identified in the DOG1 pull-down assay. Restoration of YFP fluorescence was not observed in leaves co-transformed with DOG1 and ABI2 fusion proteins despite the presence of both
proteins in the transfected tobacco leaves (Fig. 3 and Supplementary Fig. 4). The BiFC assay showed interaction of DOG1 with PDF1 in the cytosol. Interaction of PDF1 and DOG1 in the cytosol might explain the weaker interaction observed in Y2H since this system requires translocation of the reconstituted complex into the nucleus to activate histidine autotrophy. Overall, these results confirmed and validated the selective interaction of DOG1 with RDO5, AHG1/3 and PDF1.

**DOG1-interacting phosphatases control seed dormancy.** DOG1 is a key seed dormancy gene and interacting proteins that are important for its function are likely to influence dormancy. Dormancy phenotypes have not been described for *pdf1* and thus we analysed its germination during seed storage. An insertion mutant in Columbia (Col) background, *pdf1*, showed enhanced seed dormancy (Fig. 4a and Supplementary Fig. 5a) compared to wild-type Col. The *pdf1* and *dog1-2* mutants were combined by crossing and selection. This double mutant completely lacked dormancy, similar to the *dog1-2* single mutant (Fig. 4a). Therefore, *dog1-2* appears to be epistatic to *pdf1*, suggesting that DOG1 functions downstream of PDF1.

Interestingly, both *ahg1* and *ahg3* mutants have been described to show enhanced sensitivity to ABA during germination and reduced germination speed, but their dormancy loss during after-ripening has not been previously analysed. We obtained homozygous insertion mutants for *ahg1* and *ahg3* in the Col background, which were named *ahg1-5* and *ahg3-2* and which lacked full-length transcripts of *AHG1* and *AHG3*, respectively (Supplementary Fig. 5b,c). Freshly harvested seeds of wild-type Col germinated ~70%, whereas *ahg1-5* and *ahg3-2* seeds germinated 10 and 20%, respectively (Fig. 4b). The wild-type seeds fully after-ripened after 2 weeks of storage, whereas the *ahg* single mutants needed 5–6 weeks of after-ripening to reach a germination percentage close to 100%. This demonstrated their enhanced dormancy phenotypes. Because AHG1 and AHG3 are closely related and the two highest expressed PP2C phosphatases of clade A in seeds (Supplementary Fig. 2a), we tested their redundancy by constructing the double-mutant *ahg1-5 ahg3-2*. It has been described that this double mutant shows enhanced sensitivity to ABA compared to the single mutants. We observed a very strong dormancy phenotype of the *ahg1-5 ahg3-2* double mutant. Freshly harvested seeds were fully dormant and after extended storage of 22 weeks only 30% of the seeds germinated (Fig. 4b). These seeds still had the capacity to germinate because imbibition of the seeds in 100 μM GA₄+7 led to 100% germination (Fig. 4c). These results indicated that AHG1 and AHG3 have a largely redundant function in the negative regulation of seed dormancy. Since DOG1 interacts with AHG1 and AHG3 whose mutations have been shown to confer ABA hypersensitivity, we also analysed the ABA sensitivity of *dog1* mutants. A small reduction in ABA germination sensitivity had previously been shown for the *dog1-1* mutant in the NIL DOG1 background. Analysis of fully after-ripened seeds in the Col background confirmed the ABA hypersensitive germination phenotype of *ahg1-5* and *ahg3-2* single mutations and showed a decrease in ABA sensitivity for the *dog1-2* mutant (Fig. 4d).

In contrast to the other three interacting phosphatases, mutations in RDO5 lead to strongly reduced dormancy, indicating an opposite role. This could be related with the lack of phosphate activity of the RDO5 protein.

**AHG1 and AHG3 phosphatases are required for DOG1 function.** The opposite extreme dormancy phenotypes of *dog1-2* and the *ahg1-5 ahg3-2* double mutant provide an ideal background to study their genetic relation by combining their mutations (Supplementary Fig. 6). The double mutants *dog1-2 ahg1-5* and *dog1-2 ahg3-2* were completely non-dormant. In contrast, the triple mutant *dog1-2 ahg1-5 ahg3-2* showed a very strong dormancy phenotype similar to the *ahg1-5 ahg3-2* double mutant (Fig. 4b). The triple mutant was able to germinate 100% after imbibition on 100 μM GA₄+7 (Fig. 4c). Thus, the genetic analysis demonstrated that DOG1 requires the phosphatases AHG1 and AHG3 for its function and that both phosphatases function redundantly downstream of DOG1. Seeds with high DOG1 protein levels or mutations in AHG1 or AHG3 show enhanced dormancy, suggesting that DOG1 negatively influences the action of AHG1 and AHG3. Strikingly, as for the double-mutant *ahg1 ahg3*, the triple mutant with *dog1-2* did not gradually release seed dormancy, but remained at ~10–30% germination. This suggests that AHG1 and AHG3 are essential factors in the release of dormancy by after-ripening controlled by DOG1. In addition, using stratified seeds, we could show that the triple-mutant *ahg1-5 ahg3-2 dog1-2* had a similar sensitivity to ABA as the double-mutant *ahg1-5 ahg3-2 dog1-2*, indicating that the *ahg1 ahg3* double mutant is epistatic to *dog1* for both dormancy and ABA sensitivity (Fig. 4d).

The phosphate activity of most of clade A PP2Cs is inhibited in the presence of ABA by the formation of a stable complex consisting of ABA, a member of the PYR/PYL/RCAR family of ABA receptors, and PP2C. In this complex, the receptor bound to ABA hinders the active site of the phosphate by mimicking the SnRK class II activation loop. Interestingly, the seed-specific
DOG1 could directly influence the catalytic activity of AHG1 or AHG3 using recombinant protein and in vitro phosphatase activity assays (Supplementary Fig. 7). Incubation of DOG1 with AHG1 or AHG3 did not lead to any significant change in their phosphatase activities even in the presence of ABA and/or the pseudophosphatase RDO5 (Supplementary Fig. 7b). It is likely that our in vitro experimental system, using purified components from *Escherichia coli*, cannot mimic the in vivo situation in seeds. A missing factor (for instance other proteins, metabolites or post-translational modifications) or a specific context (limitation in water and/oxygen availability) putatively prevent a reliable in vitro investigation of the relationship between DOG1 and PP2Cs.

**Discussion**

DOG1 has been identified as an essential gene for seed dormancy in *Arabidopsis*. DOG1 homologues regulating dormancy have been found in various species across the plant kingdom. The lack of homology of DOG1 with proteins that have a known function hindered attempts to understand its molecular function. Several laboratories proposed different hypotheses for a role of DOG1 in dormancy, which were mainly based on observations at the transcript level. These included the inhibition of expression of GA-regulated genes encoding cell wall remodelling proteins and alterations in seed GA metabolism, reduced expression of genes associated with microRNA processing leading to reduced levels of miR156, and a role in seed maturation by interference with ABA signalling. In this work we identified two protein phosphatases (AHG1 and AHG3) that interact with DOG1 in the seed and that are essential for its function. This provides an important direct insight into the mechanism by which DOG1 controls seed dormancy and we propose that previously identified mechanisms might function downstream of this direct mechanism.

The phosphatases AHG1, AHG3, RDO5 and PDF1 were found among the proteins that interact with DOG1 in seeds. Plants with mutations in the corresponding genes showed altered seed dormancy. Three of these phosphatases belong to the PP2C family while PDF1 encodes one of the three scaffolding subunits of the PP2A family. Relatively little is known about these scaffolding units. One of them (RCN1) has a major role in the regulation of phosphatase activity and its mutant showed several defects, mainly in relation with hormone signalling. In contrast, mutants in the other two units (*pdf1/pp2a22* and *pdf2/pp2aa3*) did not show obvious phenotypes. Here, we identified a negative role for PDF1 in seed dormancy. Our genetic analysis suggests that PDF1 acts upstream of DOG1 because the *dog1* mutant phenotype is epistatic to *pdf1*. RDO5 belongs to the PP2C phosphatase family but lacks phosphatase activity, therefore it might act as a pseudophosphatase. In agreement with a role as pseudophosphatase, the *rdo5* mutant showed reduced dormancy in contrast to the other three identified phosphatases interacting with DOG1 that are functional and which mutants all showed enhanced dormancy.

Most importantly, DOG1 interacts in seeds with the PP2C phosphatases AHG1 and AHG3, which have been previously identified based on their ABA hypersensitive mutant phenotypes. In this work, we demonstrated that AHG1 and AHG3 have redundant roles in seed dormancy because the phenotype of the double mutant is much more severe than that of the single mutants (Fig. 4b). The double mutant shows extreme dormancy and did not germinate more than 20% after 6 months of storage. These low germination rates are not due to a loss of viability of the seeds because stored *ahg1 ahg3* double-mutant seeds could germinate 100% when imbibed in GA$_{4+7}$. Overall, this indicates a crucial role for AHG1 and AHG3 in the
release of seed dormancy, while they are not required for germination per se.

We further demonstrated that these two phosphatases are essential for DOG1-dependent seed dormancy control. Our genetic data indicate that the interaction of DOG1 with AHG1 and AHG3 can negatively affect the function of these PP2Cs rather than that these phosphatases control DOG1 activity by phosphorylation. The triple mutant dog1 ahg1 ahg3 showed the same dormancy and ABA sensitivity phenotypes as the ahg1 ahg3 double mutant, indicating that DOG1 functions upstream of these PP2Cs. Enhanced DOG1 protein amounts and absence of AHG1 and AHG3 (in the ahg1 ahg3 double mutant) both lead to increased dormancy. Therefore, our genetic analysis suggests that DOG1 acts as a suppressor of AHG1 and AHG3 action in dormancy release.

ABA and DOG1 are both required for seed dormancy. Genetic and physiological experiments suggested that they act independently although they are likely to have common downstream targets. The mechanistic basis of the crosstalk between ABA and DOG1 has been mentioned as one of the outstanding open questions in seed biology. In this work, we provide data indicating that the DOG1 and ABA pathways are connected at the clade A PP2Cs.

The clade A PP2Cs function as key negative regulators of the ABA signalling pathway. In accordance with their role in ABA signalling and their expression pattern, mutants in the clade A PP2Cs show varying seed-dormancy phenotypes. Most of the nine phosphatases belonging to this clade are inhibited by the PYR/PYL/RCAR family of ABA receptors in the presence of ABA. Interestingly, the seed-specific clade A PP2C AHG1 was shown to be resistant to inhibition by ABA, although it does not positively regulate the release of seed dormancy and among them AHG1 and AHG3 have major roles. Seed-specific proteins are indicated in red. Clade A PP2C proteins are encircled. Proteins that form a complex with DOG1 in seeds are underlined.

We propose that the ABA and DOG1 pathways converge at the level of the clade A PP2C phosphatases. The inhibition of the clade A PP2Cs by both ABA and DOG1 is supported by the reduced sensitivity to ABA of the dog1 mutant (Fig. 4d). The functional redundancy of AHG1 and AHG3 in seed dormancy and their common inhibition by DOG1 but different inhibition by ABA could explain previous observations about the relation between DOG1 and ABA. Enhanced expression of DOG1 in ABA biosynthesis mutants cannot induce dormancy. This is probably due to the activity of PP2Cs that are not or only poorly inhibited by DOG1 and that are still able to promote germination. Similarly, the lack of dormancy of mutants with enhanced ABA levels in a dog1 mutant background can be explained by the activity of AHG1, which is probably not inhibited by the conventional ABA pathway.

ABA has multiple functions in plant development and stress resistance, including seed maturation and dormancy. The main function of DOG1 is to promote seed dormancy, although a role in seed maturation has recently also been demonstrated. The inhibition of germination by ABA and DOG1 through both common and separate PP2Cs would enable a flexible but robust way to shape dormancy.

The role of DOG1 in seed dormancy was shown to be conserved between several dicot and monocot species. Therefore, it is likely that the mechanism of DOG1 action that we propose for Arabidopsis will be conserved within the plant kingdom and could have evolved as an early adaptation of seed plants to survive seasonal conditions.

**Methods**

**Plant material and growth conditions.** The dog1-1 mutant is in the NIL DOG1 background, which is a near isogenic line that contains the DOG1 allele from Cvi in a Landsberg erecta (Ler) background. The dog1-2 mutant is in the Col background. AHG1, AHG3 and PDF1 insertion lines were obtained from the NASC collection with the following seed stock numbers: ahg1-5, SALK_049885C; ahg3-2, SALK_028132; pdf1, SALK_037093. The gene-specific primer sequences were obtained from the SALK SIGnAL database. PCR with reverse transcription with DNA and RNA isolated from dry or imbibed seeds was performed to confirm the homozygous mutant lines (Supplementary Fig. 5 and Supplementary Table 2). Double mutants were constructed by crosses between the single mutants dog1-2, ahg1-5 and ahg3-2 and selected from the self-pollinated progeny of F2 plants using PCR to confirm their homozygosity. The triple-mutant dog1-2 ahg1-5 ahg3-2 was obtained by crossing the double mutants dog1-2 ahg1-5 and dog2-1 ahg3-2 (Supplementary Fig. 6).

Seed batches were obtained from plants cultivated on soil in a growth chamber with a 16-h-light/8-h-dark cycle (22/16 °C) with the exception of the seed batches used in Fig. 4a that were obtained from plants cultivated at lower temperature (16/14 °C), conditions that enhance dormancy. Freshly harvested seeds were immediately used for experiments or stored under constant conditions (21 °C, 50% humidity, in the dark) for after-ripening treatment. All comparative germination assays presented in this work use seed batches obtained at the same time from the same environment.

**Germination assays.** About 50 seeds were plated on a filter paper moistened with demineralized water, 100 µM GA₄₉, 0.2–3 µM ABA or 5–10 µM fluridone in Petri dishes and incubated in a growth chamber (12 h light/12 h dark, 25/20 °C cycle). Stock solutions of ABA, fluridone and GA were dissolved in ethanol (final concentrations in the assays were 0.05%). When imbibed on chemicals, the control corresponds to 0.05% ethanol in water. Radicle emergence was scored after 7 days.

**Construction of YFP-DOG1 transgenic lines.** Binary constructs were prepared using the Gateway Technology (Invitrogen). A chimeric DNA fragment of YFP and a genomic DOG1 fragment from Cvi (ATG to 1.1 kb downstream of the stop codon of alpha/delta splicing variants) was generated by fusion PCR using the adapter sequence 5′-ccagcacgcatcggaccccttc-3′ and cloned into the pENTR/D-TOPO vector. The DOG1 promoter region from Cvi of 2.17 kb (corresponding to the upstream region of Ler genomic fragment) was inserted in the entry clone pENTRYFP-DOG1_Cvi using the unique NotI site. The binary construct pGW16:ProDOG1: YFP:DOG1_Cvi: was produced from the above mentioned entry clone and the destination vector pGW134 by LR reaction. The resulting pDOG1_Cvi:YFP-DOG1_Cvi construct was introduced by electroporation into Agrobacterium tumefaciens strain GV3101, which was subsequently used to transform dog1-1 mutant.
plants by floral dipping. Independent homozygous single insertion lines were selected based on their antibiotics resistance and genotyping by PCR. 

**Gene constructs.** Full-length coding DNA sequence (CDS) for AHG1, AHG3 and PDF1 were amplified from cDNA of dry Ler seeds using gene-specific primers including or not the stop codon. The PCR product was extended using the attB adapter sequences and cloned into pDONR201 or 207 (Invitrogen) using BP reactions. The pDONR207: RDOS plasmid containing the CDS (including stop) of Ler RDOS as well as the cloning of N-terminus truncated AHG3 (starting at amino acid position N88) was already described. To obtain the DOG1 expression clone for recombinant protein production, the full-length CDS of β-DOG1 from Cvi was amplified from the entry clone using gene-specific primers including a BamHI site extension in the reverse primer. A synthetic double-strand DNA fragment containing an NcoI restriction site, a STREP-tag and a linker sequence was generated by denaturation and annealing (from 98 °C to room temperature) of complementary primers. DOG1 PCR product and the reconstituted tag sequence were digested with BamHI and Ncol respectively and the restricted fragment was purified from agarose gel. The chimeric STREP-DOG1 fragment was generated by blunt end ligation of digested proteins and cloned into pET16b (Novagen) using Ncol and BamHI sites. Sequences of all constructs were verified by Sanger sequencing.

**Yeast two-hybrid assay.** The CDS (including stop) of AHG1, AHG3, RDOS and PDF1 were recombined from entry clones in the pAS2-gateway (GAL4 BD fusion) vectors (modified from Clontech) using LR reactions. The pACT2-gateway (GAL4 AD fusion) vector was seed cloned by pACT2 DOG1 and pAS2-DOG1 containing the β-isofoms of DOG1 from Cvi were previously described. GAL4-DNA-binding domain fusion proteins (prey) and GAL4 activation domain fusion proteins (bait) vectors were transformed in the yeast strain YEp64-αUra with a LiA/SS carrier DNA/PEG method. Co-transformed colonies were selected on selective medium (-LW) lacking Leu (-L) and Trp (-W). Interaction tests were performed on -LW medium (-LW) lacking L, W and His (H) with 5 mM 3-aminotriazole. Yeast was grown at 30 °C for 7 days. β-galactosidase assay was performed by pressing filter papers on the plates with yeast colonies. The filters were frozen in liquid nitrogen and subsequently incubated at 30 °C in a solution containing 0.1% X-gal.

**Bimolecular fluorescence complementation.** The full-length AHG1, AHG3, AB12, PDF1 and RDOS coding sequences (without stop codon) were recombined from entry clones in the pbatTL-B-YFPc gateway vector (gift from Joachim Uhrig; MPI IPZ Cologne, Germany) using LR reactions. The pbatTL-B-YFPc containing DOG1 beta was already described. Constructs were transformed into the Agrobacterium strain GV3101. Overnight cultures were diluted to an OD<sub>590</sub> of 0.5 in resuspension buffer (10 mM MgCl₂, 10 mM MES (pH 5.7) and 100 μM acetoacysertone and injected into 4 papers on the plates with yeast colonies. The paper on the plates with yeast colonies. The exact same leaves were used for imaging were frozen in liquid nitrogen and used to extract total protein for western blotting.

**Production and purification of recombinant protein.** Full-length CDS of AHG1, or N terminus truncated CDS of AHG3 were recombined from entry clones in the pDEST-HIS-MBP or pDEST17 gateway vector, respectively. The CDS (including stop) of AHG1, AHG3 and PDF1 were amplified from cDNA of dry Ler seeds using gene-specific primers including or not the stop codon. The PCR product was extended using the attB adapter sequences and cloned into pDONR201 or 207 (Invitrogen) using BP reactions. The pDONR207: RDOS plasmid containing the CDS (including stop) of Ler RDOS was incubated for 20 min at room temperature in 50 mM HEPES buffer pH 7.5; 10 mM MgCl₂, 1 mM EDTA; 1 mM EGTA; 0.25% (w/v) Triton X-100; 5 mM DTT; 20 µM M. After 10 min, proteins were solubilized in a buffer containing 50 mM HEPES pH 7.5; 2.5% (w/v) SDS; 5 mM DTT. Both soluble and total protein extraction buffers were supplemented with 1% (v/v) of protease inhibitor cocktail special (Sigma) and 1% (v/v) phosphatase inhibitor cocktail 2 (Roche). Protein fractions from seed extracts were pooled by precipitation using acetosyringone and stored at −80 °C until protein extraction. Cell pellets were resuspended in either 0.1 M Tris-HCl pH 8.0, 1 mM CaCl₂. Trypsin digestion (1:100 enzyme-to-substrate ratio) was performed in a buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 5 mM DTT, 1 mM PMSF, and 25 mM imidazole or in STREP lysis buffer: 50 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 5 mM DTT, 1 mM PMSF, and 25 mM imidazole. Yeast was grown at 30 °C for 7 days. β-galactosidase assay was performed by pressing filter papers on the plates with yeast colonies. The filters were frozen in liquid nitrogen and subsequently incubated at 30 °C in a solution containing 0.1% X-gal.

**In vitro phosphatase assays.** In vitro phosphatase activity of recombinant proteins was assayed using the Serine/Threonine Phosphatase Assay System (Promega). The reaction was started by the addition of the substrate (a substrate in a final volume of 50 μl in half-area, flat-bottom 96 wells plate. Assays were performed as follows: 0.5 μM of purified recombinant 6xHis-MBP-AHG1 or 6xHis-N88AHG3 were incubated for 20 min at room temperature in 50 mM HEPES buffer pH 7.5; 10 mM MgCl₂, in the absence or presence of purified recombinant MBP-PP2C fusion (starting at amino acid position N88). After 10 min of reaction time the activities were stopped by addition of 50 μl of molybdate dye solution. Dye was developed for 30 min and absorbance was read at 330 nm on a multi-scan spectrometer. Urea concentration was adjusted to 2 M by addition of 4.5 M Tris-HCl pH 8.0, 1 mM CaCl₂, Tryptsin digestion (1:100 enzyme-to-substrate ratio). Western blotting. Detection of histone H3 was performed using anti-H3 antibody produced in rabbit (Abcam ab9110) and MYC tag antibody raised in mouse (MA1 980 ThermoScientific) at a dilution of 1:1,000. Detection of C-terminal HA-αYFP and MYC-N/αFusion protein in tobacco leaf extracts was performed using HA tag antibody produced in rabbit (Abcam ab9110) and MYC tag antibody raised in mouse (MA1 980 ThermoScientific) at a dilution of 1:10,000.

**Protein gel and immuno-detection methods.** Proteins (10 μg of total extract unless otherwise stated in the figures) were separated using NuPAGE® Bis-Tris gels (ThermoFischer) and MES buffer. Protein gels were stained using either Oriole™ fluorescent gel stain (Biorad), or colloidal Coomassie Brilliant Blue. For Western blot, proteins were transferred on immobilation-P PVDF membrane (Milipore) using semidry transfer. After transfer proteins were stained with Ponceau S before blocking with 3% BSA. Detection of YFP-tagged DOG1 in seed extracts was performed using anti-GFP (7.1 and 13.1 mixture) monoclonal antibody produced in mouse (Roche #11814460010) at a dilution of 1:1,000. Detection of C-terminal HA-αYFP and MYC-N/αFusion protein in tobacco leaf extracts was performed using HA tag antibody produced in rabbit (Abcam ab1791) at a dilution of 1:12,000. Horse radish peroxidase conjugated secondary antibodies were goat anti-mouse IgG (Sigma A3562) or goat anti-Rabbit IgG (Sigma A0545) both at a dilution of 1/1,500. Amersham ECL™ Plus Western Blotting Detection Reagents (GE Healthcare, U.K.) were used for detection of the activity. Images were recorded using Chemidoc X MP imager (Biorad). Full scan images are available in Supplementary Fig. 9.

**YFP-DOG1 pull-down procedure.** Native seed protein extracts from 5 and 29 weeks stored Cdol1#4 and dog1-1 dry and 24 h imbedded seed were used for the pull-down assays. Pull-downs were performed from three independent biological replicates each consisting of a mix of seeds from three different plants. For each pull-down, 25 μl of agarose beads coupled to a GFP antibody (GFP-Trap_A Chromotech gta-10) corresponding to 10 μg GFP-binding capacity was equilibrated extensively in native extraction buffer. For each replicate, an adjusted quantity of 4 mg of total protein (in 1 ml final volume) was incubated with the beads under constant rotation for 2 h at 4 °C. After incubation, beads were separated from the native extract by centrifugation and washed three times with native extraction buffer without Triton X-100, DNase 1 and RNase A. Bound proteins were eluted by incubation for 5 min with 0.1% (v/v) trifluoroacetic acid. Elutions were recovered from the beads by centrifugation and immediately neutralized by an equal volume of neutralization buffer (1 M Tris-HCl pH 8.0). All steps of the enrichment procedure were monitored by SDS-PAGE analysis and western blotting (Supplementary Fig. 1b).

**Mass spectrometry sample preparation and analysis.** Eluted proteins for pull-down assays were reduced, alkylated and digested in solution. Cysteines were reduced by adding DTT to a final concentration of 5 mM and incubation for 30 min. Subsequently, alkylation was performed by adding chloroacetamide to a final concentration of 14 mM and incubation for 30 min. The reaction was quenched by addition of DTT. Urea concentration was adjusted to 2 M by dilution with 0.1 M Tris-HCl pH 8.0, 1 mM CaCl₂. Tryptsin digestion (1:100 enzyme-to-substrate ratio).
protein ratio) was performed over night at 37 °C and stopped by addition of 1% formic acid. Peptides were desalted with StageTips43 (Empore C18, 3 M), dried and directly subjected to MS measurement.

**Mass spectrometry data processing.** Raw data were processed using MaxQuant software (version 1.5.1.2, http://www.maxquant.org/) with label-free quantification (LFQ) enabled44. Tandem mass spectrometry spectra were searched by the Andromeda search engine against the Arabidopsis TAIR10_pep_201001214 database (ftp://ftp.arabidopsis.org/home/tair/Proteins/TAIR10_protein_list/). In addition, the protein sequences of the three putative splicing variants46 of the YFP-DOG1 (Cvi allele) fusion proteins were added to avoid bias due to DOG1 polymorphisms between Cvi and Col. Sequences of 248 common contaminant proteins and decoy sequences were automatically added during the search. Trypsin specificity was required and a maximum of two missed cleavages was allowed. The minimal peptide length was set to seven amino acids. Carbamidomethylation of cysteine residues was set as a fixed modification. iTRAQ and TMT modifications were set as variable modifications. De novo search was performed using the software (version 1.5.1.2, http://www.maxquant.org/) with label-free quantification. Two replicates of the YFP-DOG1 fusion proteins were added to avoid bias due to DOG1 protein phosphorylation induced by the plant hormone abscisic acid. Proc. Natl Acad. Sci. USA 107, 15986–15991 (2010).

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**Data availability.** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository48 with the data set identifier PXD006347. The authors declare that all other data supporting the findings of this study are available within the manuscript and its Supplementary Information files or are available from the corresponding author upon request.

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Author contributions
G.N., I.F. and W.J.J.S. planned this study. G.N., K.N., B.Y., Y.X., E.M. and W.J.J.S. carried out the experimental work. K.K. performed MS measurements. G.N., K.K. and I.F. performed MS data analysis. G.N. and W.J.J.S. wrote the paper.

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