**Short Title:** A Pseudophosphatase Controls Dormancy Variation

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**Title:**
Sequence Polymorphisms at the Reduced Dormancy 5 Pseudophosphatase Underlie Natural Variation in Arabidopsis Dormancy

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**One sentence summary:**
DELAY OF GERMINATION 18 QTL encodes a pseudophosphatase, involved in seed dormancy regulation.

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Seed dormancy controls the timing of germination, which regulates the adaptation of plants to their environment and influences agricultural production. The time of germination is under strong natural selection and shows variation within species due to local adaptation. The identification of genes underlying dormancy quantitative trait loci (QTL) is a major scientific challenge, which is relevant for agricultural and ecological goals. In this study, we describe the identification of the *DELAY OF GERMINATION 18 (DOG18)* QTL, which was identified as a factor in natural variation for seed dormancy in *Arabidopsis thaliana*. *DOG18* encodes a member of the clade A of the type 2C protein phosphatases family, which we previously identified as the *REDUCED DORMANCY 5 (RDO5)* gene. *DOG18/RDO5* shows a relatively high frequency of loss-of-function alleles in natural accessions restricted to northwestern Europe. The loss of dormancy in these loss-of-function alleles can be compensated by genetic factors like *DOG1* and *DOG6*, and by environmental factors such as low temperature. RDO5 does not have detectable phosphatase activity. Analysis of the phosphoproteome in dry and imbibed seeds revealed a general decrease in protein phosphorylation during seed imbibition that is enhanced in the *rdo5* mutant. We conclude that RDO5 acts as a pseudophosphatase that inhibits dephosphorylation during seed imbibition.
Survival of plants depends on their control of seed germination timing because this indirectly determines the conditions during all subsequent phases of the life cycle. Seed dormancy prevents germination under (temporary) favorable conditions and can delay seedling establishment until the onset of the growth season. This makes it an important factor in the adaptation of plants to their local environment. The level of seed dormancy is influenced by environmental conditions experienced by the mother plant and by the seeds during storage in the seed bank, in particular temperature, humidity, light, and nitrate. In *Arabidopsis thaliana* (Arabidopsis), low temperatures experienced by the mother plant during seed maturation are known to enhance seed dormancy. In contrast, low temperatures during seed imbibition promote germination (Footitt et al., 2011; Kendall et al., 2011; Graeber et al., 2012; He et al., 2014). Changes in dormancy are coupled with an altered balance between the levels of the plant hormones abscisic acid (ABA) and gibberellin (GA), which are also reflected by the transcript levels of genes controlling hormone synthesis and breakdown. ABA is required for the induction of dormancy, whereas GA is necessary for germination. The biosynthesis and signalling pathways of both hormones negatively influence each other (Nambara et al., 2010; Graeber et al., 2012). Members belonging to clade A of the type 2C protein phosphatases (PP2Cs) have important roles in ABA-dependent dormancy by inhibiting the activity of SNF1-related kinases (SnRKs) via dephosphorylation (Park et al., 2009).

*Arabidopsis* grows in large areas of the northern hemisphere and is adapted to different environments. This is reflected in seed dormancy variation between accessions and its underlying genetic variation (Springthorpe and Penfield, 2015). Therefore, it is not surprising that analyses of natural variation in seed dormancy between *Arabidopsis* accessions yielded several dormancy QTLs (Alonso-Blanco et al., 2003; Bentsink et al., 2010). A major QTL, *DELAY OF*
GERMINATION 1 (DOG1), was identified in the progeny of a cross between the low dormant accession Landsberg erecta (Ler) and the highly dormant accession Cape Verde Islands (Cvi). DOG1 has been cloned and turned out to be a conserved key regulator of seed dormancy (Bentsink et al., 2006). The amount of DOG1 protein in freshly harvested seeds showed a strong positive correlation with dormancy levels. Interestingly, DOG1 transcript and protein levels are increased by low temperatures during seed maturation, corresponding with enhanced dormancy levels (Kendall et al., 2011; Nakabayashi et al., 2012).

Here, we report the cloning and analysis of a gene underlying the seed dormancy QTL DOG18, which was identified in crosses of Ler with three other accessions (Bentsink et al., 2010). DOG18 encodes a family member of the PP2C phosphatases named REDUCED DORMANCY 5 (RDO5). We had previously identified this gene based on the low dormancy level of its mutant (Xiang et al., 2014). We now show that RDO5 functions as a pseudophosphatase that influences the seed phosphoproteome. DOG18 has extensive sequence variation among accessions and a high frequency of potential loss-of-function alleles with a geographic distribution limited to northwestern Europe. The influence of DOG18 on seed dormancy can be modified by other dormancy QTLs like DOG1 and DOG6, and by the temperature experienced by the mother plant during seed maturation.
RESULTS

Fine-Mapping of DOG18

The seed dormancy QTL DOG18 has been identified in recombinant inbred line (RIL) populations derived from crosses between Ler and the accessions Antwerpen-1 (An-1), Santa Maria da Feira-0 (Fei-0), and Kashmir-2 (Kas-2) (Bentsink et al., 2010). DOG18 is a relatively weak QTL in comparison with DOG1 and its Ler allele enhances dormancy in all three populations. DOG18 has been located in a 2 cM region on chromosome 4 (Supplemental Figure 1A; Bentsink et al., 2010). The Near-Isogenic Line DOG18-Fei-0 (NIL DOG18) contains an introgression of Fei-0 encompassing the DOG18 QTL in the Ler background and shows reduced seed dormancy compared to Ler (Figure 1A). Fine mapping of DOG18 using progeny from a cross between NIL DOG18 and Ler narrowed down its location to a 700 Kb region (Supplemental Figure 1B). A recently cloned dormancy gene, RDO5, is located in this region making it a strong candidate for the gene underlying the QTL (Xiang et al., 2014). A comparison of the RDO5 genomic sequence between the four accessions showed that An-1 and Fei-0 contain an identical one bp deletion in the second exon, leading to a frame shift and early STOP codon. The Kas-2 allele of RDO5 still encodes a full-length protein, but has four amino acid changes in comparison to the Ler allele (Figure 1B and Supplemental Figure 1C). Recently, the same gene was also identified to underlie the germination QTL IBO, which was detected in a cross between the accessions Eilenburg-0 (Eil-0) and Loch Ness-0 (Lc-0). The Lc-0 allele enhances dormancy and has a, thus far unique, single bp change causing an A to E substitution in a highly conserved PP2C motif (Amiguet-Vercher et al., 2015).

To obtain additional evidence that RDO5 is the gene underlying DOG18, complementation experiments were carried out. We transformed NIL DOG18 with a construct containing the RDO5
promoter and gene from *Ler*, fused with a C-terminal HA tag. Six independent transformants with a single introgression event were selected. All of these showed enhanced dormancy compared to *NIL DOG18* (Figure 1A). Moreover, the amount of RDO5:HA protein in the transformants

**Figure 1.** Complementation of DOG18 by *RDO5*.  
(A) Germination after different periods of dry storage of seeds from *Ler*, *NIL DOG18* and six independent transgenic *NIL DOG18* lines containing the *RDO5* gene from *Ler*. Shown are means ± SE of six to eight independent batches of seeds for each genotype.  
(B) Gene structure of *RDO5* and natural polymorphisms identified in the An-1, Fei-0 and Kas-2 accessions compared to *Ler*. Exons are shown as black boxes and introns as lines.  
(C) Immunoblot analysis of RDO5:HA protein accumulation in seeds of the six *NIL DOG18* complementation transgenic lines from (A). Coomassie Brilliant Blue (CBB) staining was used as a loading control.
correlated with their seed dormancy level confirming previous observations (Xiang et al., 2014). Lines 2 and 5 showed dormancy levels similar to the Ler accession and contained less RDO5:HA protein in their seeds than the other four transformants. Line 6 with a high RDO5:HA abundance showed a high dormancy level (Figure 1A, C). Additionally, the Fei-0 allele of RDO5 was introduced into the rdo5-1 mutant, which has very low dormancy levels (Xiang et al., 2014). Three independent homozygous single insertion transformants all showed a complete lack of complementation, indicating that the RDO5 Fei-0 allele is not functional (Supplemental Figure 1D).

**Loss-of-function RDO5 Alleles are Frequently Found in Northwestern European Accessions**

We were interested to find out whether the RDO5 Fei-0/An-1 specific allele is more widespread among Arabidopsis accessions. Therefore, a dCAPs marker was designed for this one bp deletion. A population of about 360 diverse accessions from the haplotype map collections (Li et al., 2010) was screened and the deletion was found in two additional accessions, Cam61 and Lac-3 (Supplemental Figure 2). Fei-0 and Lac-3 are moderately dormant, but An-1 and Cam61 have low dormancy levels. We investigated whether the seed dormancy level of An-1 and Cam61 could be enhanced by introducing a genomic fragment containing the functional RDO5 allele from Ler driven by its native promoter. Three independent homozygous single insertion transformants with enhanced RDO5 transcript levels in seeds were obtained for both accessions (Supplemental Figure 3A-B). All transformants showed increased dormancy levels compared to the wild-type An-1 and Cam61 accessions (Figure 2A-B). We have previously shown that the reduced dormancy phenotype of the rdo5 mutant requires enhanced transcript levels of the Arabidopsis PUMILIO 9 (APUM9) gene, which encodes a protein belonging to the conserved PUF family of RNA binding
proteins (Xiang et al., 2014). Interestingly, introduction of the wild-type Ler RDO5 gene in the An-1 and Cam61 accessions caused a significant decrease in APUM9 transcript levels in both dry seeds and those imbibed for six hours (6HAI) (Figure 2C-D). This suggests that a reduction in APUM9 transcript levels by introduction of a functional RDO5 gene contributes to the enhanced dormancy levels in these two accessions.

To identify additional alleles of RDO5, we analyzed sequences from nearly 870 accessions of the 1001 genome project with a world-wide distribution (http://1001genomes.org). Forty-two of these accessions contained RDO5 alleles that were predicted to have lost their function because of a single bp change leading to a STOP codon in the second exon, small deletions causing frame shifts and early STOP codons, large deletions, or a splice site mutation causing a frame shift (Figure 3

Figure 2. Complementation of An-1 and Cam61 by RDO5. (A) and (B), Germination after different periods of dry storage of seeds from transgenic lines containing the RDO5 Ler allele in An-1 (A) or Cam61 (B) background. Shown are averages ± SE of six to eight independent batches of seeds for each genotype. (C) and (D) qRT-PCR analysis of APUM9 transcript levels in dry and 6HAI seeds in An-1 (C), Cam61 (D) and their transgenic lines. The expression values were normalized using ACT78 as control. n = 3 biological replicates; error bars represent SE.
and Supplemental Table 1). Most of the accessions with nonsense mutations and indels are predicted to encode truncated proteins with a similar length as the protein encoded by the Fei-0 allele, for which we showed that it has lost its ability to induce dormancy (Supplemental Figure...
Interestingly, nearly all of the 42 accessions were located in a relatively small region of Europe consisting of the United Kingdom, northern France and southwestern Germany, as well as coastal regions of Sweden (Figure 4A). These regions have a mild oceanic climate with evenly dispersed rainfall and relatively small temperature changes during the year. Ri-0 and POG-0 originated from Canada and LP3413.41 originated from the USA. The accession Fei-0 originated from Portugal, but was collected in a very humid environment that is atypical for that region (personal communication, C. Alonso-Blanco). The genetic relationships among the potential loss-of-function RDO5 accessions were determined using a structure analysis, which indicated that the loss-of-function alleles of RDO5 had three different origins (Figure 4B) and can be divided in three main haplotypes representative for Sweden, Western Europe and the United Kingdom (Supplemental Figure 4). Accessions Ri-0 and POG-0 clustered with the haplotypes from Western Europe and LP3413.41 with the UK, suggesting that these accessions originated from these regions (Figure 4B). A haplotype network analysis of 132 different accessions, including the 32
predicted loss-of-function RDO5 accessions revealed the existence of 14 independent haplotypes including one cluster of 22 identical alleles with a single bp change causing a STOP codon and a second cluster of 3 alleles with a 1bp deletion including Fei-0 and An-1 alleles. In addition, multiple independent deletions causing frame shifts occurred (Figure 4C). Therefore, independent RDO5 loss-of-function alleles seem to have arisen from different parental alleles.

Factors Enhancing Seed Dormancy in Accessions Containing RDO5 Loss-of-function Alleles

We analyzed seed dormancy in 11 accessions with potential RDO5 loss-of-function alleles and found considerable variation. Several accessions showed high dormancy levels (Figure 5A), which was not expected considering the very low seed dormancy of the loss-of-function rdo5-1 mutant (Xiang et al., 2014). In addition, we observed extensive variation in dormancy within F2 progenies of crosses between the rdo5-2 mutant (in Col background) and the Fei-0 and Lac-3 accessions that contain RDO5 loss-of-function alleles (Supplemental Figure 5). These observations indicated that a loss of RDO5 can be compensated by other genetic factors. Besides DOG18, ten additional QTLs for seed dormancy have been found that could be candidates for these factors; among them, the two strongest are DOG1 and DOG6 (Bentsink et al., 2010).

To study the role of DOG1 on dormancy in the absence of functional RDO5, DOG1 protein levels were analyzed in a set of RDO5 loss-of-function accessions with varying amounts of seed dormancy. In general, low dormant accessions showed a low abundance of DOG1 protein, whereas highly dormant accessions showed high levels of DOG1. A few exceptions, like Cam61 and Fr-2 showed relatively high DOG1 protein levels with low dormancy levels, whereas HR-10 showed relatively low DOG1 protein level and a high dormancy level (Figure 5B). A comparison of the DOG1 alleles showed that two of the accessions (An-1 and Fr-2) contained the DSY
haplotype, which has a strongly reduced function compared to the ECCY DOG1 haplotype that was present in the other 8 accessions (Nakabayashi et al., 2015) (Figure 5B). When we considered the functionality of the DSY DOG1 allele to be 1/10 of that of the ECCY DOG1 allele, a positive correlation was found between DOG1 protein level and dormancy level ($R^2=0.53$, $p=0.01$) (Figure 5C). Therefore, high DOG1 protein accumulation is one possible way for seeds to hide the phenotypic effect of RDO5 loss-of-function alleles.

The restriction of natural loss-of-function RDO5 alleles to a relatively small region of
northwestern Europe could be related to temperature. We tested germination of the \textit{rdo5-1} mutant and wild-type seeds maturing at higher (22/16°C) and lower (16/14°C) temperatures. The \textit{rdo5-1} mutant completely lacked dormancy at higher temperatures, but showed increased dormancy at the...
lower seed maturation temperature (Figure 6A). This might be caused by enhanced accumulation
of DOG1 induced by low temperatures (Kendall et al., 2011; Nakabayashi et al., 2012). Indeed, the
enhanced dormancy level of rdo5-1 mutant seeds matured at low temperatures was lost in the
dog1-1 mutant background. This indicated that DOG1 is required to enhance seed dormancy at
low temperatures in the rdo5 mutant (Figure 6A). Interestingly, in contrast with DOG1, the
transcript levels of RDO5 were not affected by low temperatures (Figure 6B).

Because the RDO5 function is associated with decreased APUM9 transcript levels (Xiang et al.,
2014), we analyzed APUM9 mRNA levels at both temperature regimes. As previously
demonstrated (Xiang et al., 2014), APUM9 expression level is highly increased in the rdo5-1
background, especially in 6HAI seeds. The higher dormancy levels of seeds matured at lower
temperatures was associated with reduced APUM9 transcript levels (Figure 6C). However, the
relation between reduced dormancy and enhanced APUM9 transcription was not observed in the
dog1-1 mutant background, both in the presence and absence of rdo5-1. This suggests that DOG1
influences dormancy independently of APUM9.

The relationship of RDO5/DOG18 with DOG6 was assessed by combining the rdo5-1 mutant (in a
Ler background) with an introgression from the Shahdara accession containing a strong DOG6
allele (Figure 5D). Analysis of germination of freshly harvested seeds showed that DOG6 can
enhance the dormancy level of rdo5-1, indicating an additive relation between DOG6 and
RDO5/DOG18. We also analyzed the APUM9 transcript levels in the NIL DOG6 lines.
Interestingly, we found that the enhancement in seed dormancy caused by the NIL DOG6
introgression was associated with strongly reduced APUM9 transcript levels, both in the wild type
and the rdo5-1 mutant background (Figure 5E). This indicated that APUM9 is not specific for the
RDO5 pathway, but has a more general role in seed dormancy.
RDO5 Functions as a Pseudophosphatase

RDO5 is a member of the PP2C phosphatase family and enhances seed dormancy (Xiang et al., 2014). Although RDO5 has strong homology with PP2Cs, it does not show complete sequence conservation in amino acids essential for phosphatase activity. Amiguet-Vercher et al described that in particular, RDO5 lacks conserved residues for metal coordination and phosphate binding (Amiguet-Vercher et al., 2015). Interestingly, the D111 residue in RDO5 is a conserved G in active PP2Cs. The replacement of this G to D in the clade A PP2C phosphatases HAB1, ABI1, ABI2 and AHG3 caused loss of activity (Rodriguez et al., 1998; Gosti et al., 1999; Robert et al., 2006; Yoshida et al., 2006). RDO5 also lacks about 40 amino acids in front of the MAPK docking site, which is important for protein interaction (Supplemental Figure 6, 7 A-B). Gosti et al identified five amino acid substitutions in this region in ABI1 that led to loss of activity indicating that this domain is also important for activity (Gosti et al., 1999). In addition, RDO5 lacks a tryptophan residue that is involved in the interaction with ABA in most ABA responsive PP2Cs. This may explain why RDO5 does not affect ABA sensitivity (Xiang et al., 2014).

These observations suggest that RDO5 is not a catalytically active protein phosphatase. In accordance, Amiguet-Vercher et al demonstrated very low phosphatase activity for RDO5 using \textit{in vitro} phosphatase assays (Amiguet-Vercher et al., 2015). We further confirmed these observations and did not detect any phosphatase activity for RDO5 using the synthetic phospho-peptide RRA(phosphoT)VA as substrate in the presence of either Mn\textsuperscript{2+} or Mg\textsuperscript{2+} as metal cofactor (Figure 7A). We were interested whether the phosphatase activity of RDO5 could be restored by introducing back mutations to recover all the missing residues known to affect PP2C activity (RDO5bm). Introduction of these back mutations into the RDO5 sequence indeed restored the
Figure 7. RDO5 functions as a pseudophosphatase. 
(A) Phosphatase activity is restored in RDO5 after backmutations. Phosphatase activity of RDO5 and RDO5 back-mutation (RDO5bm) proteins was measured in vitro using the RRA(phosphoT)VA peptide as a substrate. ΔC-terN88AHG3 was used as a positive control. Data are averages ± SE from three replicates. 
(B) Venn diagram analyses showing common and differential distribution of phosphorylated sites identified in NIL DOG1 and rdo5-1 after six hours imbibition compared with dry seeds. 
(C)-(E) Localization in the SeedNet network of differentially phosphorylated proteins from wild-type NIL DOG1 (C), rdo5-1 (D) and differentially phosphorylated proteins present in rdo5-1 but not in NIL DOG1 (E) after six hours imbibition compared with dry seeds. The regions outlined in green correspond to clusters associated with dormancy (region 1) or germination (regions 2 and 3). The red dots represent proteins with decreased phosphorylation levels and the blue dots represent proteins with increased phosphorylation levels. 

phosphatase activity of RDO5 (Figure 7A, Supplemental Figure 6). RDO5bm displayed a specific activity of 29.1 pmol.min⁻¹.mg⁻¹ and 20.2 pmol.min⁻¹.mg⁻¹ with Mg²⁺ or Mn²⁺ as cofactors respectively. This activity is in a similar range as that of AHG3. These results suggest that RDO5
probably evolved from a functional PP2C phosphatase but has lost its phosphatase activity. Proteins homologous to phosphatases lacking essential amino acids for activity have previously been described as pseudophosphatases or anti-phosphatase proteins that might prevent specific residues from becoming dephosphorylated or phosphorylated by protecting them from real phosphatases or kinases (Reiterer et al., 2014). Thus we studied whether RDO5 could be a pseudophosphatase indirectly implied in the control of protein phosphorylation levels by comparing the phosphoproteome of the rdo5-1 mutant with its wild-type NIL DOG1 in dry and 6 HAI seeds. A label-free quantitative mass spectrometry analysis on three biological replicates for each genotype and treatment identified 1527 phosphorylation sites in 875 protein groups. Imbibition of seeds caused a general decrease in phosphorylation, which was enhanced in the rdo5 mutant (Figure 7B). In the wild-type NIL DOG1 background 380 phosphorylation sites in 238 proteins were significantly decreased after imbibition for 6 hours, whereas 26 phosphorylation sites in 20 proteins increased in phosphorylation status compared to dry seed. In the rdo5-1 mutant 529 phosphorylation sites in 289 proteins were significantly decreased and 23 phosphorylation sites in 17 proteins increased in phosphorylation status compared with dry seed (Supplemental Table 2-3). In total, we found 229 phosphorylation sites with decreased phosphorylation levels and 12 phosphorylation sites with increased phosphorylation levels in rdo5-1 compared to wild type (Figure 7B). These results indicated a general decrease in protein phosphorylation during seed imbibition that is enhanced in the rdo5 mutant. We used SeedNet, a topological model of transcriptional interactions controlling dormancy and germination in Arabidopsis (Bassel et al., 2011), to study the relationship between the transcriptome and protein phosphorylation in imbibed seeds. The majority of differentially phosphorylated proteins after 6 HAI are located within region 1 that is associated with dormancy and in a section of region 2 that does not contain germination
associated genes. (Figure 7C-D) (Bassel et al., 2011). Proteins with altered phosphorylation levels
specific in the rdo5 mutant showed the same pattern (Figure 7E). These results suggest that the
regulation of protein phosphorylation plays a role in the release of seed dormancy during early
imbibition of seeds. Proteins directly involved in ABA signaling were not found among those
with significant changes in phosphorylation level in rdo5-1 seeds compared to the wild-type. This
confirmed our previous observations that RDO5 is probably not part of the ABA pathway (Xiang
et al., 2014).
RDO5 controls protein phosphorylation levels despite its lack of phosphatase activity. This control
might occur by protecting proteins from dephosphorylation during imbibition because we
identified more proteins with decreased phosphorylation levels in the rdo5 mutant than in the wild
type. Thus, our biochemical and proteomic results suggest that RDO5 acts as a
pseudophosphatase.
In contrast to the changes observed in phosphoprotein abundance, from 5017 quantified protein
groups only 106 (41 up-regulated and 65 down-regulated with a log2-fold change > 1) were
significantly altered in abundance in wild type after 6h imbibition in comparison to dry seeds. In
the rdo5 mutant 132 proteins groups (48 up-regulated and 84 down-regulated with a log2-fold
change > 1) were significantly altered upon imbibition (Supplemental Data set 1). Interestingly,
only 29 of these regulated proteins overlapped between both genotypes. A SeedNet analysis
showed that differentially expressed proteins are evenly located in the network (Supplemental
Figure 8). Therefore, regulation of protein modifications seems to be more prominent during early
seed imbibition compared to regulation of protein levels.
DISCUSSION

Seed dormancy has a high adaptive value for plants. Hence, it is not surprising that extensive natural variation exists for this trait. Exploring and identifying the underlying genes of this variation is important to understand plant adaptation and helpful to study the molecular mechanisms of dormancy. In Arabidopsis, several seed dormancy QTLs have been found. A major dormancy QTL locus, *DOG1*, was cloned nearly ten years ago and has been shown to be conserved within the plant kingdom (Ashikawa et al., 2010; Graeber et al., 2010). Here we report the identification of the underlying gene of a second dormancy QTL, *DOG18*. 

*DOG18* encodes a member of the type 2C protein phosphatase family that we previously identified as *RDO5*, which is a positive regulator of seed dormancy (Xiang et al., 2014). Natural variation at this gene has recently been described by the identification of a specific allele, IBO, which has a unique mutation in the Loch Ness accession, probably causing enhanced activity (Amiguet-Vercher et al., 2015).

We identified predicted loss-of-function *RDO5* alleles at a relatively high frequency in Arabidopsis accessions from northwestern Europe but could not detect any from other parts of the world (Figure 4A). Absence of RDO5 function could be an adaptation to the northwestern European climate of mild temperatures with low fluctuations and evenly dispersed rainfall. Interestingly, the non-dormant phenotype of the *rdo5-1* loss-of-function allele can be partially rescued when plants are grown at low temperatures. In addition, RDO5 enhances dormancy but is not essential for it, since accessions with loss-of-function *RDO5* alleles vary considerably in their dormancy levels (Figure 5A). This is consistent with the relatively weak effect on seed dormancy of the *DOG18* QTL compared with *DOG1* and *DOG6* (Bentsink et al., 2010). We assume that *RDO5* might have a neutral role or could even be under negative selection under specific environmental conditions prevalent in northwestern Europe. This could have led to the occurrence...
of natural loss-of-function alleles that subsequently spread through the population. Interestingly, a QTL analysis of dormancy in two accessions from north-central Sweden and central Italy identified DOG18 under greenhouse conditions but not in populations grown in the field. This is likely due to the lower temperatures in the field compared to the greenhouse, which mask the role of DOG18 in dormancy (Postma and Agren, 2015).

We have identified two dormancy QTLs, DOG1 and DOG6, that can partially rescue the rdo5 mutant phenotype. Therefore, these QTLs contribute to explain differences in dormancy level between RDO5 loss-of-function accessions. These results also confirm that different QTLs have additive roles in seed dormancy as previously suggested by Bentsink et al (Bentsink et al., 2010). Interestingly, in contrast to DOG18, naturally occurring loss-of-function alleles have never been identified for DOG1. This is consistent with the inability to induce seed dormancy in dog1 mutant plants grown at low temperatures during seed maturation and the absence of loci that enhance dormancy in dog1 loss-of-function alleles.

Despite its similarity to PP2C phosphatases, RDO5 is unlikely to be catalytically active as a phosphatase because it lacks several conserved residues. In accordance, we could not detect any phosphatase activity for RDO5. Amiguet-Vercher et al also reported a very low phosphatase activity for IBO/RDO5 (Amiguet-Vercher et al., 2015). It is likely that RDO5 has evolved from a functional phosphatase because we could recover its phosphatase activity by introducing amino acid changes to restore critical residues needed for activity (Figure 7 A, Supplemental Figure 6). These observations led us to propose that RDO5 could function as a pseudophosphatase.

Pseudophosphatases have been described in humans and nematodes. For instance, human TAK-binding protein 1 (TAB1) is a member of the PPM family of protein phosphatases that does not show phosphatase activity. TAB1 possibly functions by binding to and controlling the
accessibility of phosphorylated residues on a kinase protein or its downstream substrates, thereby regulating pro-inflammatory signaling pathways (Conner et al., 2006). Pseudophosphatases, as well as pseudokinases, are increasingly seen as regulators of signaling pathways that can act as modulators, competitors or anchors of kinases and phosphatases (Reiterer et al., 2014).

Our comparison of the phosphoproteomes between wild-type NIL DOG1 and the rdo5 mutant showed that RDO5 influences the phosphorylation status of hundreds of proteins, despite its lack of phosphatase activity. This is in agreement with its function as a pseudophosphatase. We suggest that RDO5 may bind to its target proteins to prevent them from dephosphorylation by other active protein phosphatases. Such a role for RDO5 would suggest an opposite function compared to other phosphatases. This hypothesis is supported by the positive role of RDO5 in seed dormancy (Xiang et al., 2014), which is contrasting with the negative roles of clade A PP2Cs like ABI1, ABI2, AHG1, and HAI2 (Rodriguez et al., 1998; Gosti et al., 1999; Nishimura et al., 2007; Kim et al., 2013). Amiguet-Vercher recently proposed a role for a unique natural allele of RDO5/IBO (from the Lc-0 accession) in ABA signaling because of its inhibitory influence on ABI phosphatase activity (Amiguet-Vercher et al., 2015). However, our phosphoproteome analysis did not give support for a general role of RDO5 in the ABA pathway because proteins involved in ABA signaling were not found among those with significant changes in phosphorylation level in rdo5-1 mutant seeds. In addition, mutations in RDO5 neither affected ABA levels in dry and imbibed seeds nor ABA sensitivity during germination (Xiang et al., 2014). We observed a massive dephosphorylation during seed imbibition, which was enhanced in the rdo5 mutant. Therefore, RDO5 probably controls seed dormancy by preventing dephosphorylation during seed imbibition. Interestingly, changes in protein levels were relatively minor during imbibition compared to changes in phosphorylation levels.
We have previously identified the mRNA binding protein APUM9 to act downstream of RDO5 (Xiang et al., 2014). In this work, we identified a general role for APUM9 in seed dormancy that is not restricted to the RDO5 pathway. Factors that enhance dormancy like low seed maturation temperatures and the DOG6 QTL reduce APUM9 transcript levels (Figure 6C and 5E). Therefore APUM9 might be a common downstream factor of these dormancy pathways. However, APUM9 is probably not part of the DOG1 dormancy pathway because its transcript levels are not affected by DOG1 (Figure 6C). This again confirms regulation of dormancy by independent mechanisms.

The first two identified dormancy QTLs, DOG1 and RDO5, are both only expressed in seeds and their mutants exclusively show dormancy and germination defects without pleiotropic phenotypes (Bentsink et al., 2006; Xiang et al., 2014). It is probably no coincidence that these two modifiers of natural variation for seed dormancy are seed-specific. Sequence polymorphisms leading to changes in the expression levels of these genes or their protein function represent an excellent way for plants to adapt their dormancy levels to local environments without simultaneously altering other traits. The properties of DOG1 and RDO5 make them highly suitable target genes to manipulate and control seed dormancy levels in crop plants.

CONCLUSIONS

Germination timing is an important adaptive trait for plants and is controlled by seed dormancy. Arabidopsis accessions collected in nature show a high variation for dormancy, making it a typical quantitative trait. Finding the genes underlying dormancy quantitative trait loci is a major scientific challenge, which is relevant for agricultural and ecological goals. Here we identified the gene RDO5 to underlie the dormancy QTL DOG18. We show that the RDO5 protein can function as a pseudophosphatase. We found a relatively high number of predicted natural loss-of-function
alleles for *RDO5* that were restricted to northwestern Europe. The loss of RDO5 function could be compensated by low temperatures and by strong alleles of the dormancy QTLs *DOG1* and *DOG6.*

**MATERIALS AND METHODS**

**Plant Materials**

NIL DOG6 and NIL DOG18 were a gift from Leónie Bentsink (Wageningen University, the Netherlands). *RDO5* loss-of-function accessions were obtained from the collection of Maarten Koornneef (MPIPZ Cologne, Germany) and originated from the Nottingham Arabidopsis Stock Centre. The *rdo5-1* mutant was described before (Xiang et al., 2014) and has been obtained in a NIL DOG1 background consisting of the Ler accession with an introgression on chromosome 5, containing the *DOG1* gene from Cvi (Alonso-Blanco et al., 2003). The *rdo5-1* mutant allele in Ler background was obtained by crossing *rdo5-1* with Ler and selection against the Cvi introgression fragment in the progeny. Germination tests were performed as described previously (Xiang et al., 2014). Information of primers used in this study can be found in Supplemental Table 4.

**DOG18 Map Based Cloning and Complementation Analysis**

*DOG18* mapping was performed by backcrossing NIL DOG18-Fei-0 with Ler. Genotyping F2 plants with cleaved-amplified polymorphic sequence markers narrowed the *DOG18* interval into a 700 kb region on chromosome 4, which included the *RDO5* gene. For NIL DOG18-Fei-0 complementation, a genomic fragment, including a 2.7-kb promoter sequence and the *RDO5* coding sequence fused with the HA tag, was amplified from Ler using gene-specific primers (the HA tag was designed in the reverse primer), cloned into the pDONR207 vector, and transferred into pFASTR01 (Shimada et al., 2010). The binary construct was introduced by
electroporation into *Agrobacterium tumefaciens* strain GV3101, which was subsequently used for transformation by floral dipping (Clough and Bent, 1998). T3 homozygous lines containing single insertion events were used for expression level detection and phenotyping.

**Analysis of DOG18 loss-of-function Natural Variants**

The *RDO5* sequence from 855 available accessions was downloaded from Salk *Arabidopsis thaliana* 1001 Genomes. The different types of loss-of-function *RDO5* alleles were confirmed by Sanger sequencing including non-sense mutations (POG-0, HR-10, NFA-8, NFA-10 and Vind-1), Indels (Cam61, Lac-3, An-1, Fei-0 and Durh-1), deletions (Vaar2-6), gene loss (Fr-2), and splicing site mutations (Boot-1). The whole *RDO5* cDNA was sequenced in the accessions of the last four types to confirm the absence of mutations that might restore RDO5 function. For the structure analysis, population structure was inferred using model-based clustering algorithms implemented in the software STRUCTURE, based on 149 genome-wide SNP markers (Lewandowska-Sabat et al., 2010; Platt et al., 2010), using the haploid setting and running 20 replicates with 50,000 and 20,000 MCMC (Markov chain Monte Carlo) iterations of burn-in and after-burning length, respectively (Hubisz et al., 2009). To determine the K number of significantly different genetic clusters, we applied the ΔK method in combination with the absolute value of ln P (X|K) (Evanno et al., 2005) implanted in STRUCTURE HARVESTER (Earl and Vonholdt, 2012). For Haplotype Network analysis, the RDO5 nucleic acid sequence was translated into amino acid sequence according to the standard genetic codes basing on the TAIR 10 ORF. The haplotype network of RDO5 was constructed using Network 4.6 that implements a Reduced Median method (Bandelt et al., 1995).
RNA Extraction, RT-PCR, Protein Extraction and Western Blotting were performed as described previously (Xiang et al., 2014).

### 3-Dimensional Protein Modeling

A 3D model of full length RDO5 sequence (UniProtKB entry Q9T010 295 amino acids) was constructed using Swiss model software (Arnold et al., 2006). The crystal structure of probable protein phosphatases from rice (UniProtKB entry Q0JLP9 / PDB entry 4oic chain B) was used as template. The model was superimposed on the crystal structure of the complex HAB1/SnRk2,6 (PDB entry: 3ujg chain B) (Soon et al., 2012) using PDBBeFold (http://www.ebi.ac.uk/msd-srv/ssm/).

### Protein Purification and PP2C Activity Assay

The coding sequences of RDO5 from Ler and Fei-0, were amplified from cDNA using gene specific primers. The full length of AHG3 was poorly soluble in E.coli, therefore an N terminal deleted AHG3 (ΔNter-N88AHG3) protein was cloned using gene specific primers. N terminal deleted PP2C are reported to be active (Dupeux et al., 2011). RDO5 Ler and ΔNter-N88AHG3 coding sequence were introduced in pDEST17 expression vector to generate an expression clone with proteins fused with a 6xHis tag in N-terminal. For back mutation (RDO5bm), mutated RDO5 sequences were synthesized by the Life Technologies Company. To improve solubility of the back-mutated RDO5 protein we generated a construct where RDO5bm is fused with HIS: MBP: TEV in N-terminal. For this purpose, the synthesized sequence was elongated by PCR using gene specific primers to introduce N-terminal fusion TEV cleavage site. Then the PCR product was recombined in pDONR207 and subsequently in pDEST-HIS-MBP (Addagene), constructs were
introduced in a BL21-plyss strain. Fusion proteins were induced by addition of 0.3mM IPTG in the medium. After overnight culture at 23 °C, cells were harvested by centrifugation and pellets were stored at –80 °C until purification. Pellets were re-suspended in 50mM HEPES (pH 7.5), 500 mM NaCl, 10 % (v/v) Glycerol, 5 mM DTT, 1 mM PMSF and 25 mM Imidazole, and cells were disrupted by sonication. The soluble fraction was obtained after centrifugation and applied on HisTrap column 1 mL (GE health, USA); the purification process was performed and monitored using ÄKTaPrime and Primeview software (GE Health, USA). Elution was performed with the resuspension buffer supplemented with 500mM imidazole. Imidazole was removed from purified protein by gel filtration on PD-10 columns (GE Health, USA) and the protein fraction was concentrated to about 2 mg.ml⁻¹ and stored at –80°C. Purified His:MBP:TEV tagged recombinant protein was incubated with HIS:TEV protease and subsequently applied on Ni-NTA agarose (Qiagene, USA) to remove the majority of the His:MBP:TEV tag and HIS:TEV protease. The purity of purified proteins was assessed by SDS-PAGE (Supplemental Figure 7C). Phosphatase activity was measured using the RRA(phosphoT)VA peptide as a substrate. The Serine/Threonine Phosphatase Assay system (Promega, USA) was used for the phosphopeptide assay. Phosphatase assays were performed in 50 μl volumes in half-area, flat-bottom 96 wells plate containing 50 mM Hepes buffer (pH 7.5), 10 mM MnCl₂ or MgCl₂, 1 mM dithiothreitol (DTT), 0.5μM purified recombinant PP2C proteins and 100 μM peptide substrate. Reaction were performed at 30 °C for 30 min and stopped by addition of 50 μL of molybdate dye solution. Absorbance was read at 630 mM on a multiscanspektrum (Thermo) 96 well plate reader.

Proteolytic Digestion and Desalting

Total protein was extracted in buffer containing 100mM TrisHCl (pH 7.5), 3% SDS, 100mM
DTT, 1X protease inhibitor cocktail, Phosphatase Inhibitor Cocktail 2 and 3 (Sigma, USA). Seed protein extract (2 mg) were processed using the FASP method (Wisniewski et al., 2009) as described in detail in Hartl et al. (2015). Cysteines were alkylated by incubation with 55 mM chloroacetamide in UA buffer (8 M urea, 0.1 M Tris/HCl pH 8.5) for 30 min at room temperature in the dark followed by three washing steps with UA buffer. Incubation with LysC in UA buffer (1:50 enzyme-to-protein ratio) was performed overnight at room temperature. The sample was diluted with ABC buffer (50 mM ammonium bicarbonate) to a final urea concentration of 2 M urea. Trypsin digestion (1:100 enzyme-to-protein ratio) was performed for 4 h at room temperature. The sample was passed through the filter by centrifugation at 14,000 g for 10 min and residual peptides eluted with 50 µl ABC. Formic acid was added to a final concentration of 0.5% and the sample was desalted using Sep-Pak SPE 1 cc/100 mg (Waters). Solvent was passed through by gravity flow. Columns were conditioned by successive addition of methanol, buffer B (80% acetonitrile (ACN) and 0.5% formic acid (FA)), and buffer A (0.5% FA). The sample was loaded and washed three times with buffer A and eluted twice with buffer B. Eluates were concentrated in a centrifugal evaporator to remove acetonitrile. One tenth of the eluted peptides were processed for whole proteome analysis, the rest was subjected to titanium dioxide enrichment. For total proteome analysis, peptides were desalted and pre-fractionated prior LC-MS/MS into three fractions using the Empore Styrenedivinylbenzene Reversed Phase Sulfonate material (SDB-RPS, 3M) as described in detail by Kulak et al. (2014).

**Phosphopeptide Enrichment**

Phosphopeptides were enriched with a modified version of the protocol of Melo-Braga et al. (2015). Samples were diluted to 900 µl with 50% ACN, 1% TFA, then 100 µl loading buffer 200
mg/ml DHB in 50% ACN, 1% TFA was added. Incubation steps were performed under gentle agitation at room temperature. 6 mg TiO₂ bead (5 mm; GL Science) were suspended in 40 µl loading buffer (20 mg/ml DHB, 50% ACN, 1% TFA) and incubated for 15 min. The first half of the suspension was added to the sample and incubated for 15 min. For removal of liquid, beads were pelleted by centrifugation at 3000 rpm for 1 min. The supernatant was transferred to the second half of the beads suspension and incubated for 15 min. TiO₂ beads from both incubations with bound phosphopeptides were resuspended with loading buffer and combined on C8 StageTips (Empore, 3M). Liquid was passed though the tips by centrifugation at 800 g. The beads were washed twice with 80% ACN, 1% TFA and twice with 10% ACN, 0.1% TFA. Phosphopeptides were eluted three times with 5% ammonia and once with 30% ACN by centrifugation at 300 g. Eluates were combined and concentrated in a centrifugal evaporator to remove ammonia. Samples were acidified by adding 1/10 volume of 20% ACN, 10% TFA and desalted with StageTips (Empore C18, 3M) as described as Rappsilber et al. (2007).

**LC-MS/MS Data Acquisition**

Dried peptides were redissolved in 2% ACN, 0.1% TFA for analysis and adjusted to a final concentration of 0.18 µg/µl. Samples were analyzed using an EASY-nLC 1000 (Thermo Fisher) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher). Peptides were separated on 16 cm frit-less silica emitters (New Objective, 0.75 µm inner diameter), packed in-house with reversed-phase ReproSil-Pur C18 AQ 3 µm resin (Dr. Maisch). Peptides (1 µg) were loaded on the column and eluted for 120 min using a segmented linear gradient of 0% to 95% solvent B (solvent A 5% ACN, 0.5% FA; solvent B 100% ACN, 0.5% FA) at a flow-rate of 250 nL/min. Mass spectra were acquired in data-dependent acquisition mode with a TOP15 method. MS spectra were
acquired in the Orbitrap analyzer with a mass range of 300–1750 \( m/z \) at a resolution of 70,000 FWHM and a target value of \( 3 \times 10^6 \) ions. Precursors were selected with an isolation window of 1.3 \( m/z \). HCD fragmentation was performed at a normalized collision energy of 25.

MS/MS spectra were acquired with a target value of \( 10^5 \) ions at a resolution of 17,500 FWHM and a fixed first mass of \( m/z \) 100. Peptides with a charge of +1, greater than 6, or with unassigned charge state were excluded from fragmentation for MS2, dynamic exclusion for 30s prevented repeated selection of precursors.

**MS Data Analysis**

Raw data were processed using MaxQuant software (version 1.5.3.12, http://www.maxquant.org/) with label-free quantification (LFQ) enabled (Cox et al., 2014). MS/MS spectra were searched by the Andromeda search engine against the Arabidopsis TAIR10_pep_20101214 database (ftp://ftp.arabidopsis.org/home/tair/Proteins/TAIR10_protein_lists/). 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 allowed. Minimal peptide length was set to seven amino acids. Carbamidomethylation of cysteine residues was set as fixed, oxidation of methionine and protein N-terminal acetylation as variable modifications. Phosphorylation of serine, threonine, and tyrosine were added as variable PTMs only for the TiO2 enriched samples. Peptide-spectrum-matches and proteins were retained if they were below a false discovery rate of 1%. Subsequent quantitative statistical analyses were performed in Perseus (version 1.5.2.6, http://www.maxquant.org/). Identified proteins and phosphorylation sites were processed separately as follows: Hits were only retained if they were quantified in at least two of three replicates in any of the four conditions. LFQ intensities (proteins) and peptide intensities
(phosphorylation sites) were log2 transformed. Two-sample t-tests were performed with a p-value of 1% as cut-off. Log2 ratios were calculated by replacing missing intensity values with zero.

**Distribution of Proteins in SeedNet Network**

Differently phosphorylated or expressed proteins were submitted to the cytoscape software loading SeedNet network data files, which is publicly available at [http://www.vseed.nottingham.ac.uk](http://www.vseed.nottingham.ac.uk).

**Accession Numbers**

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).

**Supplemental data**

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

**Supplemental Figure 1.** Map based cloning of *DOG18*.

**Supplemental Figure 2.** *DOG18* Fei-0 allele screening.

**Supplemental Figure 3.** *RDO5* transcript level detection in transgenic lines.

**Supplemental Figure 4.** Structure estimation of populations for K ranging from one to fourteen by delta K-values.

**Supplemental Figure 5.** Germination of *rdo5* segregating populations.

**Supplemental Figure 6.** *RDO5* sequence analysis.

**Supplemental Figure 7.** *RDO5* topology structure analysis and SDS PAGE gel of purified
proteins.

Supplemental Figure 8. Localization of differently expressed proteins in the SeedNet network after 6 hours imbibition in wild-type NIL DOG1 (A), rdo5-1 (B) and potential RDO5 specific targets (C).

Supplemental Table 1. Information of 42 loss-of-function DOG18 accessions including mutation type, accession name, ID, country, origin, habitat, latitude and longitude of collection site.

Supplemental Table 2. Overview of quantitative mass spectrometry results.

Supplemental Table 3. Overview of regulated phosphosites and phosphorylated protein groups.

Supplemental Table 4. Primers used in this study.

Supplemental Data Set 1. Mass spectrometry results from the phosphoproteome analysis of rdo5-1 and NIL-DOG1.

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FIGURE LEGENDS

Figure 1. Complementation of DOG18 by RDO5.

(A) Germination after different periods of dry storage of seeds from Ler, NIL DOG18 and six independent transgenic NIL DOG18 lines containing the RDO5 gene from Ler. Shown are means ± SE of six to eight independent batches of seeds for each genotype. (B) Gene structure of RDO5
and natural polymorphisms identified in the An-1, Fei-0 and Kas-2 accessions compared to Ler. Exons are shown as black boxes and introns as lines. (C) Immunoblot analysis of RDO5:HA protein accumulation in seeds of the six NIL DOG18 complementation transgenic lines from (A). Coomassie Brilliant Blue (CBB) staining was used as a loading control.

**Figure 2. Complementation of An-1 and Cam61 by RDO5.**

(A) and (B), Germination after different periods of dry storage of seeds from transgenic lines containing the RDO5 Ler allele in An-1 (A) or Cam61 (B) background. Shown are averages ± SE of six to eight independent batches of seeds for each genotype. (C) and (D) qRT-PCR analysis of APUM9 transcript levels in dry and 6HAI seeds in An-1 (C), Cam61 (D) and their transgenic lines. The expression values were normalized using ACT8 as control. n = 3 biological replicates; error bars represent SE.

**Figure 3. Natural loss-of-function mutations in the DOG18 gene.**

Natural mutations causing predicted DOG18 loss-of-function alleles are divided into five groups that are shown in separate rows. The location of the mutations for the individual accessions are indicated. Exons (black boxes) are connected with horizontal lines representing intronic regions of DOG18. The location of the predicted PP2C domain is indicated at the top.

**Figure 4 Geographic distribution, population structure and haplotype network of accessions containing RDO5 loss-of-function alleles.**

(A) Geographical distribution of 42 accessions harboring RDO5 predicted loss-of-function alleles. (B) Population structure of 95 accessions containing functional or predicted loss-of-function
**Figure 5 Genetic modifiers of the RDO5 phenotype.**

(A) Dormancy level of 11 accessions with predicted loss-of-function RDO5 alleles. Col and Cvi are used as low and high dormancy controls. POG-0, HR-10, NFA-8, Vind-1 and NFA-10 belong to the non-sense mutation group, An-1, Fei-0, Lac-3, Cam61 and Durh-1 belong to the indels group, and Fr-2 belongs to the gene loss group. Shown are means ± SE of six to eight independent batches of seeds for each genotype. DSDS50, days of seed dry storage required to reach 50% germination. (B) Immunoblot analysis of DOG1 protein accumulation using a DOG1 antibody in the 11 accessions with predicted loss-of-function RDO5 alleles. A band from the CBB staining was used as a loading control. The lower panel indicates the DOG1 haplotype of the accessions, ECCY is a strong allele and DSY is a weak allele (Nakabayashi et al., 2015). (C) The correlation of dormancy level and DOG1 protein abundance (quantified from Figure 5B and normalized by the loading control). (D) A strong DOG6 allele from the Shahdara accession enhances dormancy of the *rdo5-1* mutant (in *Ler* background). Germination percentages were determined in freshly harvested seeds. Shown are means ± SE of six to eight independent batches of seeds for each genotype. **P < 0.01 (E) qRT-PCR analysis of *APUM9* transcript levels in *Ler, rdo5-1* (*Ler* background), NIL DOG6 and *rdo5-1* NIL DOG6 dry and 6HAI seeds. Inset, *APUM9* expression in dry seeds from the different genotypes at a magnified scale. The expression values were
normalized using ACT8 as control. n = 3 biological replicates; error bars represent SE.

Figure 6. Maternal temperature affects the RDO5 dormancy phenotype.

(A) Germination of NIL DOG1, rdo5-1, dog1-1 and rdo5-1 dog1-1 freshly harvested seeds that matured under a day/night regime of 22/16°C or 16/14°C. Shown are means ± SE of six to eight independent batches of seeds for each genotype. (B) qRT-PCR analysis of RDO5 transcript levels in NIL DOG1 dry and 6HAI seeds that matured under a day/night regime of 22/16°C or 16/14°C. The expression values were normalized using ACT8 as control. n = 3 biological replicates; error bars represent SE. (C) qRT-PCR analysis of APUM9 transcript levels in NIL DOG1, rdo5-1, dog1-1 and rdo5-1 dog1-1 dry and 6HAI seeds that matured under a day/night regime of 22/16°C or 16/14°C. The expression values were normalized using ACT8 as control. n = 3 biological replicates; error bars represent SE.

Figure 7. RDO5 functions as a pseudophosphatase.

(A) Phosphatase activity is restored in RDO5 after backmutations. Phosphatase activity of RDO5 and RDO5 back-mutation (RDO5bm) proteins was measured in vitro using the RRA(phosphoT)VA peptide as a substrate. ΔC-terN88AHG3 was used as a positive control. Data are averages ± SE from three replicates. (B) Venn diagram analyses showing common and differential distribution of phosphorylated sites identified in NIL DOG1 and rdo5-1 after six hours imbibition compared with dry seeds. (C)-(E) Localization in the SeedNet network of differentially phosphorylated proteins from wild-type NIL DOG1 (C), rdo5-1 (D) and differentially phosphorylated proteins present in rdo5-1 but not in NIL DOG1 (E) after six hours imbibition compared with dry seeds. The regions outlined in green correspond to clusters associated with
dormancy (region 1) or germination (regions 2 and 3). The red dots represent proteins with decreased phosphorylation levels and the blue dots represent proteins with increased phosphorylation levels.


