

# **REDUCED DORMANCY5 Encodes a Protein Phosphatase 2C That Is Required for Seed Dormancy in *Arabidopsis***

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Seed dormancy determines germination timing and contributes to crop production and the adaptation of natural populations to their environment. Our knowledge about its regulation is limited. In a mutagenesis screen of a highly dormant *Arabidopsis thaliana* line, the *reduced dormancy5* (*rdo5*) mutant was isolated based on its strongly reduced seed dormancy. Cloning of *RDO5* showed that it encodes a PP2C phosphatase. Several PP2C phosphatases belonging to clade A are involved in abscisic acid signaling and control seed dormancy. However, *RDO5* does not cluster with clade A phosphatases, and abscisic acid levels and sensitivity are unaltered in the *rdo5* mutant. *RDO5* transcript could only be detected in seeds and was most abundant in dry seeds. *RDO5* was found in cells throughout the embryo and is located in the nucleus. A transcriptome analysis revealed that several genes belonging to the conserved PUF family of RNA binding proteins, in particular *Arabidopsis PUMILIO9* (*APUM9*) and *APUM11*, showed strongly enhanced transcript levels in *rdo5* during seed imbibition. Further transgenic analyses indicated that *APUM9* reduces seed dormancy. Interestingly, reduction of *APUM* transcripts by RNA interference complemented the reduced dormancy phenotype of *rdo5*, indicating that *RDO5* functions by suppressing *APUM* transcript levels.

## **INTRODUCTION**

The moment when a seed germinates is a crucial decision during the life cycle of plants because it determines all subsequent events. Seed dormancy is defined as the failure of a viable seed to germinate under favorable conditions (Bewley, 1997) and plays an adaptive role in nature by optimizing germination at the most suitable time. Seed dormancy is induced during maturation of seeds on the mother plant and released by dry storage (after-ripening) or imbibition at low temperatures. A tight control of dormancy is important in many crop species, including rapeseed (*Brassica napus*), wheat (*Triticum* spp), barley (*Hordeum vulgare*), and rice (*Oryza sativa*). Low levels of dormancy can cause pre-harvest spouting, which can cause economic losses to cereal production including reductions in seed quantity and quality. On the other hand, low seed dormancy is required for uniform and fast germination after sowing (Gubler et al., 2005; Holdsworth et al., 2008; Graeber et al., 2012). An improved control of seed dormancy and germination requires understanding of its molecular mechanisms.

Several genes regulating dormancy have been identified during the last decades. Many of these are involved in the biosynthesis and signaling of hormones among which abscisic acid (ABA)


plays a major role (Gubler et al., 2005; Holdsworth et al., 2008; Graeber et al., 2012). Mutant analyses of ABA biosynthesis and metabolism genes showed the essential role of ABA in the induction and maintenance of seed dormancy. Seeds of ABA-deficient mutants have lower ABA and dormancy levels than those of the wild type (Koorneef et al., 1982; Lefebvre et al., 2006). By contrast, seeds of ABA catabolic pathway mutants accumulate higher ABA levels than those of the wild type and exhibit enhanced dormancy levels (Millar et al., 2006; Okamoto et al., 2006). Genes involved in the ABA signal transduction pathway also influence seed dormancy (Colucci et al., 2002; Shen et al., 2006; Raghavendra et al., 2010). In particular, members of the Protein Phosphatase 2C (PP2C) family were identified as components of ABA signaling from work with the *aba insensitive 1-1* (*abi1-1*) and *abi2-1* mutants (Koorneef et al., 1984; Rodriguez et al., 1998). At least four *Arabidopsis thaliana* PP2Cs, including ABI1, ABI2, ABA HYPERSENSITIVE GERMINATION1 (AHG1), and HIGHLY ABA-INDUCED PP2C GENE2 (HAI2), are key regulators of ABA signaling and function as negative regulators of seed dormancy (Rodriguez et al., 1998; Gosti et al., 1999; Nishimura et al., 2007; Kim et al., 2013). Members of the PYR/PYL/RCAR family of ABA receptors bind and inactivate these PP2C proteins in an ABA-dependent manner, allowing SNF1-related protein kinase subfamily 2 (SnRK2) protein kinases to phosphorylate downstream substrates, thereby acting as positive regulators of seed dormancy (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Soon et al., 2012).

Besides ABA, gibberellins (GAs), ethylene, strigolactone, and brassinosteroid also influence seed dormancy and germination (Steber and McCourt, 2001; Gubler et al., 2005; Finkelstein et al., 2008; Nelson et al., 2011; Graeber et al., 2012). In general, these hormones reduce seed dormancy and promote seed germination. For example, mutants severely defective in GA biosynthesis such as *ga requiring1* show deep seed dormancy and fail to germinate


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in the absence of exogenous GA (Debeaujon and Koornneef, 2000; Ogawa et al., 2003). Ethylene can regulate dormancy by affecting ABA levels and signal transduction (Linkies et al., 2009).

Apart from plant hormones, several chromatin factors have recently been shown to be involved in the regulation of seed dormancy. HISTONE MONOUBIQUITINATION1 and REDUCED DORMANCY2 have been identified in a screen for reduced dormancy mutants (Peeters et al., 2002). Both proteins probably influence seed dormancy by regulating transcription elongation of seed dormancy genes during maturation (Liu et al., 2007, 2011). Two other chromatin factors, SIN3-LIKE1 (SNL1) and SNL2, positively regulate seed dormancy by modifying the ABA-ethylene antagonism through histone acetylation (Z. Wang et al., 2013). A role for histone acetylation in seed dormancy was recently also demonstrated by the reduced dormancy phenotype of the *histone deacetylase9* mutant (van Zanten et al., 2014).

Finally, modifications of proteins and transcripts influence dormancy and germination. Oxidative processes affecting both proteins and transcripts during the storage of dry seeds have been shown to influence the release of dormancy (Oracz et al., 2007; Bazin et al., 2011; Gao et al., 2013). Posttranslational modifications during seed imbibition, such as oxidation, phosphorylation/dephosphorylation, ubiquitination, and acetylation, affect protein function and are probably involved in the control of germination (Le et al., 1998; Buchanan and Balmer, 2005; Lu et al., 2008; Arc et al., 2011).

The above-mentioned regulators are not specific for dormancy but also influence other plant traits. By contrast, *DELAY OF GERMINATION1* (*DOG1*) has been identified as a dormancy-specific gene in *Arabidopsis* (Bentsink et al., 2006). *DOG1* encodes a protein with unknown function and is part of a small gene family. *DOG1* homologs have been found in both monocots and dicots and for several of them a conserved function in seed dormancy has been demonstrated (Ashikawa et al., 2013; Graeber et al., 2014). The abundance of *DOG1* in freshly harvested seeds highly correlates with dormancy levels. *DOG1* becomes modified during seed storage, leading to its reduced functionality (Nakabayashi et al., 2012).

Most lab accessions of *Arabidopsis* have low seed dormancy levels, decreasing the efficiency of mutagenesis screens for reduced dormancy. Here, we describe the isolation of the *reduced dormancy5* (*rdo5*) mutant in a screen of a highly dormant *Arabidopsis* genotype containing a strong *DOG1* allele. *RDO5* encodes a PP2C protein that positively regulates seed dormancy without influencing ABA and *DOG1* levels. The *rdo5* mutant phenotype requires expression of the *Arabidopsis* *PUMILIO9* (*APUM9*) and *APUM11* genes that encode RNA binding proteins. *RDO5* represents a novel dormancy pathway that probably regulates seed dormancy at the posttranscriptional level.

## RESULTS

### *RDO5* Is Required for Seed Dormancy

To identify genes with a role in seed dormancy, the highly dormant near isogenic line NIL-*DOG1* was mutagenized by  $\gamma$ -irradiation. The genotype of NIL-*DOG1* consists of the *Arabidopsis* accession

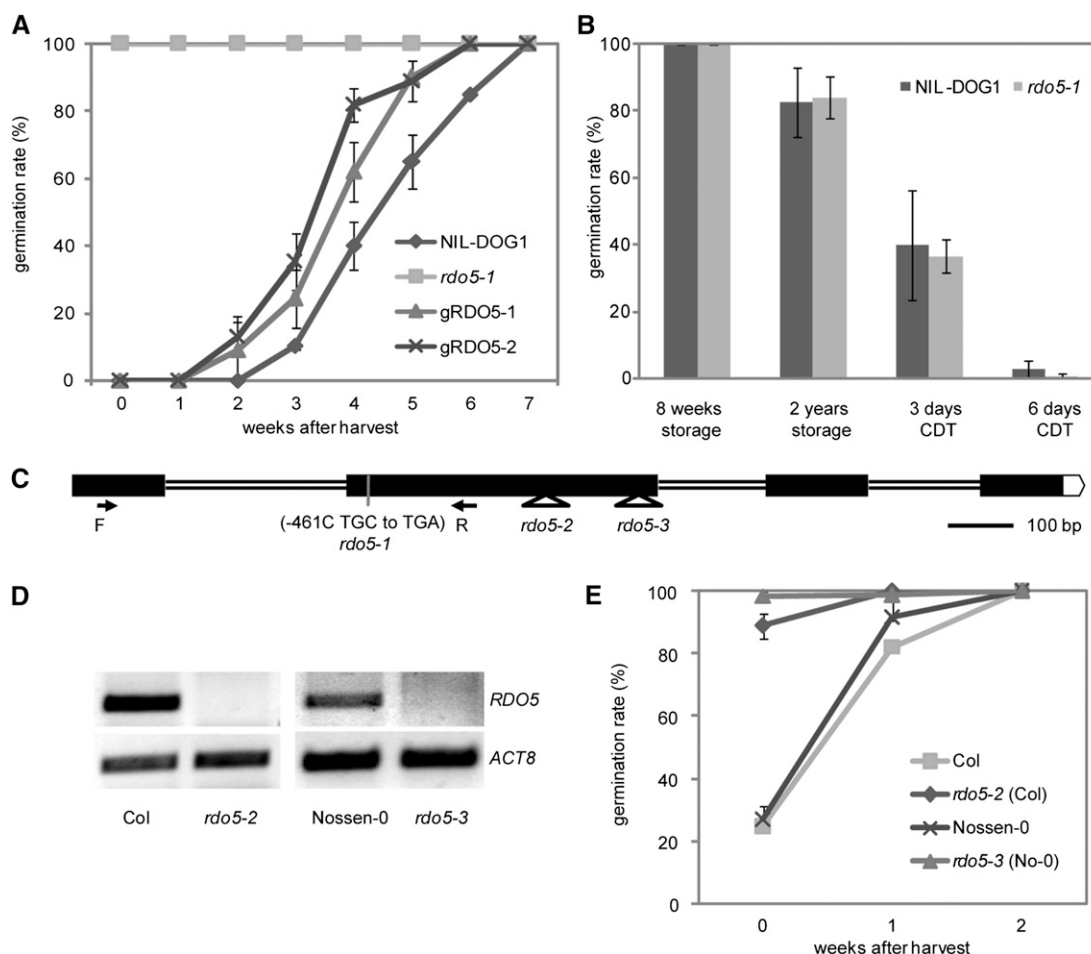
*Landsberg erecta* (*Ler*) containing a 4.5-Mb introgression from the Cape Verde Islands (*Cvi*) accession in chromosome 5, including a strong *DOG1* allele (Alonso-Blanco et al., 2003). *DOG1* has been identified as a major regulator of seed dormancy in *Arabidopsis* (Bentsink et al., 2006). Several mutants with abolished or reduced seed dormancy were isolated in this mutagenesis screen, among them a nondormant mutant that was later identified as *dog1-1* (Bentsink et al., 2006). Another isolated mutant with strongly reduced seed dormancy was named *rdo5*. We analyzed the germination of seeds from homozygous *rdo5* mutant plants in detail and confirmed their reduced dormancy phenotype (Figure 1A). Besides the absence of dormancy, the *dog1* mutant is also characterized by a reduction in seed longevity (Bentsink et al., 2006). Analysis of the germination of *rdo5* seeds after controlled deterioration or long-term storage showed a similar seed longevity as wild-type seeds (Figure 1B). In addition, *rdo5* mutant plants did not show any other obvious pleiotropic phenotypes (Supplemental Figure 1), suggesting that the function of *RDO5* is specific for seed dormancy.

### *RDO5* Encodes a PP2C Phosphatase

To identify the *RDO5* gene, we followed a map-based cloning approach. The *rdo5* mutant was crossed with the Columbia (*Col*) accession to produce a mapping population. Plants containing the *Cvi* *DOG1* allele were selected in the F2 progeny of this cross and separated in a dormant and a nondormant group according to their germination phenotype. Hybridization of the bulk DNA of these two groups to the *Arabidopsis* SNP Genotyping and Tiling Array showed that *RDO5* is localized on chromosome 4 (Supplemental Figure 2A). Further fine mapping narrowed down the location of *RDO5* to a 17-kb region corresponding to the BAC clone T22B4 containing five predicted open reading frames. We sequenced this region in wild-type NIL-*DOG1* and the *rdo5* mutant and detected a single base pair deletion in the *rdo5* ORF4, corresponding to the gene At4g11040. This mutation was located in the second exon of *RDO5* and resulted in a stop codon (Supplemental Figure 2B). At4g11040 encodes a member of the PP2C gene family in *Arabidopsis*. This family consists of 10 clades named A to J. Most of the proteins belonging to clade A are involved in ABA signaling and seed dormancy regulation. However, *RDO5* has a unique composition and could not be clustered within one of the clades (Schweighofer et al., 2004).

To confirm that At4g11040 is *RDO5*, we complemented the *rdo5* mutant with a 6-kb *Ler* genomic fragment including a 2.7-kb promoter sequence, the At4g11040 coding sequence, and a 0.9-kb sequence after the stop codon. T3 seeds from two independent homozygous single insertion lines showed similar dormancy levels as seeds from wild-type NIL-*DOG1* plants, indicating that the 6-kb fragment is sufficient to complement the reduced dormancy phenotype of the *rdo5* mutant (Figure 1A).

We named our original mutant allele *rdo5-1* and obtained additional insertion mutant alleles for *RDO5* in the *Col* and Nossen-0 background that were named *rdo5-2* and *rdo5-3*, respectively. Both insertions are located in the second exon of *RDO5* as shown in Figure 1C. RT-PCR analysis, using *RDO5* gene-specific primers, showed that *RDO5* transcript could not be detected in the two insertion mutants (Figure 1D). Similar to



**Figure 1.** The *rdo5* Mutant Shows Reduced Dormancy.

**(A)** Germination after different periods of dry storage of *rdo5-1*, its wild-type background NIL-DOG1, and two complementation lines containing a *pRDO5:RDO5* insertion in the *rdo5-1* background. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

**(B)** Seed longevity phenotype of *rdo5-1* and NIL-DOG1 after long time storage and controlled deterioration (CDT). Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

**(C)** Schematic diagram of the *RDO5* gene structure including the position of the *rdo5-1* mutation and the two T-DNA insertions. Exons are shown as black boxes and introns as lines. The positions of the primers used for RT-PCR analysis are indicated below the structure (arrows).

**(D)** RT-PCR analysis of the *RDO5* transcripts in freshly harvested seeds of wild-type and T-DNA insertion mutants. *ACTIN8* (*ACT8*) was used as a loading control.

**(E)** Germination after different periods of dry storage of the T-DNA insertion lines *rdo5-2* and *rdo5-3* and their respective wild-type backgrounds Col and Nossen. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

the *rdo5-1* allele, *rdo5-2* and *rdo5-3* showed strongly reduced seed dormancy levels compared with their corresponding wild-type Col and Nossen-0 (Figure 1E).

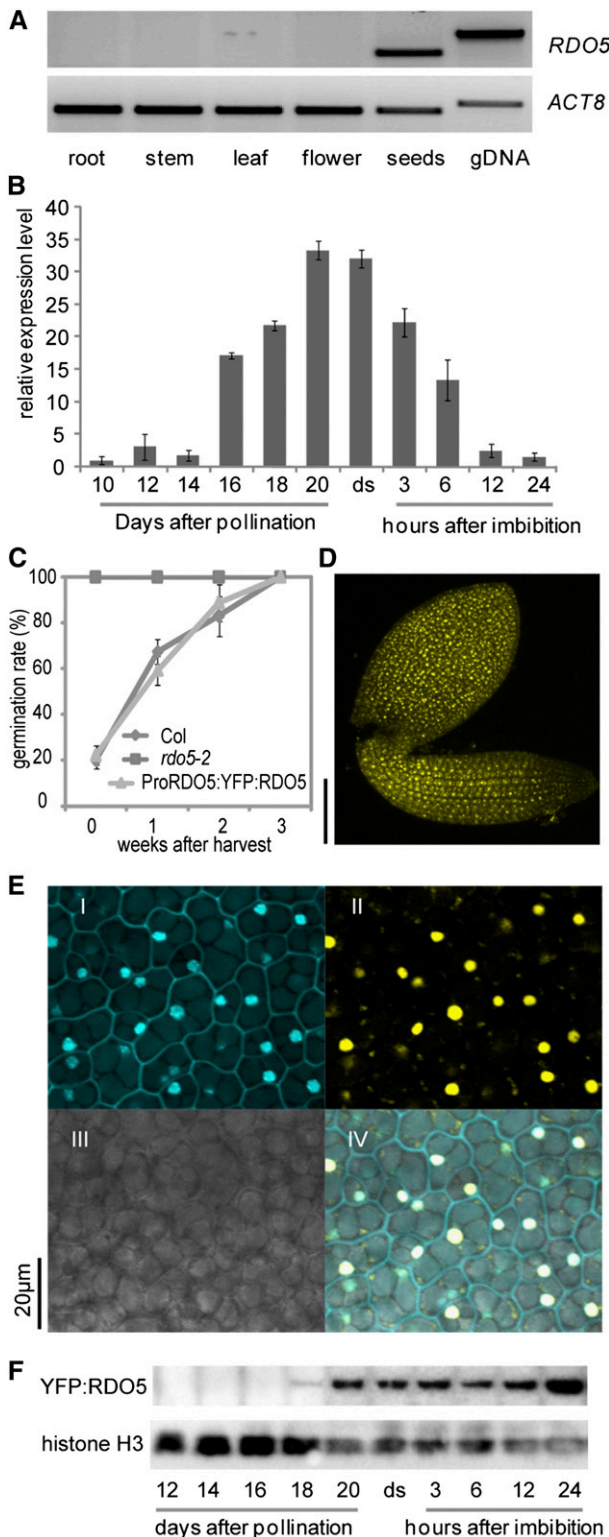
#### **RDO5 Is a Seed-Specific Protein That Is Located in the Nucleus**

The identification of *RDO5* as a dormancy gene suggests that its transcript and protein can be found in seeds. Public data about *RDO5* transcript levels are not available because this gene is not represented on the ATH1 microarray. RT-PCR analysis confirmed the presence of *RDO5* transcript in dry seeds, but it could not be detected in root, stem, leaf, or flower tissues (Figure 2A).

The seed-specific transcript pattern of *RDO5* is consistent with the location of its protein, which was only detected in seeds in a genome-scale proteomics analysis (<http://fgcz-atproteome.unizh.ch/>; Baerenfaller et al., 2008).

The temporal pattern of *RDO5* transcript levels in seeds was analyzed in detail by quantitative RT-PCR (qRT-PCR). *RDO5* transcript levels strongly increased during seed maturation from ~16 d after pollination and reached a maximum in freshly harvested dry seeds. The transcript levels quickly decreased during imbibition of seeds and became very low after 24 h (Figure 2B).

The abundance and localization of the *RDO5* protein was studied using transgenic plants in an *rdo5-2* mutant background containing the *RDO5* cDNA fragment with an N-terminal yellow



**Figure 2.** *RDO5* Transcripts Are Seed-Specific and Its Encoded Protein Is Localized in the Nucleus.

**(A)** RT-PCR analysis of *RDO5* transcript levels in various tissues. *ACT8* was used as a loading control.

fluorescent protein (YFP) tag driven by the *RDO5* native promoter. One homozygous single insertion line that complemented the *rdo5* reduced dormancy phenotype was selected (Figure 2C). The localization of RDO5 in freshly harvested seeds was studied by detection of the YFP:RDO5 protein with the confocal microscope. YFP fluorescence could be observed in the entire embryo and was confined to nuclei, indicating that RDO5 is a nuclear protein (Figures 2D and 2E). An immunoblot analysis showed that RDO5 protein started to accumulate at 16 d after pollination until the end of seed maturation, which is similar to the dynamics of *RDO5* transcript. In contrast to its transcript, protein levels slightly increased during imbibition, indicating that the RDO5 protein is very stable (Figure 2F).

### *RDO5* Expression Correlates with Seed Dormancy Levels

The phenotype of the *rdo5* mutant alleles showed that the absence of RDO5 leads to strongly reduced seed dormancy. This raised the question of whether an increase in RDO5 could enhance dormancy. We constructed transgenic Ler plants containing the *RDO5* genomic fragment with a C-terminal tandem affinity purification (TAP) tag driven by the 35S promoter. Three independent single insertion lines (*RDO5OE-6*, *RDO5OE-10*, and *RDO5OE-12*) were obtained that accumulated varying amounts of RDO5:TAP in their seeds (Figure 3A). All three lines showed enhanced seed dormancy levels that correlated with the amount of RDO5:TAP protein that they accumulated (Figure 3B).

The influence of RDO5 on seed dormancy was analyzed in more detail by temporal induction of *RDO5* expression. For this purpose, we created transgenic *rdo5-2* plants containing a single insertion of a construct consisting of the *RDO5* cDNA fused with a C-terminal HA tag, driven by a heat shock promoter. Plants were grown at 16/14°C to reduce background activity of the heat shock promoter. *RDO5* expression was induced at different times during seed maturation by applying a 3-h heat shock at 37°C for three consecutive days. An immunoblot analysis that was performed directly after the treatment showed high accumulation of RDO5 protein at all silique stages (Figure 3C). These high amounts of RDO5 protein were not stable, and freshly harvested seeds only showed high levels of RDO5 protein when this was

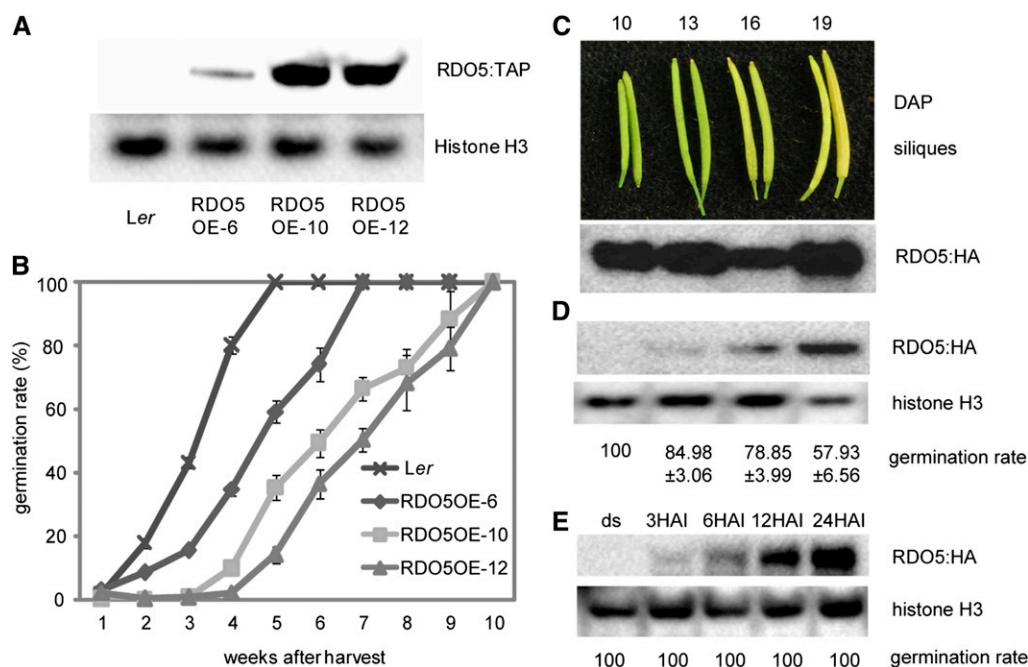
**(B)** qRT-PCR analysis of *RDO5* transcript levels during seed maturation and imbibition. The expression values were normalized using *ACT8* as control.  $n = 3$  biological replicates; error bars represent  $\pm$  SE.

**(C)** Germination after different periods of dry storage of wild-type Col, *rdo5-2*, and a transgenic *rdo5* line containing the *ProRDO5:YFP:RDO5* construct. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

**(D)** Localization of YFP:RDO5 in an embryo of a freshly harvested seed from a transgenic *rdo5* plant containing the *ProRDO5:YFP:RDO5* construct. Bar = 100  $\mu$ m.

**(E)** Subcellular localization of YFP:RDO5 in embryo cells from a transgenic *rdo5-2* seed containing the *ProRDO5:YFP:RDO5* construct. I to IV show 4',6-diamidino-2-phenylindole signal, YFP signal, transmission, and merged images, respectively. Bar = 20  $\mu$ m.

**(F)** Immunoblot analysis of YFP:RDO5 protein accumulation in the *rdo5-2* complementation line during seed maturation and imbibition. Histone H3 was used as loading control. ds, freshly harvested dry seeds.



**Figure 3.** Constitutive Expression of *RDO5* During Seed Maturation Enhances Seed Dormancy.

(A) Immunoblot analysis of *RDO5:TAP* protein accumulation in seeds of three independent *RDO5:TAP* overexpression transgenic lines.

(B) Germination after different periods of dry storage of *RDO5:TAP* overexpression transgenic lines and wild-type *Ler*. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

(C) Immunoblot analysis of *RDO5:HA* protein accumulation of transgenic *rdo5-2* lines, containing *RDO5* driven by the heat shock promoter (*ProHS*). Protein was isolated from siliques after they had received 3 h of heat induction (37°C) during three subsequent days that started at 10, 13, 16, or 19 d after pollination. The top panel shows representative siliques of the four stages.

(D) Immunoblot analysis of *RDO5:HA* protein in freshly harvested seeds from siliques that had been heat treated at different stages after pollination as indicated in (C). The germination percentage (averages  $\pm$  SE) of seeds from six independent batches for each genotype is shown at the bottom.

(E) Immunoblot analysis of *RDO5:HA* protein in after-ripened seeds after 6 h of heat induction that was given at 3, 6, 12, and 24 h after imbibition. The germination percentage (averages  $\pm$  SE) of seeds from six independent batches for each genotype is shown at the bottom.

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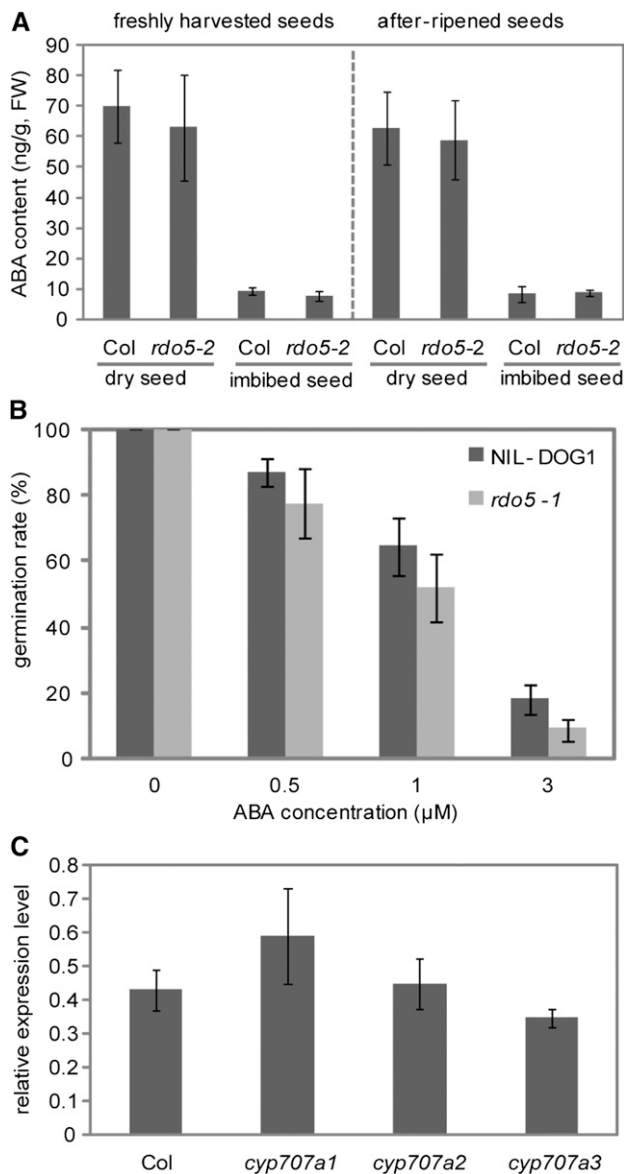
induced at the later stages of seed maturation (Figure 3D). The germination rate of seeds harvested from these siliques showed an inverse correlation with the abundance of *RDO5* protein, indicating that high levels of *RDO5* induced at the end of seed maturation prevent germination (Figure 3D). By contrast, induction of *RDO5* in after-ripened seeds during imbibition could not prevent germination of these seeds (Figure 3E). These results suggest that the *RDO5* protein is functional at the later stages of seed maturation and in dry seeds, which is consistent with the temporal pattern of *RDO5* protein accumulation in wild-type seeds (Figure 2C). The presence of *RDO5* protein at the early stages of seed maturation or during seed imbibition does not prevent germination (Figures 3C to 3E).

#### ***RDO5* Does Not Influence ABA and DOG1 Levels**

The plant hormone ABA and the dormancy protein DOG1 have been identified as major factors for the induction of seed dormancy in *Arabidopsis* (Graeber et al., 2012). We analyzed whether the reduced seed dormancy phenotype of the *rdo5-1* mutant is associated with changes in ABA or DOG1.

The ABA levels in *rdo5-2* are very similar to those of wild-type Col in freshly harvested and after-ripened seeds, as well as in dry and imbibed seeds (Figure 4A). Reduced dormancy can also be associated with decreased sensitivity to ABA. However, Figure 4B shows that the ABA sensitivity of *rdo5-1* seeds is not significantly different from wild-type NIL-DOG1 seeds. In addition, we analyzed whether enhanced ABA levels in seeds can influence *RDO5* transcript levels by studying the *cyp707a1*, *cyp707a2*, and *cyp707a3* mutants. CYP707A1, 2, and 3 are ABA 8'-hydroxylases with a key role in ABA catabolism. Mutations in these genes cause enhanced ABA levels and dormancy phenotypes in different degrees (Okamoto et al., 2006). *RDO5* transcript levels in freshly harvested seeds of these mutants did not significantly differ from those of the wild type (Figure 4C). Taken together, our results indicated that *RDO5* and ABA control seed dormancy independently.

We previously showed that the levels of *DOG1* transcript and protein in freshly harvested seeds are highly correlated with dormancy (Nakabayashi et al., 2012). This correlation is lost in the *rdo5-1* mutant, which has very low dormancy levels but high *DOG1* transcript and protein levels similar to those of wild-type



**Figure 4.** RDO5 and ABA Do Not Influence Each Other's Levels.

**(A)** ABA contents in dry and 36-h imbibed wild-type Col and *rdo5-2* seeds that were either freshly harvested or after-ripened. Shown are averages  $\pm$  SE calculated from three biological repeats.

**(B)** Germination rate of after-ripened wild-type NIL-DOG1 and *rdo5-1* seeds sown on wet filter paper containing different concentrations of ABA. Shown are averages  $\pm$  SE of more than eight independent batches of seeds for each genotype.

**(C)** qRT-PCR analysis of *RDO5* transcript levels in freshly harvested seeds of wild-type Col and the *cyp707a1-a3* mutants. The expression values were normalized using *ACT8* as control.  $n = 3$  biological replicates; error bars represent SE.

NIL-DOG1 (Figures 5A and 5B). This suggests that RDO5 does not regulate DOG1 levels, but DOG1 requires RDO5 to induce seed dormancy. DOG1 does not influence *RDO5* transcript levels because they are similar in the *dog1-1* mutant and wild-type NIL-DOG1 seeds (Figure 5C). The relation between DOG1

and RDO5 was further studied by analysis of the *rdo5-2* and *dog1-2* single and double mutants. As expected, these mutants are all nondormant. Interestingly, a detailed analysis showed that the *dog1-2* mutant germinates faster than the *rdo5-2* mutant (Figure 5D). Faster germination is usually correlated with reduced dormancy, indicating that *dog1* has a stronger influence on seed dormancy than *rdo5*. The double mutant *rdo5-2 dog1-2* showed a similar germination speed as the single *dog1-2* mutant, indicating that the high germination speed of *dog1-2* cannot be further enhanced by the *rdo5-2* mutation.

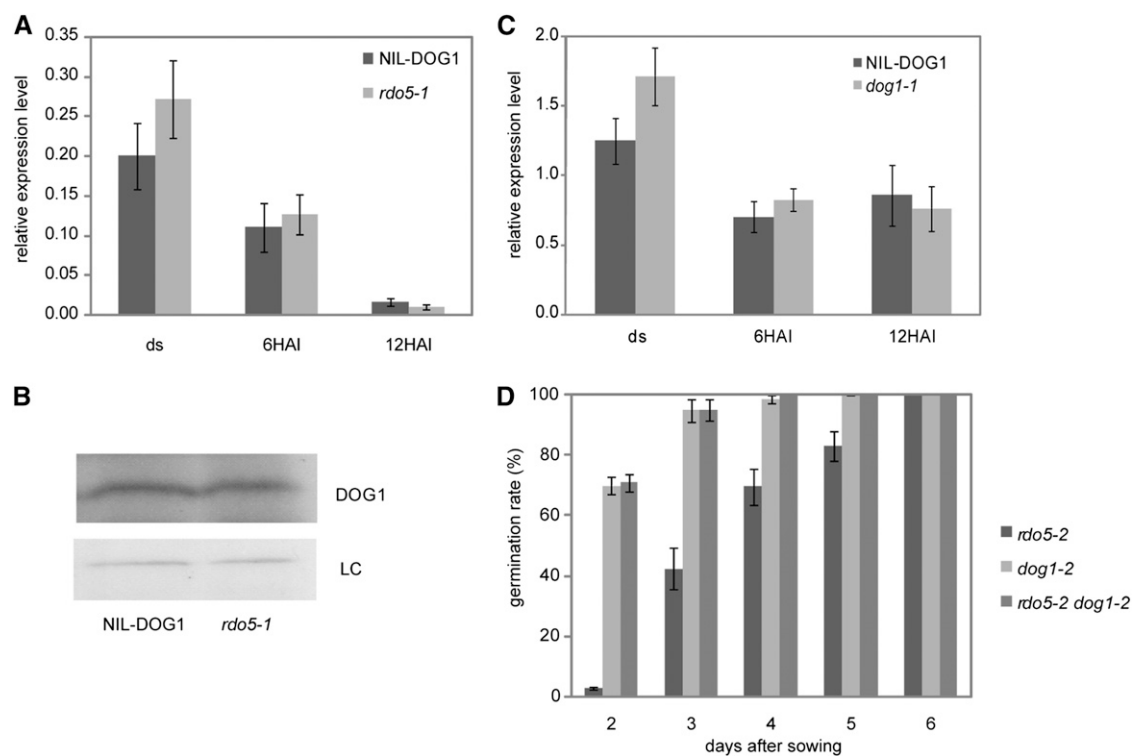
### RDO5 Influences the Transcriptome during Seed Imbibition

We identified RDO5 as a phosphatase that promotes seed dormancy, most likely independent from ABA and DOG1. To obtain an indication about the genetic and molecular pathways by which RDO5 functions, we performed a transcriptomic comparison between *rdo5-1* and its wild-type NIL-DOG1 at different seed stages, using the *Arabidopsis* Affymetrix AGRO-NOMICS1 tiling microarrays (Rehauer et al., 2010). Relatively few genes showed differential transcript levels between *rdo5-1* and NIL-DOG1 in siliques at 14 d after pollination and in freshly harvested dry seeds. However, 123 genes are upregulated in *rdo5-1* seeds after 6 h of imbibition (Supplemental Data Set 1). None of these genes are known to be involved in dormancy or ABA signaling. Interestingly, three genes belonging to the same family of RNA binding proteins, *APUM9*, *APUM11*, and *APUM19*, were upregulated in *rdo5-1* seeds that undergone 6 h of imbibition. *APUM9* showed the greatest increase in expression of all upregulated genes and expression was 43 times higher in *rdo5-1* than in wild-type NIL-DOG1. *APUM9* was also upregulated at 14 d after pollination and *APUM11* was upregulated under all three conditions. A qRT-PCR analysis confirmed the microarray data and showed that *APUM9* and *APUM11* transcript levels are greatly increased in *rdo5-1* compared with the wild type during early imbibition (Figure 6A). A similar, but less dramatic, upregulation of *APUM9* and *APUM11* was observed in the *rdo5-2* mutant (Supplemental Figure 3). The smaller increase in transcript levels in *rdo5-2* compared with *rdo5-1* could be related to the dormancy level of their background genotypes, which is much higher in *rdo5-1* (NIL-DOG1) than in *rdo5-2* (Col) (Figures 1A and 1E). The highly enhanced transcript levels in the *rdo5* mutant specifically for *APUM9* and *APUM11* suggest a role for these genes in the mechanism by which RDO5 controls seed dormancy.

### APUM Genes Are Involved in Seed Dormancy

*APUM9*, *APUM11*, and *APUM19* are predicted to encode PUF proteins, which are characterized by the presence of a conserved Pumilio homology domain (PUM-HD) that binds to RNA with sequence specificity (Francischini and Quaggio, 2009; Tam et al., 2010). These proteins have been well characterized in animal and fungal systems, but little is known about their function in plants. The *Arabidopsis* PUF family has 25 members, among which *APUM9* and *APUM11* cluster together. The *APUM9* and *APUM11* proteins have 47% identity. *APUM9* has





**Figure 5.** RDO5 and DOG1 Do Not Influence Each Other's Levels.

**(A)** qRT-PCR analysis of *DOG1* transcript levels in freshly harvested and imbibed seeds of NIL-DOG1 and *rdo5-1*. The expression values were normalized using *ACT8* as control.  $n = 3$  biological replicates; error bars represent SE.

**(B)** Immunoblot analysis of DOG1 protein in NIL-DOG1 and *rdo5-1* freshly harvested seeds. DOG1 was detected using DOG1-specific antibody. A nonspecific band of around 60 kD is used as loading control (LC).

**(C)** qRT-PCR analysis of *RDO5* transcript levels in freshly harvested and imbibed seeds of NIL-DOG1 and *dog1-1*. The expression values were normalized using *ACT8* as control.  $n = 3$  biological replicates; error bars represent SE.

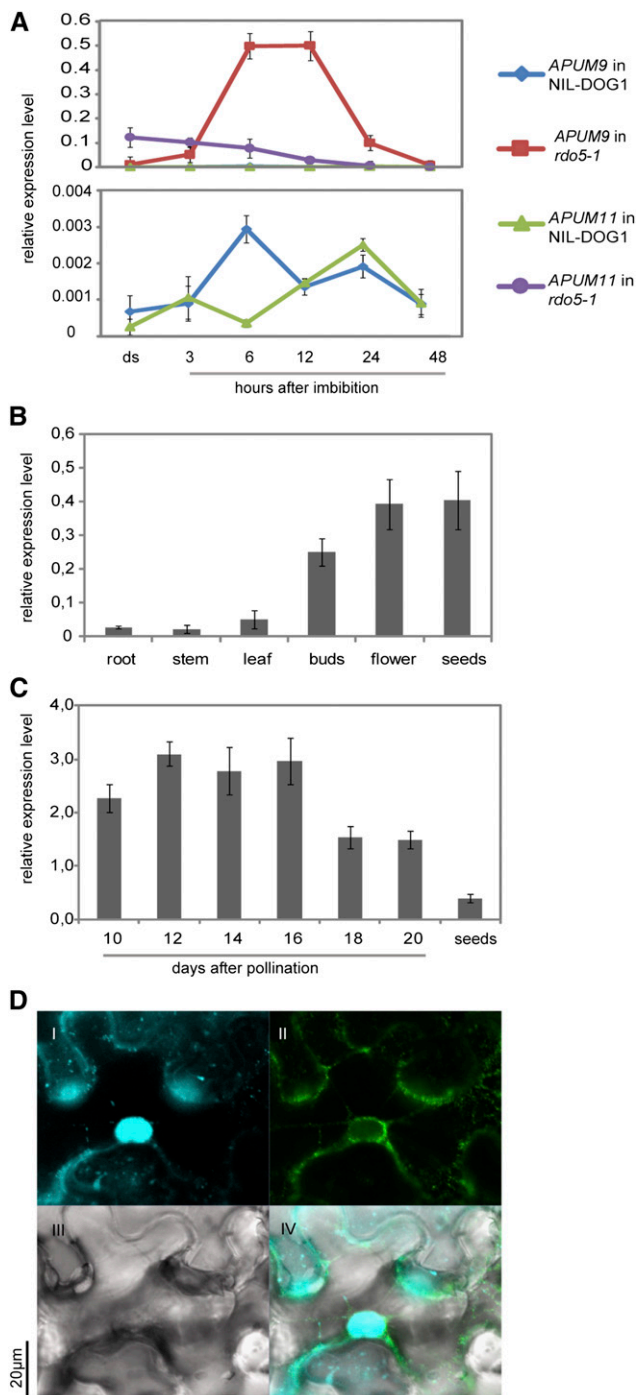
**(D)** Germination percentages of freshly harvested seeds from *rdo5-2*, *dog1-2*, and *rdo5-2 dog1-2* at 2 to 6 d after sowing. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

a tandem duplicate gene, *APUM10*, which has 74% identity at the protein level (Tam et al., 2010; Supplemental Figure 4). *APUM9*, *APUM10*, and *APUM11* are expressed at a low level in all tissues (Supplemental Figure 5). A detailed qRT-PCR analysis showed that the highest transcript levels of *APUM9* can be found in reproductive tissues (Figure 6B). *APUM9* transcript levels are especially high during the first half of seed maturation, after which they slowly reduce (Figure 6C). This transcription pattern during seed maturation is opposite to that of *RDO5*. Transient expression of *GFP:APUM9* in *Nicotiana benthamiana* leaves showed localization of the GFP:APUM9 protein in the cytoplasm, especially at the nuclear periphery (Figure 6D). *RDO5* was found in the nucleus (Figure 2E), indicating that it is not colocalized with *APUM9*. Therefore, *RDO5* probably regulates *APUM9* indirectly.

Insertion mutants of *APUM9*, *APUM10*, and *APUM11* were obtained from the NASC seed stock center. The *apum9-1* mutant contains an insertion in the third exon and full-length *APUM9* transcript could not be detected by RT-PCR (Supplemental Figures 6A and 6B). The *apum10-1* and *apum11-1* mutants contain insertions in the promoter regions of *APUM10* and

*APUM11*, which do not cause a decrease in their transcript levels compared with wild-type Col (Supplemental Figures 6A and 6C). An analysis of germination during extended seed storage showed that these three insertion lines have normal dormancy levels both in the wild-type and *rdo5-2* mutant background (Supplemental Figure 7). This lack of a dormancy phenotype could be due to redundancy (Tam et al., 2010) and (especially for *APUM10* and *APUM11*) by the absent or limited influence of the insertion on the transcript. To reduce the expression of all three *APUM* genes, an RNA interference (RNAi) construct was made based on a DNA fragment that exhibits high degrees of sequence similarity in all three genes. Three independent single insertion transgenic lines containing this construct were obtained that showed significantly lower *APUM9*, *APUM10*, and *APUM11* transcript levels compared with the wild-type control (Figure 7A). These three lines all showed enhanced dormancy levels compared with wild-type Col (Figure 7B).

In addition, transgenic plants were created in which *APUM9* was constitutively expressed using the 35S promoter (Supplemental Figure 8). Three independent single insertion



**Figure 6.** Expression Pattern and Protein Localization of *APUM9*.

**(A)** qRT-PCR analysis of *APUM9* and *APUM11* transcript levels in dry and imbibed *rdo5-1* and wild-type NIL-DOG1 seeds during imbibition. The bottom panel shows the transcript levels for *APUM9* and *APUM11* in NIL-DOG1 in more detail at a smaller scale. The expression values were normalized using *ACT8* as control.  $n = 3$  biological replicates; error bars represent SE.

**(B)** and **(C)** qRT-PCR analysis of *APUM9* transcript levels in different tissues **(B)** and during seed maturation **(C)**. The expression values were

lines all showed reduced seed dormancy compared with the Col background (Figure 7C). These results indicate a negative relation of *APUM9* transcript levels with seed dormancy, which is consistent with the high *APUM9* and *APUM11* transcript levels in the nondormant *rdo5-1* mutant.

### The Reduced Dormancy Phenotype of *rdo5* Requires *APUM9* and *APUM11*

The above described results suggest that the very low dormancy levels of *rdo5* seeds could be caused by enhanced transcript levels of *APUM9* and *APUM11*. This hypothesis was tested by downregulation of *APUM9*, *APUM10*, and *APUM11* in the *rdo5-1* background using the same RNAi constructs as described above. We obtained four independent single insertion transgenic lines. These lines complemented the *rdo5-1* phenotype. All four lines had strongly enhanced dormancy levels and two of them showed full complementation with dormancy levels similar to wild-type NIL-DOG1 (Figure 7D). We conclude that RDO5 regulates germination potential during seed imbibition by suppressing transcript levels of *APUM9* and *APUM11*.

### DISCUSSION

We identified RDO5 as a protein belonging to the PP2C phosphatases that regulates seed dormancy. *RDO5* is a seed dormancy specific gene because we did not observe additional phenotypes in the *rdo5* mutant. In accordance, *RDO5* is only expressed in seeds and exhibits peak transcript levels in freshly harvested dry seeds. Other genes that have been described to influence seed dormancy also influence other plant traits, including the key regulator of dormancy DOG1 whose mutants are reduced in seed longevity (Bentsink et al., 2006). *RDO5* could be a suitable target gene to alter seed dormancy levels in crop plants without affecting other plant traits.

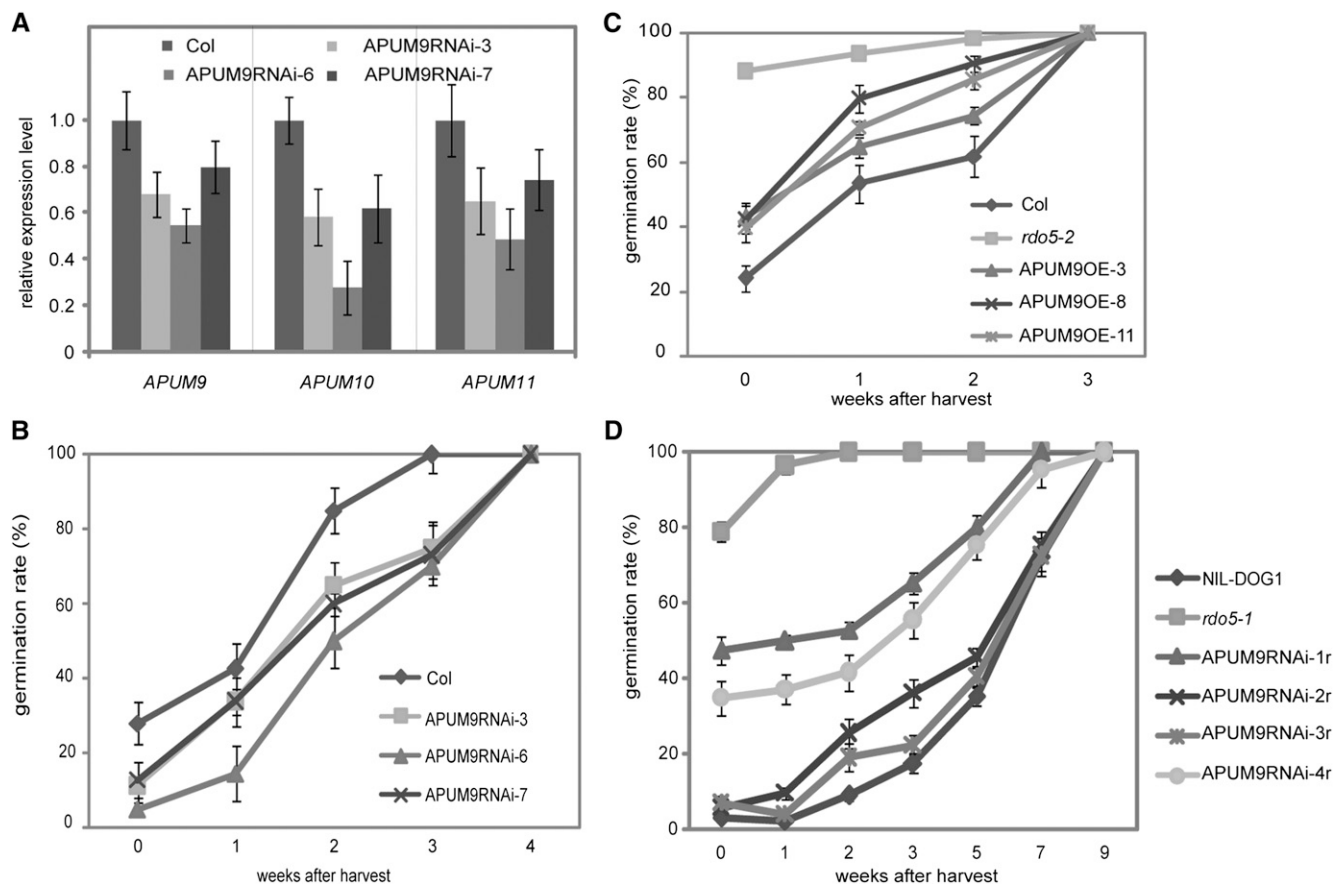
### RDO5 Does Not Influence the ABA and DOG1 Dormancy Pathways

*RDO5* encodes a PP2C protein that has not been previously analyzed. The *Arabidopsis* genome contains 76 PP2Cs, which are divided into 10 clades, except for six members that could not be clustered (Schweighofer et al., 2004). Several PP2C members belonging to clade A have been characterized as negative regulators of ABA signaling and seed dormancy, such as ABI1, ABI2, AHG1, and HAI2 (Rodriguez et al., 1998; Gosti et al., 1999; Nishimura et al., 2007; Kim et al., 2013). ABA binding to RCAR/PYR/PYL proteins promotes their association with the clade A PP2Cs, leading to the inhibition of phosphatase activity (Ma et al., 2009; Park et al., 2009). The clade A PP2C

normalized using *ACT8* as control.  $n = 3$  biological replicates; error bars represent SE.

**(D)** Protein subcellular localization of *APUM9* in *N. benthamiana* cells transiently expressing *Pro35S::GFP::APUM9*. I to IV show 4',6-diamidino-2-phenylindole signal, GFP signal, transmission, and merged images, respectively.





**Figure 7.** *APUM9* Reduces Seed Dormancy.

**(A)** qRT-PCR analysis of *APUM9*, *APUM10*, and *APUM11* transcript levels in 24-h imbibed seeds of three independent *APUM9* RNAi lines. The expression values were normalized using *ACT8* as control.  $n = 3$  biological replicates; error bars represent SE.

**(B)** Germination after different periods of dry storage of three independent *APUM9* RNAi lines in the Col background. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

**(C)** Germination after different periods of dry storage of three independent *APUM9* overexpression lines in the Col background. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

**(D)** Germination after different periods of dry storage of four independent *APUM9* RNAi lines in the *rdo5-1* background. Germination of the dormant line NIL-DOG1 is shown for comparison. Shown are averages  $\pm$  SE of six to eight independent batches of seeds for each genotype.

proteins have similar structures with a catalytic C-terminal domain and a noncatalytic N-terminal extension. The RDO5 protein lacks the N-terminal extension and only contains the catalytic domain. RDO5 and the clade A PP2Cs both control seed dormancy, but the role of RDO5 is different because it acts as a positive regulator of dormancy. Furthermore, several observations indicated that RDO5 is not regulated by ABA. Mutations in *RDO5* neither affected ABA levels in dry and imbibed seeds nor ABA sensitivity during germination (Figures 4A and 4B). In addition, *RDO5* transcript levels were not affected by enhanced endogenous ABA (Figure 4C). Finally, we have not detected any ABA signaling genes that are differentially expressed between wild-type and *rdo5* mutant seeds during maturation and imbibition (Supplemental Table 1). Overall, we assume that the role and regulation of RDO5 is different from that of the clade A PP2Cs.

Although (loss-of-function) *rdo5* mutants show strongly reduced seed dormancy, several of our germination experiments revealed a low level of residual dormancy in freshly harvested *rdo5* seeds. This suggests that the influence of RDO5 on seed dormancy is weaker than that of DOG1, whose loss-of-function mutants are always completely nondormant. In accordance, *dog1* mutant seeds germinate faster than *rdo5* mutant seeds (Figure 5D). *RDO5* and *DOG1* both have a major role in seed dormancy and are only expressed in seeds. *DOG1* transcript levels peak half way through the seed maturation phase, whereas those of *RDO5* have the highest level in dry seeds. *DOG1* and *RDO5* are localized in the nucleus and are highly abundant in freshly harvested dry seeds. In addition, both proteins are stable during seed imbibition (Figure 2F; Nakabayashi et al., 2012). These similarities suggest that *DOG1* and *RDO5* might function in the same pathway. However, we have not

found any indication for this. First, *RDO5* and *DOG1* do not influence each other at the expression level. Transcript levels of *DOG1* are similar in wild-type and *rdo5* mutant seeds, whereas *RDO5* transcript levels are similar in wild-type and *dog1* mutant seeds (Figures 5A and 5C). In addition, *DOG1* levels and migration speed are not affected in the *rdo5* mutant (Figure 5B), suggesting that the phosphatase *RDO5* does not affect *DOG1* protein. Taken together, our results suggest that *RDO5* regulates seed dormancy independently of the ABA and *DOG1* pathways.

#### A Potential Indirect Role for *RDO5* in Posttranscriptional Regulation during Seed Imbibition by Suppression of *APUM* Genes

A transcriptome analysis showed that relatively few genes are differentially expressed in the *rdo5* mutant compared with its wild type during seed maturation and imbibition (Supplemental Data Sets 1 to 6). This suggests that *RDO5* does not have a major role in transcriptional regulation. Most differentially expressed genes were identified in seeds that had been imbibed for 6 h, among which *APUM9* and *APUM11* showed dramatically increased transcript levels in the *rdo5-1* background. Transgenic plants with enhanced or reduced expression levels of *APUM9* and *APUM11* showed respectively a decrease and increase in seed dormancy levels (Figure 7). This indicates that these two *APUM* proteins negatively regulate seed dormancy. Interestingly, transgenic *rdo5-1* plants, in which *APUM* transcripts are repressed by an RNAi construct, showed wild-type dormancy levels (Figure 7D). This strongly suggests that *RDO5* regulates seed dormancy by controlling *APUM* transcripts.

PUF proteins are a class of RNA binding protein containing a PUM-HD. These domains contain multiple tandem repeats that bind to specific recognition sequences in the 3' untranslated regions (UTRs) of mRNAs to control their stability and translation. PUF proteins are involved in posttranscriptional regulation, including mRNA repression, activation, and localization (Quenault et al., 2011). The PUF proteins are highly conserved in eukaryotes and are implicated in important biological processes in yeast, *Drosophila*, humans, and other organisms (Kusz et al., 2007; Ariz et al., 2009; Kedde et al., 2010; Chen et al., 2012; Shigunov et al., 2012; Weidmann and Goldstrohm, 2012). The family of PUF proteins is expanded in *Arabidopsis* compared with vertebrates and yeast. The *Arabidopsis* genome contains 25 *APUMs*, of which most are uncharacterized. Several *APUMs* represent examples of gene duplication events, such as *APUM1/APUM2/APUM3*, *APUM9/APUM10*, and *APUM18/APUM19*. These *APUM* gene pairs could have redundant functions (Tam et al., 2010), which could explain the lack of phenotype in the *apum9-1* single mutant.

A few *Arabidopsis* *APUMs* have recently been described and showed different functions in plant growth. *APUM23* is a constitutively expressed gene that is required for normal growth patterning in *Arabidopsis*. Analysis of rRNA processing showed that *APUM23* is involved in the processing and/or degradation of 35S pre-rRNA (Abbasi et al., 2010). *APUM5* was isolated in a mutant screen for reduced susceptibility to *Cucumber mosaic virus* infection. *APUM5* directly binds viral RNAs and works as a negative regulator for *Cucumber mosaic virus* replication (Huh et al., 2013).

Additionally, *APUM5* was shown to be involved in the abiotic stress response and bound to the 3' UTR of abiotic stress-responsive genes (Huh and Paek, 2014). All the *APUMs* that have been characterized in *Arabidopsis* are therefore involved in different processes. This diversion in function of individual *APUMs* could be caused by their specific binding to UTRs of different mRNAs as well as by their expression patterns.

#### The Function of *RDO5* and *APUMs* in the Regulation of Seed Dormancy and Germination

Microarray analyses have revealed that more than 10,000 different mRNA species are present in *Arabidopsis* dry seeds, including genes for all functional categories (Nakabayashi et al., 2005; Dekkers et al., 2013). In addition, mass spectrometry analyses revealed the presence of more than 3700 proteins in mature seeds (Baerenfaller et al., 2008). The functions of these stored mRNAs and proteins in dry seeds are still not clear. It has been demonstrated that germination of *Arabidopsis* seeds can be inhibited by a cycloheximide treatment, which is an inhibitor of protein synthesis, but not by  $\alpha$ -amanitin, which blocks Pol II-mediated transcription. These experiments indicated that transcription is dispensable for germination and that translatable mRNAs and proteins stored in dry seeds are sufficient for germination (Rajjou et al., 2004; Kimura and Nambara, 2010). A recent study of the transcriptome in dormant and nondormant sunflower (*Helianthus annuus*) embryos during imbibition demonstrated a selective recruitment of mRNAs to polysomes (Layat et al., 2014). In our study, we identified two seed dormancy regulators, the phosphatase *RDO5* and the RNA binding *APUM* proteins (in particular *APUM9* and 11) that could be part of the molecular bases of the selective association of transcripts with polysomes during imbibition. Based on the predicted function of their homologs in other species and their enhanced transcription levels during imbibition, we hypothesize that the *APUMs* are involved in the translation efficiency of specific stored mRNAs in seeds during imbibition. The subsequently produced proteins could determine whether a seed will germinate or not. We conclude that *RDO5* regulates this process because our genetic and transgenic analyses indicated that *RDO5* requires the *APUM* genes for its function. However, *RDO5* probably has an indirect role because its protein is located in the nucleus (Figure 2E), while the *APUM9* protein was detected in the cytoplasm and around the nucleus (Figure 6D). In addition, *RDO5* and *APUM9* seem to function at different times. Inducible expression of *RDO5* with the heat shock promoter indicated that *RDO5* functions at the end of seed maturation and in dry seeds (Figures 3D and 3E), whereas the enhanced transcript levels of *APUM9* and *APUM11* in the *rdo5* mutant were mainly observed during seed imbibition (Figure 6A). However, the *RDO5* protein remained stable during seed imbibition (Figure 2F) and could therefore function by repressing *APUM* transcripts during early imbibition in wild-type plants.

Protein phosphatases like *RDO5* dephosphorylate proteins, leading to their activation or inactivation. In the ABA signaling transduction pathway, PP2C phosphatases from clade A can physically interact with subclass III SnRK2s in an ABA-independent manner. PP2Cs inactivate SnRK2 kinase activity by direct

dephosphorylation. In the presence of ABA, the ABA receptors inhibit PP2Cs, leading to the activation of SnRK2 kinases, which in turn activate transcription factors and some other targets (Umezawa et al., 2009; P. Wang et al., 2013). Interestingly, in human cancer cells, it was shown that Pumilio-1 (PUM1) is upregulated and phosphorylated for optimal induction of its RNA binding activity toward the tumor suppressor, p27-3' UTR (Kedde et al., 2010). This suggests that the activity of APUM proteins could be regulated by phosphorylation. Most likely, the phosphatase RDO5 regulates APUM proteins indirectly because we have not detected any physical interaction and the proteins are not located in the same cell compartment. In analogy to the clade A PP2Cs, the relation between RDO5 and the APUM proteins could involve a kinase phosphorylating the APUM proteins and regulating their activity.

## METHODS

### Plant Materials and Growth Conditions

Plant materials used in this study were all derived from the *Arabidopsis thaliana* accessions Ler and Col. NIL-DOG1 is derived from the Ler background with an introgression on chromosome 5, containing the *DOG1* gene from Cvi (Alonso-Blanco et al., 2003). *RDO5* and *APUM* insertion lines were obtained from the NASC and RIKEN collections with the following seed stock numbers: *rdo5-2*, SALK\_073656; *rdo5-3*, pst18302; *apum9-1*, SALK\_028441; *apum10-1*, SALK\_022907; and *apum11-1*, SALK\_089493. The gene-specific primer sequences were obtained from the SALK SIGnAL database. RT-PCR or qRT-PCR with RNA isolated from dry or imbibed seeds was performed to confirm the homozygous knockout lines. All used primers are listed in Supplemental Data Set 7.

Seeds were sown on soil and plants grown in a growth chamber with a 16-h-light/8-h-dark cycle (22°C/16°C) or in a greenhouse where the temperature was maintained close to 23°C and 16 h of light was provided daily. Freshly harvested seeds were immediately used for experiments or stored under constant conditions (21°C, 50% humidity, in the dark) for after-ripening treatment.

Plants for the heat shock induction treatment were grown in a chamber with a 16-h-light/8-h-dark cycle (16°C/14°C) during seed maturation and induced by transfer to 37°C for 3 h per day. For heat induction during seed imbibition, imbibed seeds were exposed to a 37°C heat shock for 3 h per day.

### Seed Dormancy and Longevity Measurements

Approximately 50 to 100 seeds of individually harvested plants were sown on filter paper, placed into transparent moisturized containers, and incubated in a germination cabinet in long-day conditions (12 h light at 25°C, followed by 12 h darkness at 20°C). After 7 d of incubation, the germination percentages were analyzed.

Seed longevity was determined as germination ability after long-time storage in natural conditions or after a controlled deterioration test. For controlled deterioration, we stored seeds above a saturated KCl solution (80% relative humidity) in a closed container at 37°C for 3 to 6 d, after which germination assays were performed as described above.

For ABA responsiveness tests, after-ripened seeds with fully released dormancy were sown on filter paper imbibed with water containing different ABA concentrations. For each germination test at least six to eight biological replicates were performed.

### Fine Mapping and Identification of *RDO5*

The *rdo5-1* mutant was isolated in a  $\gamma$ -irradiation mutagenesis screen of NIL-DOG1 (Bentsink et al., 2006). *RDO5* was identified by map-based

cloning in a cross with Col. F2 progeny with a homozygous Cvi-DOG1 background were selected using derived cleaved amplified polymorphic sequence markers and phenotyped for their dormancy level. After phenotyping F2 individuals, genomic DNA of highly dormant and nondormant plants was bulked in two groups. These genomic DNA bulks were hybridized with the *Arabidopsis* SNP Genotyping and Tiling Array (Affymetrix).

For *RDO5* fine mapping, plants were genotyped with simple sequence length polymorphism and cleaved-amplified polymorphic sequence markers, designed based on the Monsanto *Arabidopsis* polymorphism database. The interval was narrowed down to 17 kb on chromosome 4. Finally, the complete 17-kb genomic region of NIL-DOG1 and *rdo5-1* was sequenced.

### Constructs and Plant Transformation

All the binary constructs were prepared using Gateway technology (Invitrogen). For *rdo5* complementation, a 6-kb Ler genomic fragment, including a 2.7-kb promoter sequence, *RDO5* coding sequence, and 0.9-kb sequence after the stop codon, was amplified from Ler using gene-specific primers, cloned into the pDONR207 vector, and transferred into pGWB401 (Nakagawa et al., 2007) after sequencing. The *RDO5* cDNA was amplified from Ler with gene-specific primers, cloned into pDONR207, and transferred into pGWB429 (Nakagawa et al., 2007) after sequencing for *RDO5* overexpression in fusion with the TAP tag. *APUM9* cDNA was amplified using gene-specific primers, cloned into pDONR207, and transferred into pFASTR02 (Shimada et al., 2010) after sequencing. The genomic fragment for *APUM*-RNAi was amplified with *APUM9*-specific primers, cloned into pDONR207, and transferred into pFASTR03 (Shimada et al., 2010). The binary clone for *RDO5*-inducible expression was constructed by cloning of the *RDO5* cDNA sequence from the donor clone into the pLEELA-HSP vector, which contains the promoter from the soybean (*Glycine max*) Gmhsp 17.6L heat shock protein gene (Severin and Schöffl, 1990). The *ProRDO5::YFP::RDO5* construct was made by recombining the *RDO5* cDNA entry clone with pENSG-YFP. The 35S promoter was subsequently replaced with the *RDO5* native promoter with an *AscI* and *XhoI* digestion. Finally, the *APUM9::GFP* construct was made by transfer of *APUM9* cDNA from an entry clone into pFAST-R05 (Shimada et al., 2010). All used primers are listed in Supplemental Data Set 7.

All the binary constructs were introduced by electroporation into *Agrobacterium tumefaciens* strain GV3101 or GV3101 carrying the helper plasmid pMP90RK, which was subsequently used for transformation by floral dipping (Clough and Bent, 1998). All the transgenic lines were first selected based on their antibiotics resistance or fluorescence. T3 homozygous lines containing single insertion events were used for expression level detection and phenotyping.

### Confocal Microscopy Analysis

For the analysis of *RDO5* localization, embryos of freshly harvested seeds from transgenic plants containing *YFP::RDO5* driven by the *RDO5* promoter were dissected from the testa after a short imbibition time. YFP fluorescence was observed using a Zeiss LSM 700 confocal microscope. The *APUM9* subcellular localization was studied after transient expression of *Pro35S::GFP::APUM9* in *Nicotiana benthamiana* leaves.

### RNA Extraction and Expression Studies

Total RNA was extracted from developing *Arabidopsis* siliques or imbibed seeds using RNAqueous columns and an RNA isolation aid (Ambion) as described previously (Kushiro et al., 2004). cDNA was synthesized from 1  $\mu$ g of total RNA using the QuantiTect reverse transcription kit (Qiagen). qRT-PCR was performed with QuantiTect SYBR Green PCR (Qiagen) on

a Mastercycler Realplex2 system (Eppendorf) with gene-specific primer sets. The PCR program was as follows: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 20 s at 72°C. The relative expression value for each gene was quantified using the delta Ct value method and normalized to the *ACTIN8* control. At least three biological replicates were analyzed. All primers are listed in Supplemental Data Set 7.

### Transcriptome Analysis

RNA was extracted from siliques at 14 d after pollination, freshly harvested seeds, and seeds that had been imbibed for 6 h. The transcriptome analysis was performed using Affymetrix microarrays (*Arabidopsis* AG-ORONOMIC1 genome array) containing more than 30,000 probe sets. Labeling and hybridization were performed according to the manufacturer's instructions at the Max Planck Genome Centre Cologne. Three hybridizations of independent biological replicates were performed. Normalization, processing, and statistical analysis of the raw data were performed using RankProd (Hong et al., 2006) in R programming. Genes were only considered differentially expressed between the mutant and wild type when having a significant expression difference of at least 1.5-fold with false discover rate < 0.05.

### ABA Measurements

Extraction, purification, and measurement of ABA were performed as described previously with some modifications (Zhou et al., 2003). About 10 mg seed material was used for ABA measurements. Metabolites were extracted from ground seeds using an acetone:water:acetic acid (80:19:1) solution with the addition of ABA-d6 as internal standards for quantification. Samples were subsequently purified with 1 mL Oasis HLB column by gravity flow. After drying, samples were resuspended in water:acetic acid:formic acid (94.9:5:0.1) and analyzed by liquid chromatography-tandem mass spectrometry.

### Protein Extraction and Immunoblotting

Twenty milligrams of developing siliques or 10 mg of dry seeds was ground in liquid nitrogen and then extracted with a buffer containing 6 M urea, 2 M thiourea, 0.2% (v/v) Triton X-100, 0.2% (w/v) sarcosyl, and 2 mM DTT in 100 mM Tris-Cl, pH 7.5. After two cycles of 30 min shaking the supernatant was collected by centrifugation at 4°C, protein concentration was determined by Bradford dye reagent (Bio-Rad) using BSA as a standard. Thirty micrograms of protein samples were separated by NuPAGE 4–12% Bis-Tris protein gels (Life Technologies). Traditional wet transfer was performed using the XCell II blot module and immunological reaction was performed. Protein accumulation was analyzed by immunoblotting using GFP- (Roche), HA- (Covance), or TAP-tag (Thermo) antibodies. Histone H3 antibodies (Abcam) were used for the loading control.

### Accession Numbers

The microarray data have been submitted at the Gene Expression Omnibus database (accession number GSE62578). Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *RDO5* (At4G11040), *DOG1* (AT5G45830), *APUM1* (At2g29200), *APUM2* (At2g29190), *APUM3* (At2g29140), *APUM9* (At1G35730), *APUM10* (At1g35750), *APUM11* (AT4G08840), *APUM18* (At5g60110), *APUM19* (At5g60180), *CYP707A1* (At4g19230), *CYP707A2* (At2G29090), *CYP707A3* (At5G45340), and *ACTIN8* (At1G49240).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Phenotype of 4-Week-Old NIL-DOG1 and *rdo5-1* Plants.

**Supplemental Figure 2.** Map-Based Cloning of *RDO5*.

**Supplemental Figure 3.** Analysis of *APUM9* and *APUM11* Transcript Levels.

**Supplemental Figure 4.** Amino Acid Sequence Alignment of *APUM9*, *APUM10*, and *APUM11*.

**Supplemental Figure 5.** Expression Profiles of *APUM9*, *APUM10*, and *APUM11*.

**Supplemental Figure 6.** Insertion Site Predictions and Transcript Levels of *APUM9*, *APUM10*, and *APUM11* T-DNA Insertion Lines.

**Supplemental Figure 7.** Germination Phenotypes of the *apum* Insertion Mutants in Col and *rdo5-2* Background.

**Supplemental Figure 8.** *APUM9* Transcript Levels in *APUM9* Over-expression Lines.

**Supplemental Data Set 1.** Upregulated Genes in *rdo5-1* Siliques at 14 Days after Pollination Compared with NIL-DOG1.

**Supplemental Data Set 2.** Downregulated Genes in *rdo5-1* Siliques at 14 Days after Pollination Compared with NIL-DOG1.

**Supplemental Data Set 3.** Upregulated Genes in *rdo5-1* Freshly Harvested Seeds Compared with NIL-DOG1.

**Supplemental Data Set 4.** Downregulated Genes in *rdo5-1* Freshly Harvested Seeds Compared with NIL-DOG1.

**Supplemental Data Set 5.** Upregulated Genes in *rdo5-1* Seeds That Had Been Imbibed for 6 h Compared with NIL-DOG1.

**Supplemental Data Set 6.** Downregulated Genes in *rdo5-1* Seeds That Had Been Imbibed for 6 h Compared with NIL-DOG1.

**Supplemental Data Set 7.** Primers Used in This Study.

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### AUTHOR CONTRIBUTIONS

Y.X. and W.J.J.S. designed the research. Y.X., K.N., and L.B. performed research. Y.X., K.N., F.H., and J.D. analyzed data. Y.X. and W.J.J.S. wrote the article.

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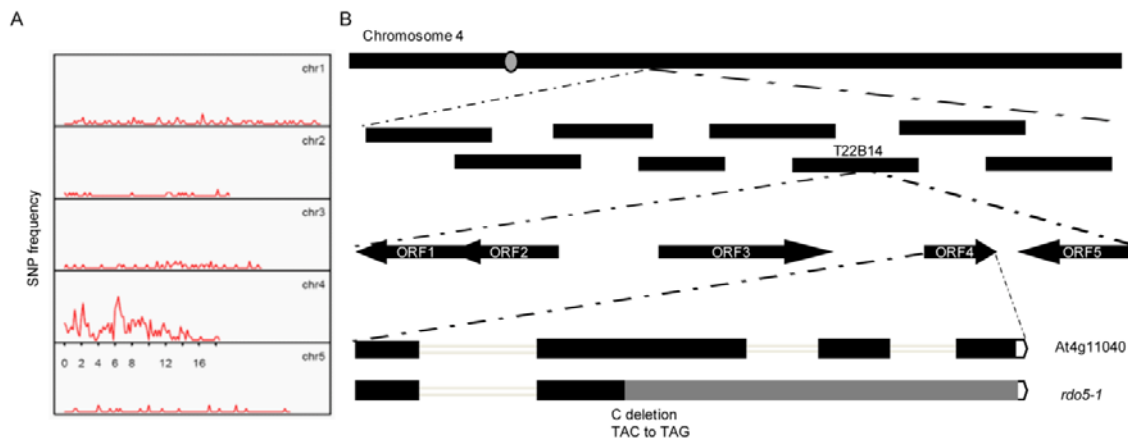
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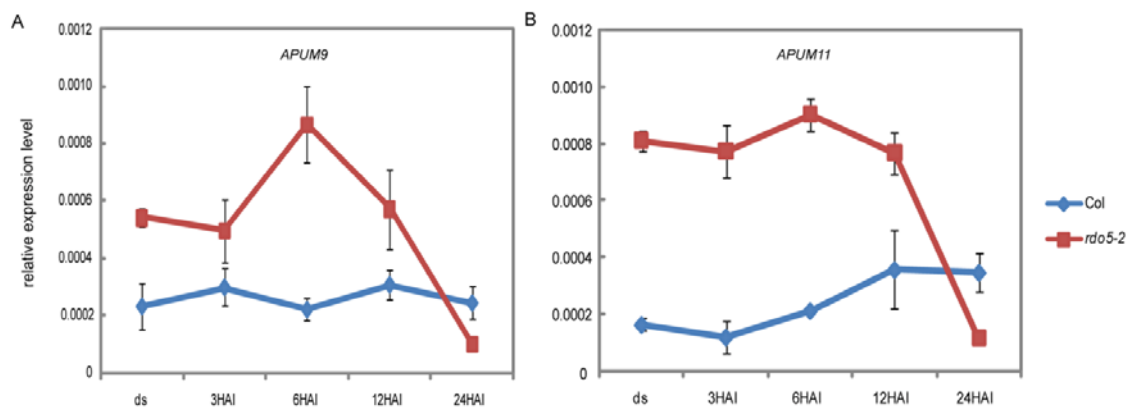
Supplemental Figure 1. Phenotype of 4 Weeks Old NIL-DOG1 and *rdo5-1* Plants. NIL-DOG1 (left) and *rdo5-1* (right) plants were grown in a greenhouse where the temperature was maintained close to 23°C and 16 h of light was provided daily.



Supplemental Figure 2. Map Based Cloning of *RDO5*.

(A) Rough mapping of *RDO5* based on hybridization of the *Arabidopsis* SNP genotyping and tiling array. The SNP frequency in the mutant phenotype bulk compared to the wild-type phenotype bulk is shown relative to their position on each chromosome. The graph indicates that the *rdo5* mutation is located on chromosome 4.

(B) Fine mapping of *RDO5*. The upper part of the figure shows the position of BAC T22B4 on chromosome 4. The middle part shows the 17-kb region containing the *rdo5* mutation that was identified after fine mapping. This region contains five open reading frames (ORFs). The lower part of the figure shows the identified mutation that was found in At4g11040, which consists of a single base pair deletion resulting in a stop mutation.



Supplemental Figure 3. Analysis of *APUM9* and *APUM11* Transcript Levels.

qRT-PCR analysis of *APUM9* (left) and *APUM11* (right) transcript levels in *rdo5-2* and wild-type Col during seed imbibition. The expression values were normalized using *ACT8* as control. ds, dry seed; HAI, hours after imbibition. n = 3 biological replicates; Error bars represent standard error (SE).

```

APUM9      MGFGGFNGDFNFRGASDHR-----PLGSDGFLPSLD--TNPFLKN---HKSVEALD-
APUM10     -----MEIFNFGQASDHRRL-----PDFGSGGFLQSLD--TNPFLKNQYNNNSVEALE-
APUM11     MDFGFFPGDLRQRGSFTDLGNGFPLTSSVNGFHFHSGDRTTNPFLNLRKLDTTSLMADG
           :. : . *.* * * *****: .: :

APUM9      ----LCKKLHKMGISCDMSIWTKEEQFRVDPSEFGARTLHGSSGFDQN-LTGASQIHDG
APUM10     ----LCKKLHKMGISCDMSIWTKEEPFRVDPGDFGAKTLHESFGFDQN-LTGASQIHDG
APUM11     VDMGLCQNLKMSISDERSNFFNHSSFSGYGQYQGRESSFHGEASSSMRGFVGVDVHR-
           **::* **.* : * : : . . : : * . . . . :.* :.*

APUM9      FRNFSSVRLQNNNFHGVSPSPGE-----MRLGRQDSFNLNG--FEEMLALKNHRDFL-
APUM10     FRNFSSVRVQNNNFHGVSPSPG-----LLGLQDSFNPNG--FEEMMAFKDHKDFL-
APUM11     FEQDLRVRASFHGESAMSSYVGDSYHRLRFLALQEASNPNCFTENMSLLNRDYMLE
           *.: ** . :. :.* * :.* **::* * * * * : : : : *

APUM9      LDQIHEPIKRPPFLRGNDALISSLMFEGNNRVSQTLAAMEASRGFYPEEDSSLLSPFHEK
APUM10     LDHINEPIKRSPFLRGNDAFKGSLMFEG-IRVSQILAAMEGSGASYPDE-----PK
APUM11     LEHFNQIIRDFSLVPQ---KSPLAFHE-ERILPPFSAMGGSRELDGSA-----KCMK
           *::: : * * * : . . * * . : : * * . * . *

APUM9      VSGKLGASYLEDTVLIGQSGYKMSPKSNNDLVSMVEIYGSVNLMAKDQIGCRVLQKLVE
APUM10     INGGL-----PLDLVSMVEIYGSVNLMAKDQIGCRALQKLVE
APUM11     NKE-----DSLDSLASMVDSYGSVYLMAKDQLGCRLLQKFVD
           . *.* **::* * * * * : * * * * * : *

APUM9      EGTfHEAKVILLAIIDHVVELSMDPFGNYIVQKLFVDSDEEQRTLIVSVLTSNPRELIRI
APUM10     EGTVLDSKVI FLEIIDHVVELSMDPLGNYIVQKLLVVSDEEQRTMIVSVLTSKPRELIKI
APUM11     EGNFVDVMIIFKEVINNVIELGTD PFGNYLIQKLEVCNEEQRTQILIRLTSKPGLLVKI
           **.. : : : : : * * * * * : * * * * * : * * * * * : * * * * *

APUM9      CLNTYGTRVVQKMIETVKTKQQIALVKSGLPGFLALVKDLNGNHVIQSLQTLGPNDNE
APUM10     CLNTNGTRVIQKMIKTVKTKQQIALVKSALEPGFLVLVNDNSNGYHVLQSCLEFLVPNDNK
APUM11     SINNYGTRVVQKLIETVTTKEQISLVKSALVPGFLSLFRELNNGNHVILNCLKFFSPNDNK
           .*. *****: * : * * * * * : * * * * * : * * * * * : * * * * *

APUM9      FVLEAATKYCAEIAIHRHGCCVLQCCISNSVGLQRERLVAEISRNSLHLSQDPFGNYVVQ
APUM10     FVVEAATEYCAQLATHQYGCYVLQCSLINTVGLQHERLVAEISRDSLRLSQDPFGNYVVQ
APUM11     FILEAATKFCIEIATTRHGCCVLQRCVSVSVGEQHEKLVDEISRNSLLLAQDPFGNYLVQ
           *::*****: * : * : * * * * : : * * * * * : * * * * * : *

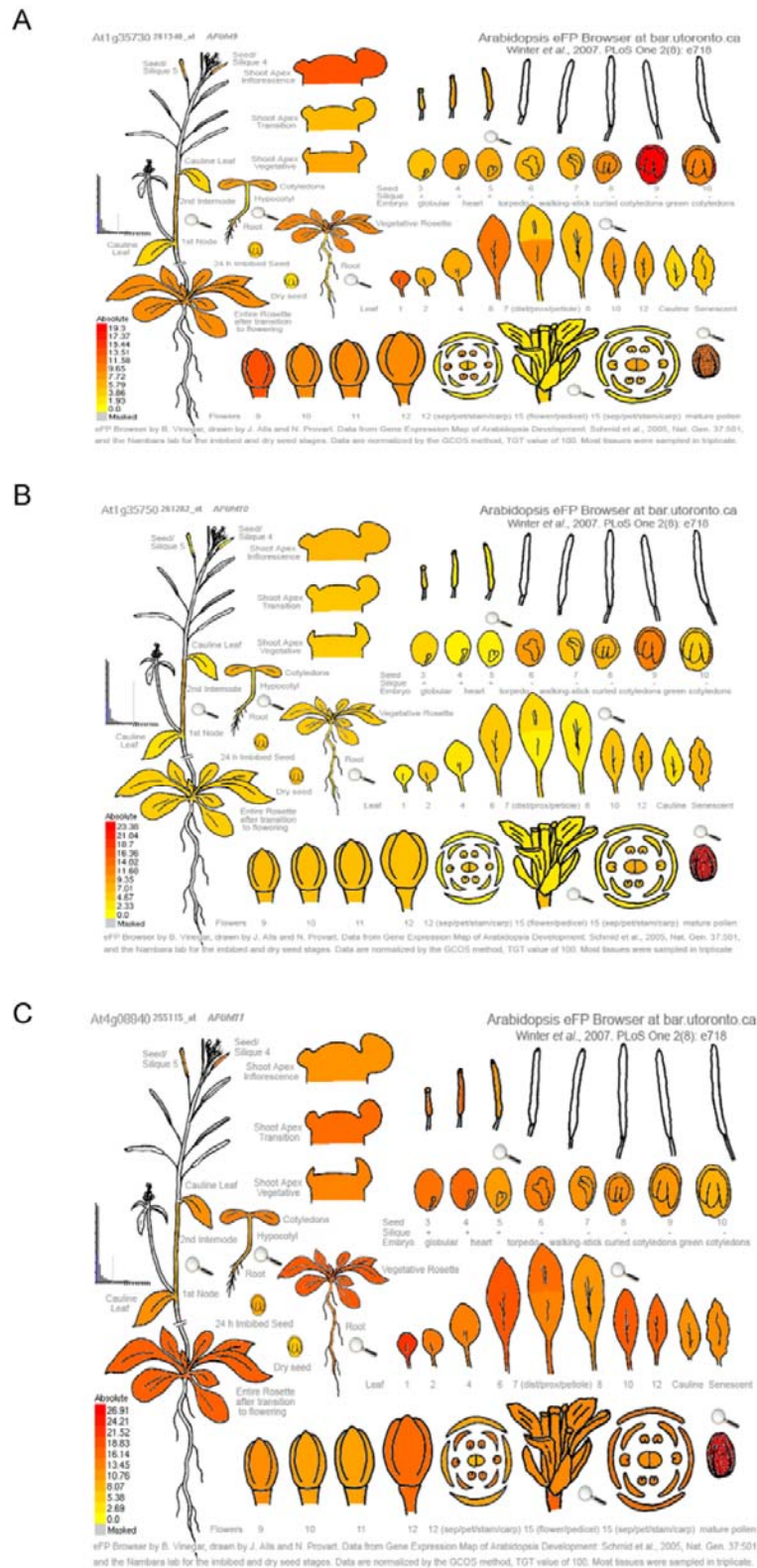
APUM9      YLIDQQVSAVKLLVQFRMHYAE LATQKFSSHVIEKCLRKYPESRAEIVRELLCVPNFEYL
APUM10     CLIDQQVSSVNL L L PFRTHCIELATQKFSSHVIEKCLRKYPESRAEIVRELLSYPNFEQL
APUM11     YIIEKKVGGVNVLFELRGNYVKLATQKFGSHVVEKCLRYYPESRSQIVNELVSVLNFYGL
           : * : * . . : * . : : * * * * * : * * * * * : * * * * * : *

APUM9      LQDPYANYVIQTALSVTKGPVRAKLVAKVYRYGKLHSSPYCKKIFSKTILKK
APUM10     LQDPYANYVIQTALSVTKGAVRARLVEKVRFGLQSNPYCKKIFSKTILKK
APUM11     LQDPYANYVIQCALSKTKGFVRASLVEKVRRYENLKMTPEYCKRIFSKNLWKK
           ***** * * * * * * * * * * : : * : * * * * * : *

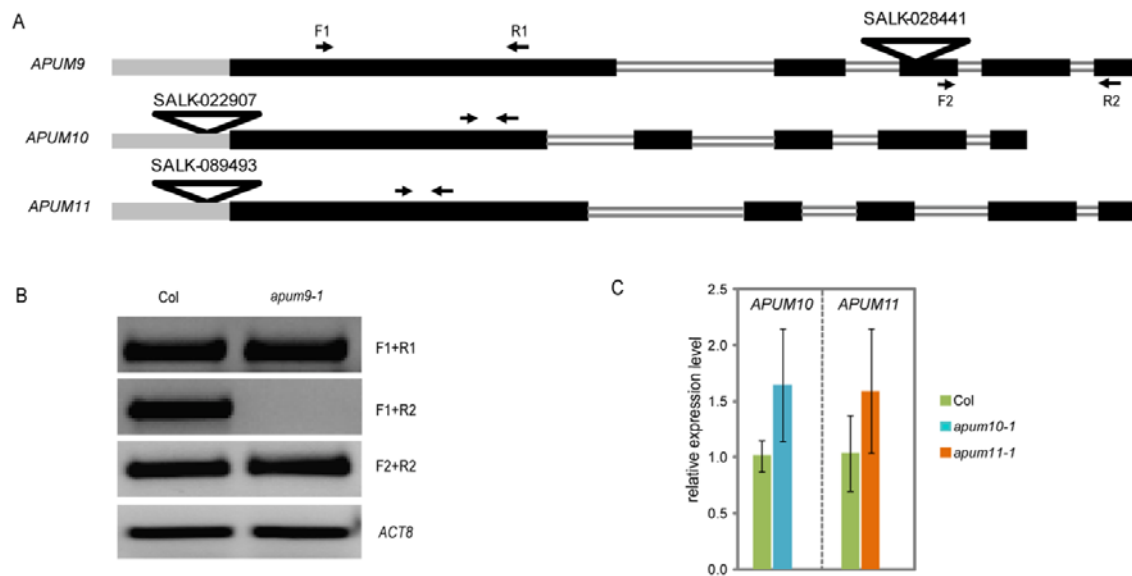
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Supplemental Figure 4. Amino Acid Sequence Alignment of APUM9, APUM10 and APUM11.

The alignment was performed using online ClusterW2 tools with default settings (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Asterisks indicate fully conserved residue. Colons and periods indicate strong and weak similarity, respectively.



Supplemental Figure 5. Expression Profiles of *APUM9*, *APUM10* and *APUM11*. Pictograms for *APUM9* (A), *APUM10* (B) and *APUM11* (C) were extracted from the *Arabidopsis* eFP browser.



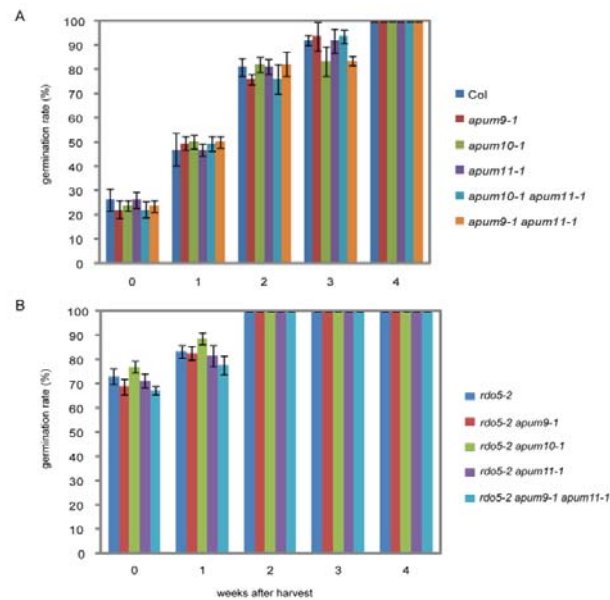
Supplemental Figure 6. Insertion Site Predictions and Transcript Levels of *APUM9*, *APUM10* and *APUM11* T-DNA Insertion Lines.

(A) Gene structures of *APUM9*, *APUM10* and *APUM11*. The predicted locations of the T-DNA insertions are indicated. The arrows represent the primers used in RT or qRT-PCR analyses.

(B) RT-PCR analysis of *APUM9* transcript in freshly harvested seeds of wild-type Col and *apum9-1* T-DNA insertion line for three different primer combinations (as shown in A). *ACT8* was used as a loading control.

(C) qRT-PCR analysis of *APUM10* and *APUM11* transcript levels in freshly harvested seeds of the *apum10-1* and *apum11-1* T-DNA insertion lines and wild-type Col. The expression values were normalized using *ACT8* as control. n = 3 biological replicates; Error bars represent standard error (SE).

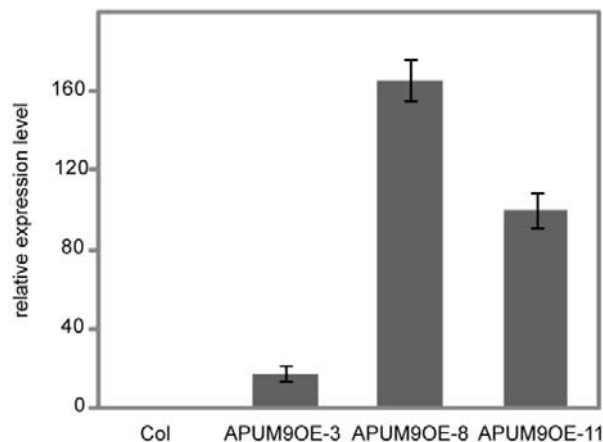




Supplemental Figure 7. Germination Phenotypes of the *apum* Insertion Mutants in Col and *rdo5-2* Background

(A) Germination after different periods of dry storage of the *apum9-1*, *apum10-1* and *apum11-1* single mutants, the *apum10-1 apum11-1* and *apum9-1 apum11-1* double mutants and the wild-type Col. Shown are averages  $\pm$  standard error (SE) of 6-8 independent batches of seeds for each genotype.

(B) Germination after different periods of dry storage of the *rdo5-2* single mutant, the *rdo5-2 apum9-1*, *rdo5-2 apum10-1* and *rdo5-2 apum11-1* double mutants and the *rdo5-2 apum9-1 apum11-1* triple mutant. Shown are averages  $\pm$  standard error (SE) of 6-8 independent batches of seeds for each genotype.



Supplemental Figure 8. *APUM9* transcript levels in *APUM9* overexpression lines. qRT-PCR analysis of *APUM9* transcript levels in freshly harvested seeds of three independent lines in which *APUM9* was constitutively expressed using the 35S promoter and wild-type Col. The expression values were normalized using *ACT8* as control.  $n = 3$  biological replicates; Error bars represent standard error (SE).



***REDUCED DORMANCY5* Encodes a Protein Phosphatase 2C That Is Required for Seed Dormancy in *Arabidopsis***

Yong Xiang, Kazumi Nakabayashi, Jia Ding, Fei He, Léonie Bentsink and Wim J.J. Soppe  
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<b>Supplemental Data</b>	<a href="http://www.plantcell.org/content/suppl/2014/10/31/tpc.114.132811.DC1.html">http://www.plantcell.org/content/suppl/2014/10/31/tpc.114.132811.DC1.html</a>
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