The control of germination by the PP2C family protein RDO5

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DECLARATION

I hereby declare that the whole PhD thesis is my own work, except where explicitly stated otherwise in the text or in the bibliography.

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Abbreviations

GA	Gibberellic acid
ABA	Abscisic Acid
IAA	INDOLE-3-ACETIC ACID
SA	SALICYLIC ACID
JA	JASMONIC ACID
BRs	BRASSINOSTEROIDS
CTKs	CYTOKININS
SLs	STRIGOLACTONES
ET	ETHYLENE
Ca	Calcium
HAI	Hours After Imbibition
DAP	Days After Pollination
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine Tetraacetic Acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kD	Kilo Dalton
-LW	Media without leucine and tryptophan
-LWH	Media without leucine, tryptophan and histidine
3'AT	3-Amino-1,2,4-triazole
M	Molar
min	Minutes
MS	Murashige-Skoog
PCR	Polymerase Gel Electrophoresis
PEG	Polyethylene Glycol
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Real time PCR
RH	Relative Humidity
RNA	Ribonucleic acid
rpm	Rotation per minute
SDS	Sodium Dodecyl Sulfate
Ser/Thr	Serine Threonine
T-DNA	Transferred DNA
Tyr	Tyrosine
U	Units
v/v	Volume/volume
w/v	Weight/volume

Gene and names	
RDO5	REDUCED DORMANCY5
DOG1	DELAY OF GERMINATION 1
CaM1	CALMODULIN1
CaM4	CALMODULIN4
SNRK	SUCROSE NON FERMENTING1-RELATED PROTEIN KINASE
AHG1	ABA-HYPERSENSITIVE GERMINATION 1
AHG3	ABA-HYPERSENSITIVE GERMINATION 3
ABI5	ABA-INSENSITIVE 5
ABF2	ABSCISID ACID RESPONSIVE ELEMENTS-BINDING FACTOR2
NCED	9′-CIS-EPOXYCAROTENOID DIOXYGENASE
CYP707A	ABA 8′-HYDROXYLASE
Col	*Arabidopsis thaliana* Columbia accession
L*er*	*Arabidopsis thaliana* Landsberg *erecta* accession

Summary

Dormancy provides a mechanism to prevent germination during unfavorable conditions, which is one of the most important process in higher plants. Apart from the phytohormones abscisic acid (ABA) and gibberellic acid (GA), two proteins namely DOG1 and RDO5 have been demonstrated to play an important role in controlling seed dormancy. *DOG1* encodes a functionally unknown protein. Recent research revealed that DOG1 regulates seed dormancy by negatively regulating the actions of AHG1 and AHG3. Meanwhile, RDO5 was identified as another important factor in controlling seed dormancy. RDO5 does not contain phosphatase activity, which suggests that it might function as a pseudo-phosphatase. Phosphoproteomics analysis of the *rdo5* mutant showed a significant alternation in the phosphorylation level of several proteins, suggesting that RDO5 affects protein phosphorylation indirectly. RDO5 pull-down and mass spectrum experiments identified some RDO5 interacting proteins include DOG1, AHG1, CaM1 and CaM4. Protein interaction experiments further showed that RDO5 can form a complex with DOG1, AHG1, CaM1 and CaM4. Genetics evidence suggested that AHG1 and AHG3 are required for the function of RDO5 in the regulation of seed dormancy. Isotope experiments indicated that CaM1/4 and RDO5 may coordinate for the regulation of in seed dormancy via inhibition of AHG1 and AHG3 function. Moreover, the seed germination assays revealed that the double mutant *dog1 rdo5* showed similar ABA sensitivity as *dog1*, and *rdo5* is less sensitive, indicating that RDO5 may act upstream of DOG1 in seed dormancy. However, we still could not exclude the possibility that DOG1 functions through RDO5 and its homologs.

1.Introduction

1.1. Characters of seed dormancy

Seed dormancy is a very complex process that is affected by a great number of genes and environmental factors (Koornneef et al., 2002; Bentsink and Koornneef, 2008). Seed dormancy is defined as the case where a viable seed cannot germinate under favorable conditions (Lin et al., 1998). However, complex inheritance of seed dormancy makes such inability in seeds difficult to measure (Takahashi, 1997). Seed dormancy mainly includes primary dormancy and secondary dormancy. Primary dormancy is when the fresh seeds released from the mother plants already maintain a dormant state. In contrast, secondary dormancy is non-dormant seeds under a unfavorable environmental condition that become dormant (Geneve, 2003). The freshly harvested seeds generally maintain a highly dormant state and the dormancy is gradually released during the seed storage or the cold temperature stratification (Nakabayashi et al., 2012a). Seed dormancy is one of most important adaptive traits for numerous seed plants to evade injury from stressful conditions (Sano et al., 2015). In addition, crop yield and quality are correlated with seed dormancy. For example, plants in a non-dormant state are problematic due to pre-harvest sprouting, which can cause the reduction of the cereal quality. In contrast, if plants are highly dormant, seed germination will be delayed. This will reduce the length of the growing season of the crop, which eventually can cause the reduction in cereal yield (Gubler et al., 2005). In the actual production process, to maintain uniform and fast germination after sowing, most crop plants with a very high seed germination rate were selected by breeders and geneticists (Nakabayashi et al., 2012a). Seed dormancy is a complex process and regulated by many phytohormones. Abscisic acid (ABA) and gibberellin (GA) are the two main plant hormones in regulating seed dormancy. The balance of the two phytohormones is considered central in regulating seed dormancy and germination (Rodríguez-Gacio et al., 2009). The function of ABA and GAs in controlling seed dormancy is conserved between dicot and monocot species. More recent research has shown important roles of the other phytohormones, such as auxin, salicylic acid (SA), jasmonic acid (JA), brassinosteroids (BRs), cytokinins (CTKs), strigolactones (SLs) and ethylene (ET) in the regulation of seed dormancy (Shu et al., 2016). For instance, auxin regulates seed dormancy through enhancing abscisic acid signaling (Liu et al., 2013b). The other plant hormones include jasmonates, brassinosteroids, and ethylene suppress seed germination by antagonizing ABA (Xi et al., 2010; Linkies and Leubner-Metzger, 2012; Jacobsen et al., 2013). In addition, it has been shown that

many genes play a crucial role in the regulation of seed dormancy in Arabidopsis. These genes include *DOG1*, *RDO5* and some of them are involved in ABA signaling, such as PP2C family proteins (ABI1, ABI2, AHG1, AHG3 etc.), SnRK2 family proteins (SnRK2.2, SnRK2.3 and SnRK2.6 etc.) and a kind of crucial ABA-dependent transcription factors (ABI3, ABI4, and ABI5). Moreover, multiple environmental factors also influence seed dormancy, such as light quality, temperature, and water availability (Socolowski et al., 2008). Overall, seed dormancy is a complex and important trait controlled by many factors and which directly affects the quantity and the quality of crops. Therefore, studies about seed dormancy can help to unravel the basis of germination and dormancy in seed biology and also provide some potential insights to improve the quantity and the quality of crops in future (Skubacz and Daszkowska-Golec, 2017).

1.2. Seed dormancy regulated by phytohormones

1.2.1. Role of ABA metabolism and signaling in maintaining seed dormancy

The plant hormone abscisic acid (ABA) is the key regulator in the induction and maintenance of seed dormancy (Kermode, 2005). ABA is produced in plants primarily from two channels: (1) from the maternal plant (around 10 d after pollination), and (2) biosynthesized de novo in the embryo. ABA biosynthesis in the embryo is important for seed dormancy maintenance (Finkelstein et al., 2002; Koornneef et al., 2002). The pathway of biosynthesis of ABA proceeds from zeaxanthin to xanthoxin via several steps catalyzed by zeaxanthin epoxidase and 9′-cis-epoxycarotenoid dioxygenase, which is subsequently converted to ABA (Taylor et al., 2000). Many factors related to ABA biosynthesis *have the* function in controlling seed dormancy in Arabidopsis. AtNCED6 and AtNCED9 are two key regulatory enzymes, which are required for ABA biosynthesis during seed germination (Lefebvre et al., 2006). Both genes showed seed-specific expression patterns in Arabidopsis. ABA levels are reduced in *Atnced6* and *Atnced9* mutant seeds and the double mutants *Atnced6 Atnced9* show reduced dormancy phenotype (Lefebvre et al., 2006). In addition, NCED5 has been confirmed as a key regulator in mediating the ABA synthesis and seed dormancy. Compared the single mutants (*nced5, nced6* and *nced9*) and double mutants (*nced5 nced6, nced5 nced9, nced6 nced9*), the triple mutant of *nced5 nced6 nced9* showed more non-dormant, which indicate *NCED5* regulates seed dormancy together with *NCED6* and *NCED9* (Frey et al., 2012). *ABA2* encodes a short-chain dehydrogenase/reductase in *Arabidopsis thaliana*. Compared with wild type plants, constitutive expression of *ABA2* in Arabidopsis leads to an increased ABA levels and delayed seed germination (Lin et al., 2007). *AtAAO3* encodes a key enzyme which catalyzes the

final step of ABA biosynthesis during seed germination. A low level of ABA and seed dormancy were found in the mutants of *aao3*, which indicates the function of *AtAAO3* in the regulation seed dormancy is mediated by ABA (Seo et al., 2004). In addition, ABA content is regulated via fine-tuning of de novo biosynthesis and catabolism in seeds (Saito et al., 2004). In the key step in ABA catabolism the 8′-position of ABA is hydroxylated by ABA 8′-hydroxylase, a cytochrome P450. Recently, CYP707A gene family proteins (CYP707A1 to CYP707A4) in Arabidopsis have been confirmed as pivotal enzymes (ABA 8′-hydroxylase) in regulating the rate of *ABA* catabolism and seed dormancy (Saito et al., 2004; Liu et al., 2013a). ABA degradation in seeds is mainly achieved by the *CYP707A2* (Rodríguez-Gacio et al., 2009). A high level of ABA and seed dormancy have been found in the dry and imbibed mutant seeds of *cyp707a2*. Interestingly, the ABA amount and the dormancy level in *cyp707a1* were much higher than the ones in *cyp707a2* (Kushiro et al., 2004a; Okamoto et al., 2006). Overall, these data suggested important functions for these genes related with ABA biosynthesis and catabolism in dormancy by controlling the amount of ABA.

The core ABA signaling includes PYR/PYL/RCAR (an ABA receptor), type 2C protein phosphates, SNF1-related protein kinase 2 and abscisic-acid-dependent basic leucine zipper (bZIP) transcription factors. ABA binds to the PYR/PYL/RCAR receptors and the complex negatively regulate PP2C phosphatases, which finally caused the activity of SnRK2 kinases' released (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). PP2C family proteins are crucial regulators in ABA signaling pathway. So far, at least 6 PP2C phosphatase proteins, namely ABI1 (ABA insensitive1), ABI2 (ABA insensitive 2), PP2C, AHG3, AHG1, HYPERSENSITIVE TO ABA1 (HAB1) and HYPERSENSITIVE TO ABA2 (HAB2) have been shown to negatively regulate the ABA signaling pathway (Gosti et al., 1999; Merlot et al., 2001; González-García et al., 2003; Saez et al., 2006; Yoshida et al., 2006c; Nishimura et al., 2007). In addition, the functions of PP2C family proteins in the regulation of seed dormancy have been reported. For instance, *ABI1* (*ABA INSENSITIVE1*) and *ABI2* encode two type-2C protein phosphatases and the gain-of-function and loss-of-function of the two genes in the regulation of seed dormancy showed insensitive and sensitive response to ABA respectively (Koornneef et al., 1984; Leung et al., 1997; Rodriguez et al., 1998; Gosti et al., 1999; Merlot et al., 2001). *AHG1* and *AHG3* encode another two PP2C family members that are key players in controlling seed dormancy. Transcriptome and histochemical experiments showed AHG1 shared partial functions with AHG3 and the two factors have functional redundancy in the regulation of seed dormancy (Nishimura et al., 2007). Sucrose nonfermenting 1 (SNF1)-related protein kinase 2s (SnRK2s) is a plant-specific protein kinase and plays an

important role in regulating plant development and stress signaling. SnRK2s protein family contains 10 members (SnRK2.1-2.10) in *Arabidopsis*. Among of them, SnRK2.2, SnRK2.3 and SnRK2.6 have been confirmed are major players in ABA signaling by directly interact and phosphorylate its substrates include SLAC1, KAT1, AtRbohF and some transcription factors, such as ABF family members, which include ABF1, ABF2 (AREB1), ABF3, ABF4 (AREB2) and ABI5 (Kulik et al., 2011). Structural analysis of SnRK2s reveal all SnRK2s contain two regions, a highly conserved kinase domain located at the N-terminal, and another important regulatory domain of SRK2E/OST1 located at the C-terminal, which is important for ABA and osmotic stress response. Moreover, the C-terminal domain was functionally subdivided into two domains, namely Domain I and II. ABA-dependent activation and interaction with the ABI1 phosphatase of SRK2E/OST1 requires Domain II, whereas Domain I was important for the ABA independent activation (Yoshida et al., 2006b). SnRK2.2, SnRK2.3 and SnRK2.6 were mainly located in the nucleus and play a crucial role in seed development and germination. Comparing with single mutants *snrk2.2*, *snrk2.3* and *snrk2.6* and the double mutant plant *snrk2.2 snrk2.3*, the triple mutant plant *snrk2.2 snrk2.3 snrk2.6* showed more non-dormant, which suggest *SnRK2.2*, *SnRK2.3* and *SnRK2.6* are functionally redundant in the regulation of seed dormancy (Nakashima et al., 2009).

ABI3, *ABI4*, and *ABI5* encode the crucial ABA-dependent transcription factors and show high levels of expression in dormant seeds. Among these ABI genes, *ABI3* plays a critical role in controlling seed dormancy. *ABI3* is a seed specific gene and the mutant seed of *abi3* is non-dormant (Clerkx et al., 2003). ABI3 regulates seed embryo development by regulating the expression level of *ABI5*. *ABI4* encodes an AP2/ERF transcription factor and the mutant *abi4* showed a low seed dormancy, which indicates an important role of ABI4 in the regulation of seed dormancy (Shu et al., 2013). *ABI5* encodes a leucine zipper transcription factor and the mutant was insensitive to ABA in seed germination inhibition and affected the expression of numerous ABA-related genes. Interestingly, transcriptomics revealed that ABI5 is required for the function of DOG1 in regulating seed dormancy from RNA level. In addition, ABI5 can be regulated by SnRK2s family proteins by phosphorylation (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001; Piskurewicz et al., 2008; Dekkers et al., 2016).

1.2.2. Role of GA metabolism and signalling in breaking of seed dormancy

Gibberellins (GAs) are important phytohormones that are involved many developmental processes in plants (Gupta and Chakrabarty, 2013). GA promotes seed germination by

counteracting the effects of ABA and have been the focus of many studies (Kucera et al., 2005). Shinjiro Yamaguchi et al. (2011) reported that the biosynthesis of GAs during seed germination takes place in two separate locations with the early step occurring in the provasculature and the later steps in the cortex and endodermis. Some genes involved in GA metabolism can affect seed dormancy. GA4 is an active Ga and the transcript of *GA4* is greatly increased during seed germination which indicates that GA4 functions in the regulation of seed dormancy (Talon et al., 1990; Derkx et al., 1994; Gallardo et al., 2002). Furthermore, two GA biosynthesis genes, *AtGA3ox1* (*GA 3-oxidase*) and *AtGA20ox3* (*GA 20-oxidase*) showed highly expressed within 8 h of imbibition, they subsequently decreased, which indicate the two genes are likely involved in the process of seed dormancy (Ogawa et al., 2003).

The core of GA signalling is a GA-GID1-DELLA complex formed by the binding of GA to its receptor GID1 (GIBBERELLIN-INSENSITIVE DWARF1). In the absence of GA, GA responses were completely blocked by DELLA proteins. Whereas in the presence of GA, the complex GA-GID1-DELLA triggers the degradation of the DELLAs via 26S proteasome, which mediated by SLY1 F-box proteins. Therefore, GA stimulates plant growth by maintaining the balance of DELLA proteins. DELLAs restrain the GA-dependent growth processes including seed germination, whereas GA relieves these activity by antagonism against DELLAs (Achard and Genschik, 2009). GID1 is a soluble receptor mediating GA signalling in rice. The triple mutant plant *gid1a gid1b gid1c* exhibits high seed dormancy, whereas and the overexpression of *GID1* leads to reduced seed dormancy. Further, the transcripts of *GID1* and protein accumulated with the dormancy-breaking treatments (cold stratification and after ripening), while after-ripening storage induced only *GID1b*. Thus, seed dormancy is regulated by the two factors differently (Hauvermale et al., 2015). DELLA is a key negative transcriptional activator and plays an important role in repressing GA signalling (Yoshida et al., 2014). The appropriate modulation of DELLA proteins is essential during seed germination. Mutations in the repressors, including GA (RGA), RGA-like 1 (RGL1), RGL2, and gibberellic acid insensitive (GAI), leads to an insensitive response to GA during seed germination, which indicate DELLA proteins play a key role in the regulation of seed dormancy (Lee et al., 2002; Cao et al., 2005). In addition, another negative regulator in GA signalling namely spindly (*SPY*), *SPY* encodes *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) and it can regulate the activity of RGA through the *O*-GlcNAc modification. The mutants of *sly1* showed reduced germination, even with the treatment of exogenous GA, which indicates that SLY1 is the crucial player in controlling seed dormancy. Another gene *COMATOSE* (*CTS*), which

encodes an ABC transporter involved in peroxisomal import of substrates for β-oxidation. The mutations of *CTS* maintain a highly seed dormant state even after stratification or after ripening, which indicate CTS is crucial factor in regulating for seed dormancy (Dave et al., 2011).

1.2.3. The roles of the other plant hormones in regulating seed dormancy

The phytohormone auxin affects diverse aspects of plant growth and development, such as axis formation, tropisms, vascular patterning and apical dominance. But recent studies showed that like ABA, auxin positively regulates seed dormancy (Shuai et al., 2016). Auxin biosynthesis mediated by the YUCCA flavin monooxygenases. The double mutant of *yuc1yuc6* showed reduced seed dormancy, which indicate the key role of auxin in regulating seed dormancy (Cheng et al., 2006). In addition, auxin response factors AUXIN RESPONSE FACTOR 10 and AUXIN RESPONSE FACTOR 16 regulate seed dormancy by controlling the transcript level of *ABI3* (Liu et al., 2013b). Auxin transporter protein1 (AUX1) is an auxin influx transporter. AUXIN RESISTANT 1 (AUX1) is identified as a key factor during seed germination, which regulated by two histone deacetylase-binding factors, SWI-INDEPENDENT3 (SIN3)-LIKE1 (SNL1) and SNL2 (Wang et al., 2016). Overall, all of these studies indicate the auxin play a key role in the regulation of seed dormancy. Recent studies showed that ethylene can affect the transcript level of some genes related with ABA metabolism or signals to induce seed germination. Ethylene receptors, ethylene triple response 1 (ETR1) and ethylene insensitive 2 (EIN2) play an important role in regulating seed dormancy. The mutants of *etr1 and ein2* show a *higher level of ABA* (Beaudoin et al., 2000), which subsequently led to increased *seed dormancy*. Overall, all these studies indicate an important function of ethylene in the regulation of seed dormancy.

BRs play a critical role in controlling cell elongation, cell division, and skotomorphogenesis. Recently, the functions of BR in regulating seed dormancy have been revealed. For example, some mutants, such as *ga1-3* and the GA-insensitive mutant *sleepy1*, which related with GA biosynthetic can be rescued by BR. Moreover, the germination of the BR biosynthetic mutants include *det2-1* and the BR-insensitive mutant *bri1-1* are greatly suppressed by ABA. Taken together, these studies revealed a role for BRs in germination induction (Steber and McCourt, 2001).

1.3. Seed dormancy affected by some genes

1.3.1. Seed dormancy regulated by DOG1

A major quantitative trait locus for seed dormancy, DOG1, was identified in a recombinant inbred line population between the lowly dormant accession Landsberg *erecta* (L*er*) and the high dormant accession Cape Verde Islands (Cvi) (Alonso-Blanco et al., 2003). The protein DOG1 belongs to a small family in *Arabidopsis thaliana* with unknown molecular function that has been shown to be a major regulator of seed dormancy in *A. thaliana* and other species. The mutants of DOG1 are completely non-dormant and do not show any obvious multiple phenotypic traits, apart from a reduced seed longevity (Bentsink et al., 2006a; Graeber et al., 2014). A transcriptomics showed DOG1 affects the expression of hundreds of genes including LATE EMBRYOGENESIS ABUNDANT and HEATSHOCK PROTEIN genes by regulating the expression of *ABI5*. In addition, metabolomics revealed that DOG1 affects the content of a subset of primary metabolites. DOG1 has five transcript variants, which only encode three protein isoforms by alternative splicing. The *dog1* mutant phenotype can be complemented by overexpressing single *DOG1* transcript variant or expressing two or more *DOG1* transcript variants from the endogenous *DOG1* promoter, which indicate that the additional isoforms are important for maintaining the protein stability (Nakabayashi et al., 2015). Moreover, the C terminus of DOG1 can be alternatively polyadenylated in *A. thaliana*, which finally produces two distinct pol-yadenylation clusters: a long DOG1 (lgDOG1) form, which has full-length mRNA; a short DOG1 (shDOG1), which has truncated mRNA. The short *DOG1* mRNA but not the long DOG1 could rescue the dormancy phenotype of *dog1*, which indicates the short DOG1 protein isoform plays an important role in the regulation of seed dormancy (Cyrek et al., 2015).

Recently, two phosphatases ABA-HYPERSENSITIVE GERMINATION 1 (AHG1) and AHG3 were identified in a DOG1 pull-down assays. Genetics experiments showed that AHG1 and AHG3 act down-stream of DOG1 and with the functional redundancy in the regulation of seed dormancy (Née et al., 2017b). Moreover, the phosphatase activity of AHG1 but not AHG3 is supressing by DOG1 (Nishimura et al., 2018). All of these data indicate that AHG1 and AHG3 are required for the function of DOG1 in the regulation of seed dormancy.

1.3.2. Seed dormancy regulated by RDO5

The seed dormancy QTL-IBO was identified in recombinant inbred line (RIL) populations from the crosses between Eilenburg-0 (Eil-0) and Loch Ness-0 (Lc-0). *IBO* namely also *RDO5* (*Reduced Dormancy 5*) or *DOG18*, which encodes a PP2C family protein but loses phosphatase activity (Amiguet-Vercher et al., 2015a). Interestingly, there is only one amino acid difference between Eil-0 and Lc-0 variant, however, the IBO protein in the Lc-0 variant

shows low germination rate. In addition, IBO protein from the Lc-0 variant can affect the phosphatase activity of ABI1 (ABSCISIC ACID INSENSITIVE 1) (Amiguet-Vercher et al., 2015a). Meanwhile, in a mutagenesis screen of a highly dormant Arabidopsis thaliana line, the *rdo5* mutant was cloned based on its strongly reduced seed dormancy in the Soppe lab. *RDO5* only expressed in seeds and its expression levels correlate with seed dormancy levels. The ABA levels and sensitivity are not altered in the mutant of *rdo5*, nor does it show altered DOG1 accumulation. Therefore, it is assumed that RDO5 regulates seed dormancy in a pathway independent from ABA and DOG1 (Xiang et al., 2014b). Additionally, a phosphatase activity assay showed RDO5 does not have phosphatase activity. RDO5 has high homology with PP2C phosphatases but lacks several conserved residues required for phosphatase activity, however, back mutations of these residues make it function as phosphatase. A RDO5 phosphoproteomics analysis revealed that there is a significantly altered phosphoproteome in the imbibed seeds of *rdo5* which indicated that RDO5 still affects protein phosphorylation and de-phosphorylation though it is a pseudo-phosphatase (Xiang et al., 2016b).

1.3.3. Seed dormancy regulated by calmodulin proteins

Calmodulin (CaM) is a small (148 residues), universal, highly conserved Ca^{2+} binding protein, which serves as a multifunctional intermediate calcium-binding messenger protein (Friedman et al., 2006). These highly conserved proteins regulate many crucial cellular processes, such as growth, gravitropism, phototropism, abiotic stress, and biotic defense responses (AL-Quraan et al., 2010). The calmodulin proteins in Arabidopsis are quite similar to vertebrate CaMs (McCormack et al., 2005a). In Arabidopsis, seven *CaM* genes encode four CaM isoforms include AtCaM1/4, AtCaM2/3/5, AtCaM6, and AtCaM7. Additionally, there are 50 CaM-like proteins (CMLs) in Arabidopsis, CMLs contain CaM-like and/or divergent $Ca2^{+}$ binding domains. Like the Calmodulin (CaM), calmodulin-like proteins (CMLs) function in Ca^{2+} binding, thereby regulating numerous plant development and stress responses by controlling calcium signals (Zeng et al., 2015). Calmodulin has two approximately symmetrical globular domains each containing a pair of EF-hand motifs (the N- and C-domain) connected by a flexible linker region for a total of four Ca^{2+} binding sites. Once calcium binds to the EF-hand motifs of calmodulin, which lead to a conformational change, the complex of calcium and calmodulin subsequently coupled with the flexible linker, allows calmodulin to interact with a wide variety of substrates such as kinases, phosphatases, transcription factors and some proteins with unknown functions to regulate their actions by influencing their activity (Chin and Means, 2000). For examples, Calmodulin proteins of CaM1 and CaM4 in Arabidopsis increase nitric

oxide and promote salt resistance through the binding and inhibition of S-Nitrosoglutathione Reductase (Zhou et al., 2016a). Receptor protein kinase 1 (RPK1), a crucial component that regulates stomatal regulation, seed germination, and stress responses by controlling *ABA signaling (Osakabe et al., 2005; Osakabe et al., 2010).* CaM1 was positively regulated by RPK1, CaM1 protein, in turn positively modulates leaf senescence, ROS production and ABA response in *Arabidopsis* by up-regulating *RbohF* gene expression (Dai et al., 2018). CaM4 can be phosphorylated by RPK1, phosphorylated CaM4, in turn, positively regulates NADPH oxidase RbohF, which is related with RPK1-mediated the production of superoxide, SIRK kinase induction, and cell death (Koo et al., 2017a). In addition, AtCaM4 has been shown to negatively regulate freezing tolerance in Arabidopsis. The mutant plants of *cam4* showed enhance freezing tolerance. Two cold-induced isoforms *AtCaM1* and *AtCaM4* encode very similar proteins; however the double-mutants of *cam4/cam1Ri* and single-mutant plants of *cam4* showed similar improvements in freezing tolerance, indicating that AtCaM4 plays major function in the regulation of freezing tolerance (Chu et al., 2018b). AtCaM2, AtCaM3 and AtCaM7 were reported with the functions in regulating pollen germination, cold and light responses in *Arabidopsis* (Zhang et al., 2009; Landoni et al., 2010; Abbas et al., 2014). In addition, accumulating evidence supports a role for calmodulin proteins in seed dormancy. For instance, CML39 is a crucial factor in regulating seed dormancy. Compared with wild type plants, the mutant *cml39* showed more rapid germination and less sensitive to hormones during seed development (Midhat et al., 2018). Moreover, CML9 (AtCML9), another calmodulin-like-protein, functions in the regulation of seed dormancy. The mutant plant of *cml9* showed a hypersensitive response to ABA during seed germination (Magnan et al., 2008). In addition, *OsMSR2* (*Oryza sativa L. Multi-Stress-Responsive gene 2*), encodes a novel calmodulin-like protein in rice and overexpression transgenic plants showed a hypersensitive response to ABA during the seed germination (Xu et al., 2011). Overall, these studies suggested that Calmodulin proteins or calmodulin-like-proteins might have an important role in controlling seed dormancy.

1.4. Objectives of the thesis

RDO5 encodes a PP2C family protein, which plays a crucial role in the regulation of seed dormancy. Phosphatase activity and phosphoproteomics revealed that although RDO5 is a pseudophosphatase, it still could prevent the protein de-phosphorylation (Yong Xiang, 2014, 2016). DOG1, AHG1 and some calmodulin proteins were identified in RDO5 pull-down and MS experiments. The DOG1 protein has been confirmed a major regulator of seed dormancy in Arabidopsis by the suppression of phosphatase activity of AHG1. Two phosphatases of

AHG1 and AHG3 with the functional redundancy were reported to play an important role in the regulation of seed dormancy. Recently, accumulating evidence has shown calmodulin proteins and calmodulin like proteins might function in the regulation of seed dormancy. In this thesis I examine how RDO5 interacts with these factors to regulate seed dormancy.

2. Materials and methods

2.1. Materials

2.1.1. Antibiotics used in this thesis

Table 2.1 Antibiotics used in this thesis

Name	Solvent	Final concentration in *E.coli* selection medium (mg/l)	Final concentration in *A. tumefaciens* selection medium (mg/l)
Ampicillin (Amp)	Water	100	-
Kanamycin	Water	50	25
Rifampicin	DMSO	-	50
Spectinomycin	Water	100	-
Gentamycin (Gen)	Water	10	10
Hygromycin	Water	50	50

2.1.2. Buffers and culture media

Buffers and culture media were prepared according to Sambrook and Russel (2001). In addition, special solutions were prepared as following:

Table 2.2 Buffers used in this thesis

DNA extraction buffer	
Tris HCl	0.2 M, pH 7.5
NaCl	0.25 M
EDTA	25 mM
SDS	0.50%
High salt solution for RNA precipitation	
Sodium citrate	1.2 M
NaCl	0.8 M
Protein extraction buffer	
Tris-HCl (pH:7.5)	50 mM
NaCl	150 mM
EDTA	1 mM
DTT	5 mM
Macrozym	1 mM
PIC	1 mM
Triton-X-100	0.25%
Blocking solution (pH= 7.5)	
Tris-HCl	

	50 mM
NaCl	150 mM
Tween 20	0.25 % (v/v)
Skim milk	5% (w/v)
Dense SDS solution	
Tris-HCl (pH=8)	100 mM
Sucrose	30% (w/v)
SDS	2% (w/v)
Mercaptoethanol	5% (v/v)
Infiltration medium	
Sucrose	25g
Silwet L-77	100 µl
Water	Up to 500 ml
Mannitol solution	
mannitol	0.4 M
KCl	20 mM
Mes pH 5.7	20 mM
PEG/Ca solution	
PEG 4000	4 g
mannitol	0.36 g
1M Ca (NO3)2	1 ml
Water	Up to 10 ml
W5 solution	
NaCl	154 mM
CaCl2*2H2O	125 mM
KCl	5 mM
Mes (pH=5.7)	2 mM
MMg solution	
Mannitol	0.2 M
MgCl2*6H20	15 mM
Mes	4 mM
Kinase buffer	
Tris-Hcl	20 mM
Mgcl2	5 mM
DTT	1 mM
AS buffer	
Mgcl2	1 M
MES (pH:5.6)	1 M
Acetosyringon	150 mM
The solutions for silver staining	
Fixing solution	40% methano, 10% acetic acid
rinse solution	30%methanol

sensitize solotion	0.02% sodium thiosulfate (Na2S2O3)
staining solution	0.1% silver nitrate (AgNO3)
developing solution	0.04% formaldehyde, 2% sodium carbonate (Na2CO3)
stop solution	1% acetic acid

2.1.3. Primers used in this thesis

Table 2.3. Primers used in this thesis

primers for cloning and expression of a gene in Gateway	**Oligonucleotide (5'-3')**
primers for full length cDNA amplification	
RDO5:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAAACGGATACTACTCT
RDO5:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGAAACGGTAGAGCTTTTGA
DOG1:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGATCTTCATCAAAGAA
DOG1:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCctactttccttcctctcctc
AHG1:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACTGAAATCTACAGAAC
AHG1:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGAGAGCTATTCTTGAGAT
AHG3:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTGGGATTTGTTGCGG
AHG3:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGACGACGCTTGATTATTCC
SnRK2.2:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATCCGGCGACTAATTC
SnRK2.2:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAGAGCATAAACTATCTCTC
SnRK2.3:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATCGAGCTCCGGTGAC

SnRK2.3:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAGAGCGTAAACTATCTCTC
SnRK2.6:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATCGACCAGCAGTGAG
SnRK2.6:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCATTGCGTACACAATCTCTC
CaM1/4:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGGATCAACTCACTGA
CaM1/4:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTAGCCATCATAATCTTGA
primers for amplification of the truncated protein	
SnRK2.6:aa158:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATCTTTAGACGAGGGGCCG
SnRK2.6:aa159:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTGTGATTTCGGATATTC
SnRK2.6:aa186:R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCAGGAGCGATGTAAGCA
SnRK2.6:aa187:F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTTTTACTAAAGAAAG
primers for genotyping	
dog1-2:LP+RP	(Née G et al., 2017)
ahg1-5:LP+RP	(Née G et al., 2017)
ahg3-2:LP+RP	(Née G et al., 2017)
rdo5-2:LP+RP	(Xiang Y et al., 2014)
snrk2.2:LP+RP	CAAGACCATACATCTGCAAGCTGG+ACACCTTGATGTTTCTTCTGTGTG
snrk2.3:LP+RP	TTGGTTTTGAGTGTTCTGCTTTTG+CACCACATGACCATACATCTGCAA
snrk2.6:LP+RP	CATATCTTTAGACGAGGGGCC+GTGAGTGGTCCAATGGATTTG
8474 (for GABI lines)	ATAATAACGCTGCGGACATCTACATTTT
LBb1.3	ATTTTGCCGATTTCGGAAC
LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
Real time PCR primers	
DOG1_F+R	AACATCGACGGCTACGAATC+GCTTGTCGAGAGCTTGATCC
RDO5_F+R	ACTCTCACGACGGAGTTGCT+TATCACTGCAGCCTTTGTCG
AHG1_F+R	TCCTCTGAGTAACGATCACAAGC+ACCACCAGCTGCTTCAATCC
AHG3_F+R	TCCTCTCTCCGTAGATCACAAGCC+ACTCCAAGAACCCTAGCTCCATC

ACT8	CTCAGGTATTGCAGACCGTATGAG+CTGGACCTGCTTCAT CATACTCTG

2.1.4. Vector plasmids used in this thesis

Table 2.4. Vector plasmids used in this thesis

pDONR207	Cloning of PCR fragments with Gateway® technology	Invitrogen, Karlsruhe, Germany
pENTR	Cloning of PCR fragments with Gateway® technology	Invitrogen
pBatTL-B-sYFPn	Split YFP assay in planta	Joachim Uhrig (MPIPZ)
pBatTL-B-sYFPc	Split YFP assay in planta, protoplast and transgenic plants	Joachim Uhrig (MPIPZ)
Pxcgg-mYFP C-terminal	protoplast	Jane parker(MPIPZ)
pAS2-attR	Yeast two hybrid	Wim Soppe (MPIPZ)
pACT2-attR	Yeast two hybrid	Wim Soppe (MPIPZ)
PDEST15	Express proteins from bacteria	Wim Soppe (MPIPZ)
PDEST17	Express proteins from bacteria	Wim Soppe (MPIPZ)
PDEST-MC2	Express proteins from bacteria	Wim Soppe (MPIPZ)

2.1.5. Bacterial and yeast strains

The *Escherichia coli* strain *DH5α* is used extensively in recombinant DNA technology (Hanahan, 1983). Rosetta™(DE3)pLys was used for expression of proteins from bacteria. Ag*robacterium tumefaciens* strain GV3101 was used for plant transformation; in addition, when plants were transformed with the vectors pBatTL-B-sYFPc and Pxcgg-mYFP, strains carrying the helper plasmid pMP90RK were used (Koncz et al., 1986; Koncz et al., 1990; Hellens et al., 2000). The yeast strain AH109 was used for yeast two hybrid experiments.

2.1.6. Antibodies used in this thesis

Table 2.5. Antibodies used in this thesis

Name	Company
Anti-HA tag antibody	Abcam (ab9110)
Anti-GFP antibody	Abcam (ab290)
TAP Tag Polyclonal Antibody	Thermo Fisher (CAB1001)
mouse	Thermo Fisher (A32723)
Rabbit	Thermo Fisher(A16110)
SnRK2.2/2.3/2.6 antibodies	AGRIAS142783

2.1.7. Plant material used in this thesis

Table 2.6. Plant material used in this thesis

Name	Background	Source
dog1-2	Col	(Née G et al., 2017)
ahg1-5	Col	(Née G et al., 2017)
ahg3-2	Col	(Née G et al., 2017)
rdo5-2	Col	(Xiang Y et al., 2014)
snrk2.2	Col	(Hiroaki Fujii et al., 2007)
snrk2.3	Col	(Hiroaki Fujii et al., 2007)
snrk2.6	Ler	(Mustilli et al., 2002)
cam1	Col	salk_202076C
cam4	Col	GABI_309E09
cam6	Col	Salk_071609
RDO5OE-10	Ler	(Xiang Y et al., 2014)
RDO5OE-10	Ler	(Xiang Y et al., 2014)
CaM1OE-3	Col	Constructed by myself
CaM1OE-5	Col	Constructed by myself
CaM1OE-8	Col	Constructed by myself

2.2. Methods

2.2.1. Plant materials and growth conditions

Arabidopsis thaliana wild-type Columbia (Col-0), the mutant *cam1* (Salk_202076C), *cam4* (GABI_309E09), *ahg1-5*, *agh3-2* (Née et al., 2017c), *dog1-2* (Nakabayashi et al., 2012b), *rdo5-2* (Xiang et al., 2014a) were used in this study. The *cam1 cam4* double mutant and *rdo5 ahg1 ahg3* triple mutants were generated by crossing and genotyping. The transgenic line 35S::RDO5:TAP seeds in L*er* background was used for IP-MS analysis. Seeds were first stratified at 4°C for 3 days and then grown on ½ MS plates containing 1 × Murashige and Skoog salt, 1% sucrose, 2.5 mM MES (pH5.8), and 0.5% agar or on soil at 22°C under 16h light/8h dark cycles. Freshly harvested seeds were immediately used for experiments or stored under constant conditions (21°C, 50% humidity, in the dark) for after-ripening treatment.

2.2.2. Germination assays

For germination and dormancy assay, approximately 50 seeds were sown onto a filter paper moistened with sterilized water, 0.1-1 μM ABA, or 100 μM GA4+7 in Petri dishes, and incubated in a growth chamber (12h/12h light/dark, 25/20°C cycle). Germination (radicle emergence) was scored after 7 days. For the ABA responsiveness tests, after-ripened seeds with

fully released dormancy were sown on filter paper, and imbibed with different concentration of ABA. For GA treatment, about 50 to 100 seeds were plated onto a filter paper moistened with demineralized water containing 100 µM GA_{4+7} in Petri dishes, and incubated in a growth chamber (12 h light/12 h dark, 25/20 °C cycle). Radicle emergence was scored after 7 days.

2.2.3. RNA Extraction and Expression Studies

Total RNA was extracted from dry or imbibed seeds using RNAqueous columns combined with the RNA isolation aid (Ambion) as described previously (Kushiro et al., 2004b). cDNA was synthesized from 1mg 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 valve method and normalized to the ACTIN8 control. At least two biological replicates were analyzed.

2.2.4. Bimolecular fluorescence complementation (BiFC) assay

The full-length CDS of *RDO5*, *CaM4*, *AHG1*, *AHG3*, *SnRK2.2*, *SnRK2.3*, *SnRK2.6*, and N-terminal, kinases domain, and C-terminal of *SnRK2.6*, and full length of *ABI2* CDS (without stop codon) were recombined from entry clones into the pBatTL-B-sYFPc or pBatTL-B-sYFPn gateway vectors using LR reactions. Constructs were transformed into the *A. tumefaciens* strain GV3101. Overnight cultures were diluted to an OD_{600} = 0.5 in resuspension buffer (10 mM $MgCl_2$, 10 mM MES pH 5.7, and 100 µM acetosyringone), and then injected into 4–6-week-old *Nicotiana benthamiana* leaves with a needleless syringe. Fluorescence within the infiltrated regions was visualized after 2 days using a confocal laser scanning microscope CLSM Zeiss LSM 700.

2.2.5. Transient expression and Co-IP

SnRK2.2, *SnRK2.3*, and *SnRK2.6* were cloned into Pxcgg-mYFP (harboring YFP-tag), and *RDO5* was cloned into pEarleyGate 201 (harboring HA-tag). The constructs were co-transformed into Arabidopsis protoplasts by the polyethylene glycol method. The protoplasts were harvested 16 h after transformation and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM EDTA, 0.1% Triton-X 100, 10% glycerol and 5 mM DTT) with freshly added proteinase inhibitor cocktail (Sigma, P9599). The lysate was centrifuged at 13,000 g, 4°C for 15 min, and the supernatant incubated with GFP-trap beads (ChromoTek) for 2 h at 4°C while rotating on a bohemian wheel. The beads were washed with lysis buffer three times,

diluted in 4 X SDS loading buffer, and boiled for 5 min before separation on SDS-PAGE. Immunoblots were probed according to standard procedures with anti-GFP (Abcam, ab290) and anti-HA (Abcam, ab9110) (Zhou et al., 2017).

2.2.6. Yeast two-hybrid assay

The full length CDS of *CaM4*, *RDO5*, *AHG1*, *AHG3* and *SnRK2.6* was recombined from entry clones into the vectors pAS2-gateway (GAL4 BD fusion) vectors (modified from Clontech) or the pACT2-gateway (GAL4 BD fusion) vectors (modified from Clontech). Gal4-DNA-binding domain fusions proteins (prey) and GAL4 activation domain fusions protein (bait) vectors were transformed in the yeast strain AH109 using a LiAc/SS carrier DNA/PEG method. Co-transformed colonies were selected on selective medium (-LW) lacking Leu (L) and Trp (W). Interaction tests were performed on –LWH medium (-LWH) lacking L, W, and His (H) with 0 or different concentration of 3-Amino-1,2,4-triazole (3-AT). Yeast was grown at 30 °C for 7 days.

2.2.7. Recombinant protein expression and purification

E. coli cells carrying expression plasmid were grown at 37°C to reach an OD_{600} of 0.5~0.6 in LB medium containing antibiotics. Recombinant protein of RDO5-MBP, CaM4-MBP, AHG1-MBP, AHG3-GST, SnRK2.6-MBP, and ABF2-MBP were induced by 0.1 mM IPTG for 16 h at 16 °C. The harvested cells were resuspended in buffer containing 20mM Tris-HCl pH 8.0, 150mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride. The cells were lysed by sonication, and the debris was removed by centrifugation at 13000 g for 60 min. The supernatant was purified with a 1 ml Glutathione Sepharose (GE Healthcare) for GST fused recombinant protein or 1 ml Amylose Resin for MBP (GE Healthcare) fused recombinant protein or 1 ml Nickel resin (GE Healthcare) for high recovery of histidine-tagged protein, the purity of the expressed proteins was confirmed by 12% SDS-PAGE.

2.2.8. Protein kinase activity assay

For the in vitro kinase assay, recombinant RDO5-MBP, CaM4-MBP, DOG1-His, AHG1-MBP, AHG3-GST, SnRK2.2/2.3/2.6-MBP and ABF2-MBP (as substrate) were purified from *E. coli* cells. All of the reactions were incubated in 20 µL of reaction buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl2, and 1 mM DTT) with 5 µCi of [γ-^{32}P] ATP at 25-30°C for 2h. The in-gel kinase assay was performed as previously described (Ding et al., 2015). Briefly, proteins were extracted from dry seeds or seeds imbibed with 25 µM ABA or mock treatment for 30 min or 5 h. 10% polyacrylamide gel (for SDS-PAGE) contains 800 µg of ABF2 and ABI5

respectively. Each lane in the gel contained 50 µg of proteins. After separation on a 10% SDS-PAGE, the gel was washed three times and renaturalized overnight. The gel subsequently was incubated in the kinase buffer which contains 80 µCi of [γ-^{32}P] ATP at room temperature for 2h. After then, the kinase reactions were stopped. The phosphorylated proteins were visualized with PhosphorImage System (FLA5100; Fujifilm).

2.2.9. Detection of SnRK2s in dry seeds

Total 50 µg of dry seeds of Col-0, *rdo5-2*, *dog1-2*, and *cam4* was grounded into fine powder in liquid nitrogen, and dissolved into 200 µl extraction buffer (50 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 25 mM NaF, 50 mM glycerophosphate, 10% glycerol, 2 mM DTT, and proteinase inhibitor cocktail). Supernatant was collected after centrifugation at 13000 g for 10 min. Protein amount was normalized by Bradford Protein Assay (Sigma). Total 50 µg of proteins were separated on 10% SDS-PAGE, and blotted on PVDF membrane. SnRK2.2/2.3/2.6 accumulation was detected by anti-SnRK2.2/2.3/2.6 polyclonal antibody (VWR, AGRIAS142783).

2.2.10. RDO5-TAP pull down

Native seed protein extract from freshly harvest seeds or after-ripened seeds and RDO5 OE-TAP freshly harvest seeds or after-ripening seeds under 6 h imbibition were used for the pull down assays. Pull downs were performed from four independent biological replicates each consisting of a mix of seeds from three different plants. For each pull down, 30 µl of IgG sepharose (GE Healthcare) was equilibrated in native extraction buffer. For each replicate, an adjusted quantity of 4 mg of total protein (in 1 ml final volume) was incubated with the beads under constant rotation for 6 h at 4°C. After incubation, beads were separated from the non-bound fraction by centrifugation and washed three times using 500 µl of native extraction buffers. Bound proteins were eluted by incubation with acetic acid pH 3.4. Eluted proteins were recovered from the beads by centrifugation and immediately neutralized by neutralization buffer (1 M Tris-HCl pH 8.0). Eluted proteins were separated on SDS-PAGE and detected by silver staining.

2.2.11. Sample preparation and LC-MS/MS data acquisition

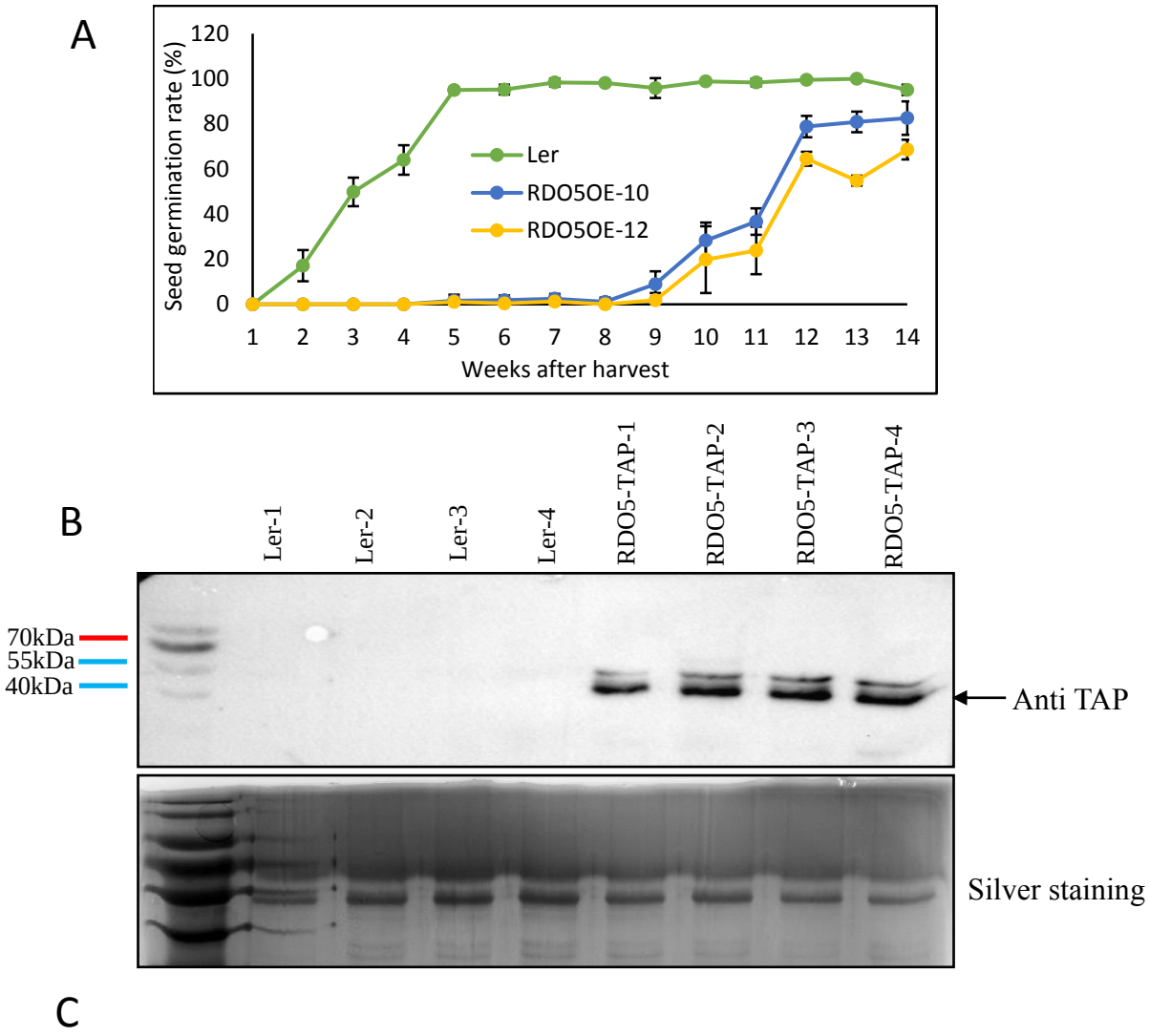
The eluted proteins were reduced with dithiothreitol, alkylated with chloroacetamide, and digested with trypsin. These digested samples were desalted using StageTips with C18 Empore disk membranes (3 M) (Rappsilber et al., 2003), dried in a vacuum evaporator, and dissolved in 2% ACN, 0.1% TFA. Samples were analysed using an EASY-nLC 1200 (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 1.9 µm resin (Dr. Maisch). Dried peptides were re-dissolved in 2% ACN, 0.1% TFA for analysis and adjusted to a final concentration of 0.1 µg/µl. Samples were analysed using an EASY-nLC 1200 (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 1.9 µm resin (Dr. Maisch). Peptides (0.5 µg) were loaded on the column and eluted for 115 min using a segmented linear gradient of 5% to 95% solvent B (0 min : 5%B; 0-5 min -> 5%B; 5-65 min -> 20%B; 65-90 min ->35%B; 90-100 min -> 55%; 100-105 min ->95%, 105-115 min ->95%) (solvent A 0% ACN, 0.1% FA; solvent B 80% ACN, 0.1%FA) at a flow rate of 300 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 3×10^6 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, a maximum injection time (max.) of 55 ms 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 MS^2, dynamic exclusion for 30s prevented repeated selection of precursors.

2.2.12. Data analysis

Raw data were processed using MaxQuant software (version 1.5.7.4, http://www.maxquant.org/) (Cox and Mann, 2008) with label-free quantification (LFQ) and iBAQ enabled (Tyanova et al., 2016). MS/MS spectra were searched by the Andromeda search engine against a combined database containing the sequences from *A. thaliana* (TAIR10_pep_20101214; ftp://ftp.arabidopsis.org/home/tair/Proteins/TAIR10_protein_lists/) and sequences of 248 common contaminant proteins and decoy sequences. 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. Peptide-spectrum-matches and proteins were retained if they were below a false discovery rate of 1%.

3.Result



Protein Name	Ac No.	Peptides	Unique peptides	SC (%)	Score	MS/MS count	LFQ intensity (35S:RDO5-TAP)				LFQ intensity (Ler)				
							rep1	rep2	rep3	rep4	rep1	rep2	rep3	rep4	P-value
RDO5	AT4G11040	11	11	44.7	98.191	38	24.3	23.9	23.3	24.3	21.8	19.2	19.0	21.7	0.00459
DOG1	AT5G45830	7	7	22	58.837	33	25.1	25.8	24.9	25.2	23.8	23.5	23.7	23.4	0.00025
AHG1	AT5G51760	2	2	6.7	13.951	6	22.0	21.8	20.9	21.7	19.6	20.1	21.4	19.8	0.026758
CaM1/ CaM4	AT1G66410/ AT1G66410	6	2	40.9	279.65	35	24.1	23.8	23.4	23.7	22.4	22.5	22.6	22.2	0.000218

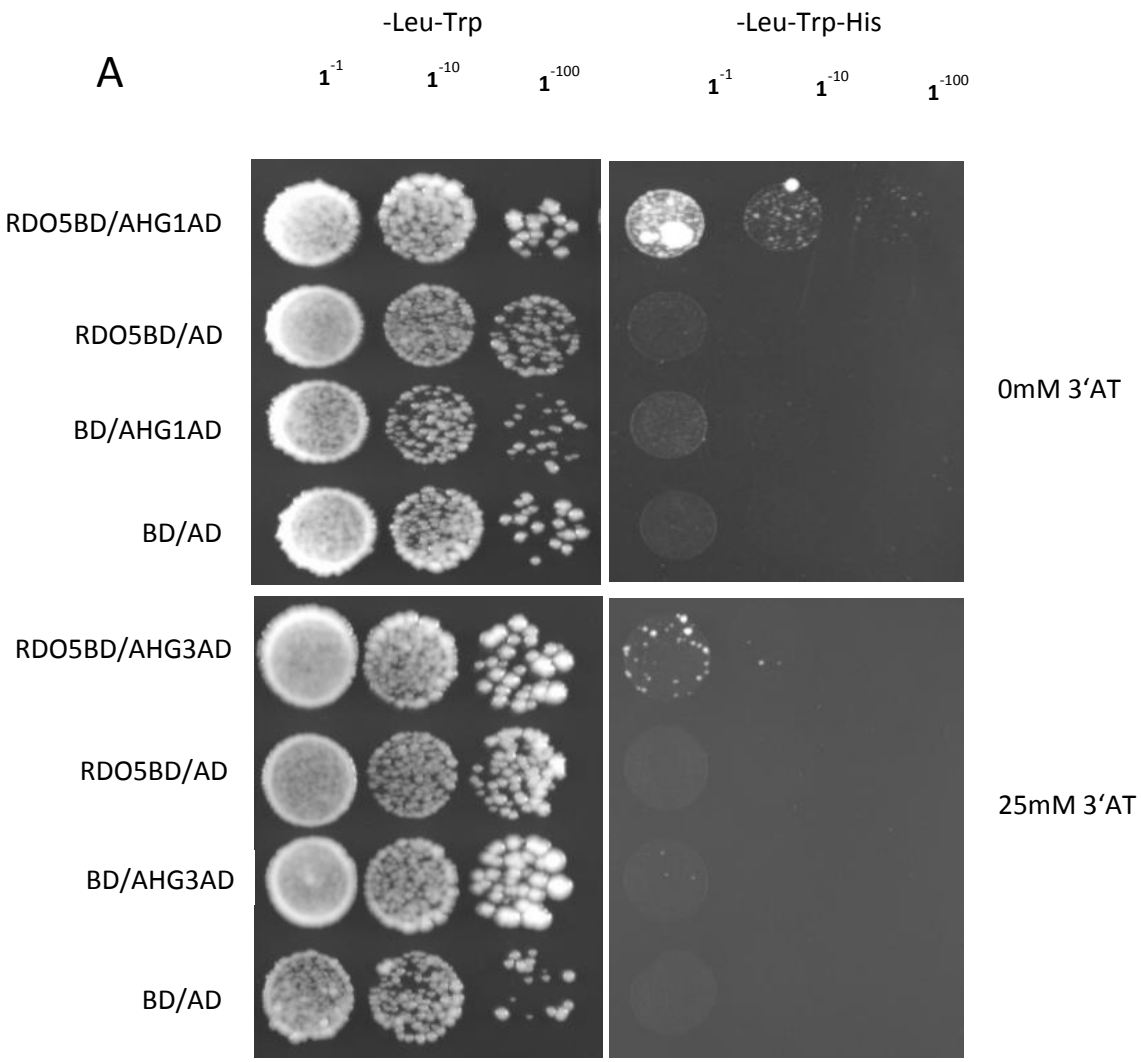
Figure 1 Proteins that were pulled-down with RDO5 from seeds

(**A**) Germination after different periods of dry storage of RDO5:TAP overexpression transgenic lines and wild-type L*er*. Shown are averages ± s.d of six to ten independent batches of seeds for each genotype. (**B**) 6 HAI (Hours after imbibition) seeds from wild type and RDO5:TAP overexpression plants were precipitated by TAP antibody. RDO5:TAP accumulation was detected by Western blot analysis with anti-TAP antibody. The precipitated proteins

were separated on SDS-PAGE and detected by silver staining. Four biological replicates of experiments were performed (indicated as rep1, 2, 3, and 4). (**C**) Overview of proteins that were pulled-down with RDO5 from seeds.

3.1. Identification of RDO5 interacting proteins in seeds

RDO5 is a pseudo-phosphatase that plays a crucial role in controlling seed dormancy (Xiang et al., 2014b; Xiang et al., 2016b). Freshly harvested seeds from *rdo5-2* show a non-dormant phenotype and constitutive expression of RDO5 with TAP tag (tandem affinity purification) causes enhanced seed dormancy (Figure 1A) (Xiang et al., 2014b). To further investigate the function of RDO5 in the regulation of seed dormancy. RDO5 pull-down and mass spectrum experiments were performed to identify its interacting proteins. Fresh and after-ripened seed samples were taken from the same batch. Proteins in their native state were extracted from the seeds after 6 hours imbibition for pull-down experiments. Western blot and silver staining experiments suggested a high efficiency and specificity for RDO5: TAP protein enrichment in RDO5 pull-down assays (Figure 1B). Proteins that co-immunoprecipitated with RDO5 were analysed by quantitative mass spectrometry. DOG1 and AHG1 were identified in RDO5 complexes under all tested conditions. *DOG1* encodes a protein with unknown function that positively regulates seed dormancy (Nakabayashi et al., 2012a; Née et al., 2017b; Nishimura et al., 2018). *AHG1* encodes a PP2C family protein, which is a seed-specific protein, which has the highest transcript level in dry seeds among all PP2C family proteins (Nishimura et al., 2007). In addition, calmodulin proteins including CaM1 and CaM4 were identified in RDO5 pull-down (Figure 1C). We subsequently focused our studies on AHG1, DOG1, CaM1 and CaM4 because of their relevance. For instance, these factors are all highly expressed in seeds, interact with RDO5:TAP in seeds and are directly or indirectly involved in protein phosphorylation and de-phosphorylation. In addition, AHG1, AHG3 and DOG1 have been implicated in either ABA signaling or dormancy.


A
-Leu-Trp
-Leu-Trp-His
1-1
1-10
1-100
1-1
1-10
1-100
RDO5BD/AHG1AD
RDO5BD/AD
BD/AHG1AD
BD/AD
0mM 3'AT
RDO5BD/AHG3AD
RDO5BD/AD
BD/AHG3AD
BD/AD
25mM 3'AT

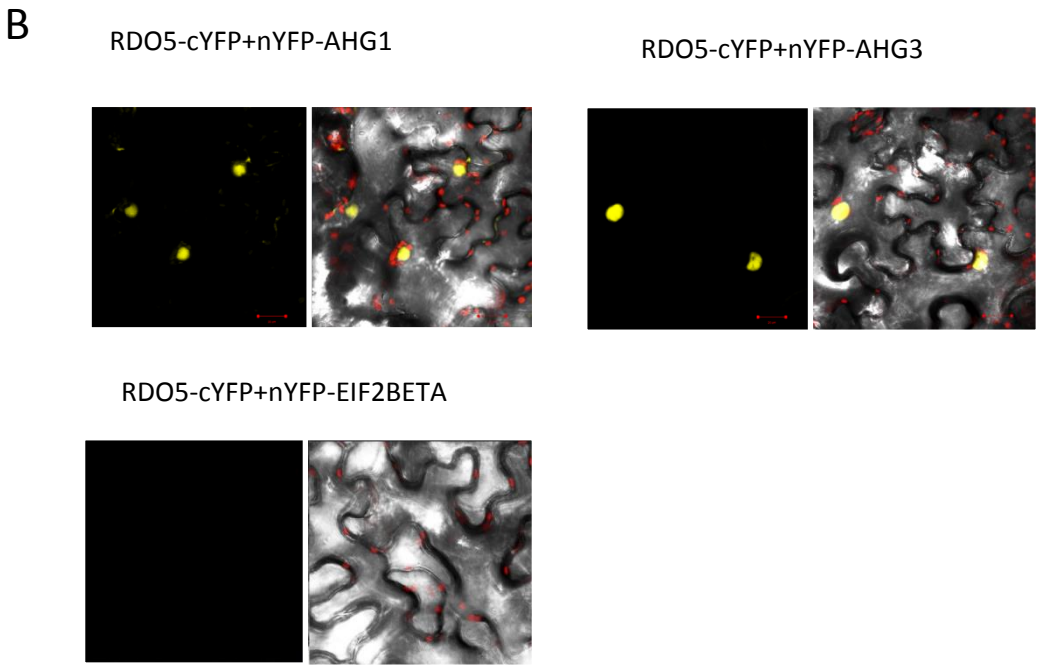

B
RDO5-cYFP+nYFP-AHG1
RDO5-cYFP+nYFP-AHG3
RDO5-cYFP+nYFP-EIF2BETA

Figure 2. RDO5 interacts with AHG1 and AHG3

(**A**) Yeast-two hybrid assay for the interaction between RDO5, AHG1 and AHG3. Yeast cells transformed with GAL4BD-RDO5, GAL4AD-AHG1 and GAL4AD-AHG3 were spotted onto YEB (-Leu/-Trp) and YEB (-Leu/-Trp/-His) plats supplied with/without 3-aminotriazole (3-AT). Yeast growth was detected after 7 days. Three independent experiments were performed exhibiting similar results. (**B**) BiFc analysis of interaction between RDO5, AHG1 and AHG3. Agrobacterium harboring RDO5-cYFP, nYFP-AHG1, nYFP-AHG3, and nYFP-EIF2Beta (Negative control) under control of 35S promoter were co-infiltrated into *Nicotiana benthamiana* leaves. Fluorescence signal was observed at 2 days post infiltration, red fluorescence represents chloroplast signal.

3.2. RDO5 forms complexes with protein phosphatases

AHG1 and *AHG3* encode two PP2C family proteins, which are regulated negatively by DOG1 in seeds (Née et al., 2017b). Phosphatase activity assays showed that AHG1 can be suppressed by DOG1 independent from ABA (Nishimura et al., 2018). Interestingly, the phosphatase of AHG1 but not AHG3 was identified in RDO5 pull-down experiments (Figure 1). Because AHG3 belongs to the same clade of PP2Cs as AHG1, we reasoned that RDO5 might interact with the two phosphatases AHG1 and AHG3. Next, a yeast two-hybrid experiment was performed to test the physical interactions between RDO5 and AHG1 and AHG3. Co-transformation of RDO5 and the two phosphatases AHG1 and AHG3 in yeast competent cells showed that RDO5 can interact with the two phosphatases AHG1 and AHG3 (Figure 2A). To confirm their interaction in planta, a bimolecular fluorescence complementation (BiFC) analysis in *Nicotiana benthamiana* epidermis was performed. This experiment indicated that RDO5 can interact with AHG1 and AHG3 and that RDO5 co-located with AHG1 and AHG3 in the nucleus (Figure 2B). Overall, these experiments confirmed that RDO5 can directly interact with AHG1 and AHG3.

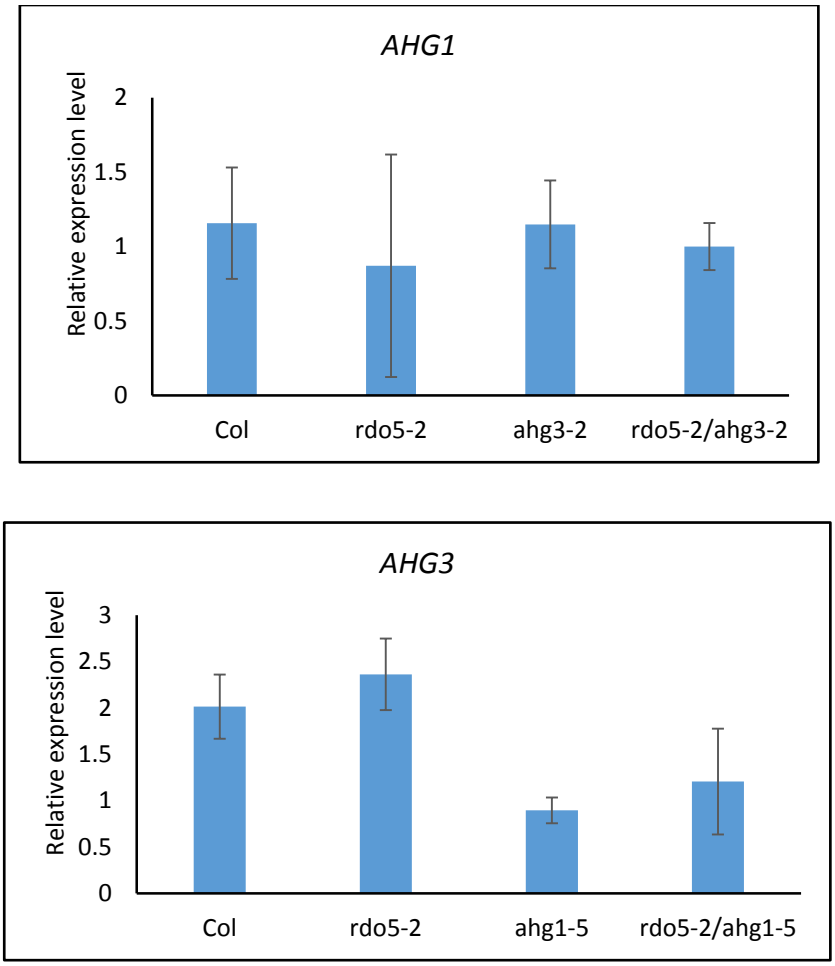
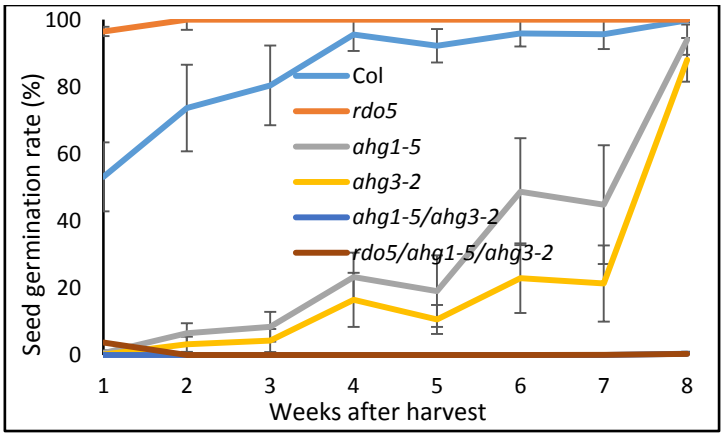


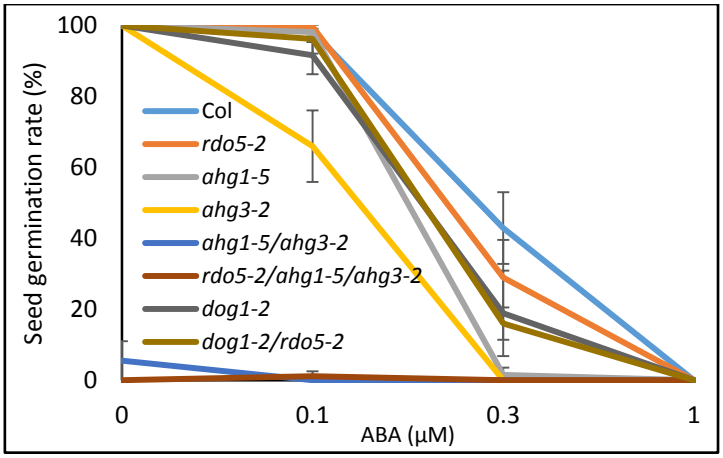
Figure 3. RDO5 does not influence *AHG1* and *AHG3* transcript levels

Transcript levels of *AHG1* in dry seeds of Col-0, *rdo5-2*, *ahg3-2*, and *rdo5-2 ahg3-2* or *AHG3* in dry seeds of Col-0, *rdo5-2*, *ahg1-5*, *rdo5-2 ahg5-2*. *ACT8* was set as an internal control. Gene expression was normalized to the expression in the dry seeds. Bars represent means and standard errors of at least two independent experiments.

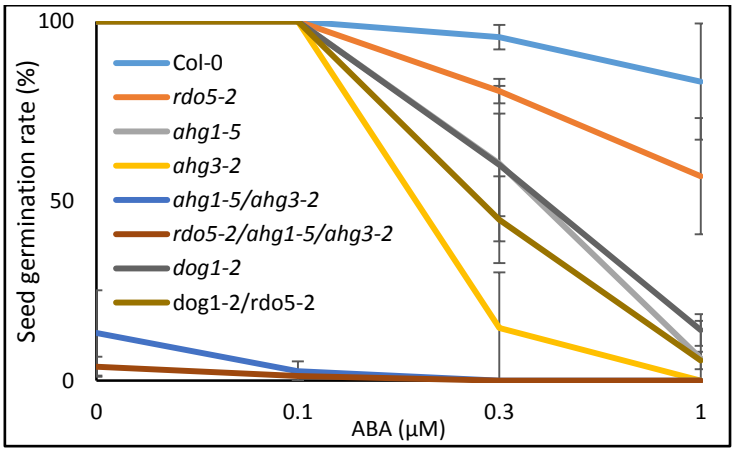
A


Seed germination rate (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Weeks after harvest
Col
rdo5
ahg1-5
ahg3-2
ahg1-5/ahg3-2
rdo5/ahg1-5/ahg3-2

B


Seed germination rate (%)
100
80
60
40
20
0
0
0.1
0.3
1
ABA (μM)
Col
rdo5-2
ahg1-5
ahg3-2
ahg1-5/ahg3-2
rdo5-2/ahg1-5/ahg3-2
dog1-2
dog1-2/rdo5-2

C


Seed germination rate (%)
100
50
0
0
0.1
0.3
1
ABA (μM)
Col-0
rdo5-2
ahg1-5
ahg3-2
ahg1-5/ahg3-2
rdo5-2/ahg1-5/ahg3-2
dog1-2
dog1-2/rdo5-2

D

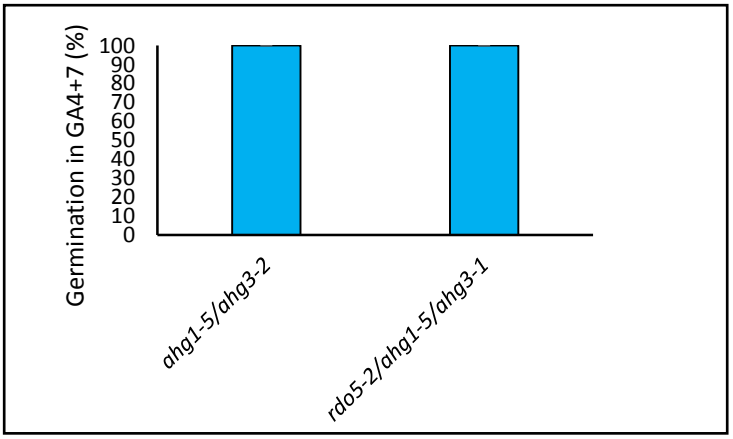

Germination in GA4+7 (%)
100
90
80
70
60
50
40
30
20
10
0
ahg1-5/ahg3-2
rdo5-2/ahg1-5/ahg3-1

Figure 4 DOG1 and the two phosphatases AHG1 and AHG3 are required for the function of RDO5 in the regulation of seed dormancy

(**A**) Seed germination of the single mutant *rdo5-2*, *ahg1-5*, *ahg3-2*, the double mutants *ahg1-5 ahg3-2*, the triple mutant *rdo5-2 ahg1-5 ahg3-2* and their wild-type background Col. Shown are averages ± s.d. of 6–10 independent batches of seeds for each genotype. B-C. Seed germination of same genotypes as in A were examined in the presence of different concentrations of ABA. Seeds were after-ripened for 10 weeks and stratified for three days (**B**) or one week (**C**) to enhance their germination potential. Shown are averages ± s.d. of 6–10 independent batches of seeds for each genotype. (**D**). Germination in 100μM GA4+7 of seeds from the double mutant *ahg1-5 ahg3-2* and the triple mutant *rdo5-2 ahg1-5 ahg3-2* that were stored for 10 weeks, Shown are averages ± s.d. of four independent plants for each genotype.

3.3. The protein DOG1 and the two phosphatases AHG1 and AHG3 are required for the function of RDO5

The two phosphatases AHG1 and AHG3 play a crucial role in the regulation of seed dormancy (Née et al., 2017b). AHG1 was identified in RDO5 pull-down experiments. Y2H and BiFc confirmed that RDO5 can directly interact with AHG1 and AHG3 (Née et al., 2017b). The *ahg1-5* and *ahg3-2* exhibits reduced dormancy compared with Col-0, whereas the *ahg1-5 ahg3-2* double mutant is extreme dormant (Figure 4A), suggesting that AHG1 and AHG3 are functionally redundant and essential for the regulation of seed dormancy. The difference between *ahg1-5* and *ahg3-2* may be due to the relative lower expression of *AHG3* in *agh1-5* background (Figure 3). Moreover, *RDO5* does not affect the transcript of *AHG1* and *AHG3*, and the *AHG1* transcript was not changed in *ahg3-2* and *ahg3-2 rdo5-2* mutants (Figure 3). To further investigate the genetic relationship between *RDO5* and *AHG1* and *AHG3*, we constructed the triple mutant *rdo5-2 ahg1-5 ahg3-2* (Figure 4 and Figure 5). Seed germination experiments showed that the *rdo5-2* mutant was completely non-dormant, whereas the double mutant *ahg1-5 ahg3-2* was quite dormant. In contrast to the single mutant of *rdo5-2*, the triple mutant *rdo5-2 ahg1-5 ahg3-2* showed a very strong dormancy phenotype, similar to the *ahg1-5 ahg3-2* double mutant, which indicates that AHG1 and AHG3 act redundantly downstream of RDO5 and are required for the function of RDO5 in the regulation of seed dormancy (Figure 4A). Seeds with enhanced accumulation of RDO5 protein and loss-of-function mutants with *ahg1-5*, *ahg3-2* as well as their double mutants show enhanced dormancy, suggesting that RDO5 negatively regulates the action of AHG1 and AHG3. Interestingly, the *ahg1 ahg3* double-mutant and the *ahg1-5 ahg3-2 rdo5-2* triple mutant always maintain a dormant state, which suggests that AHG1 and AHG3 play an important role in the release of dormancy.

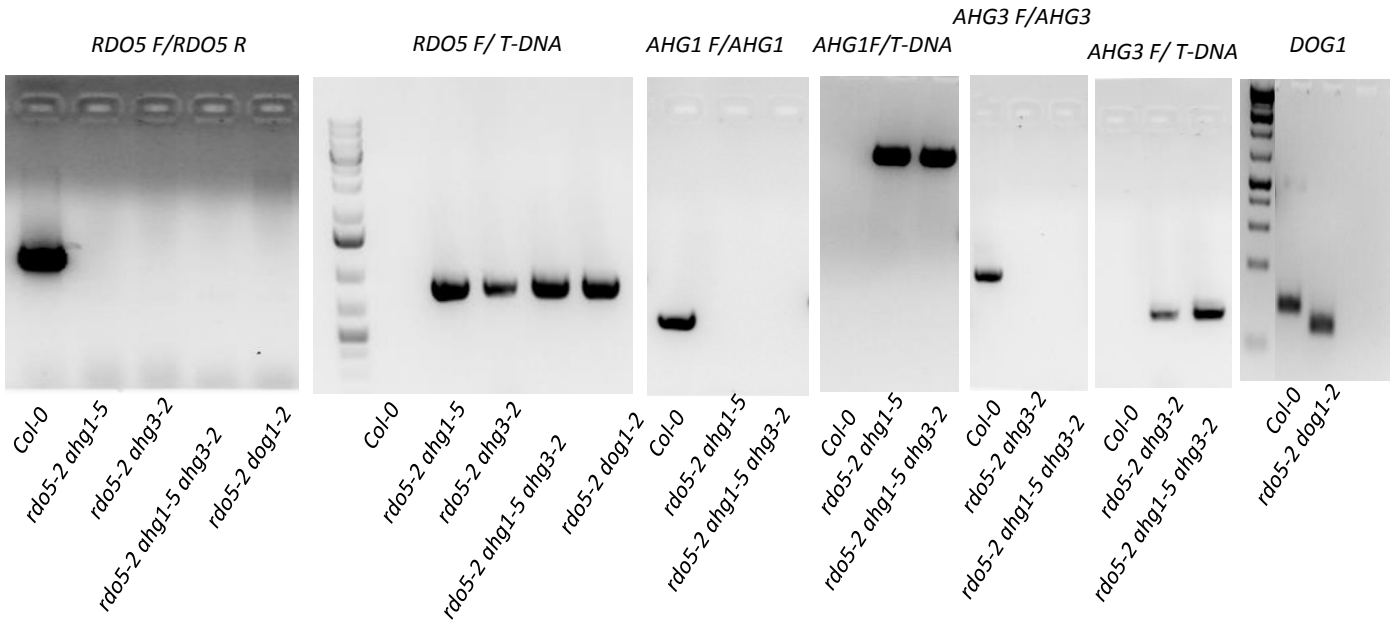
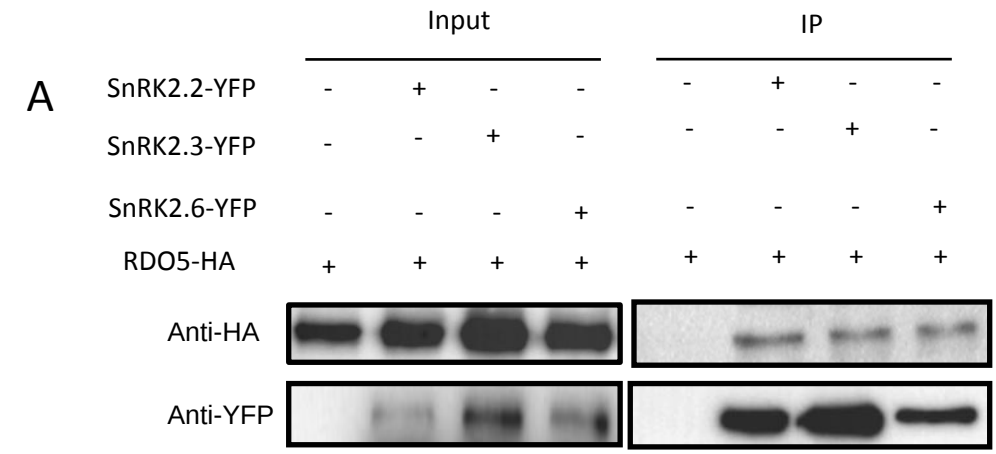
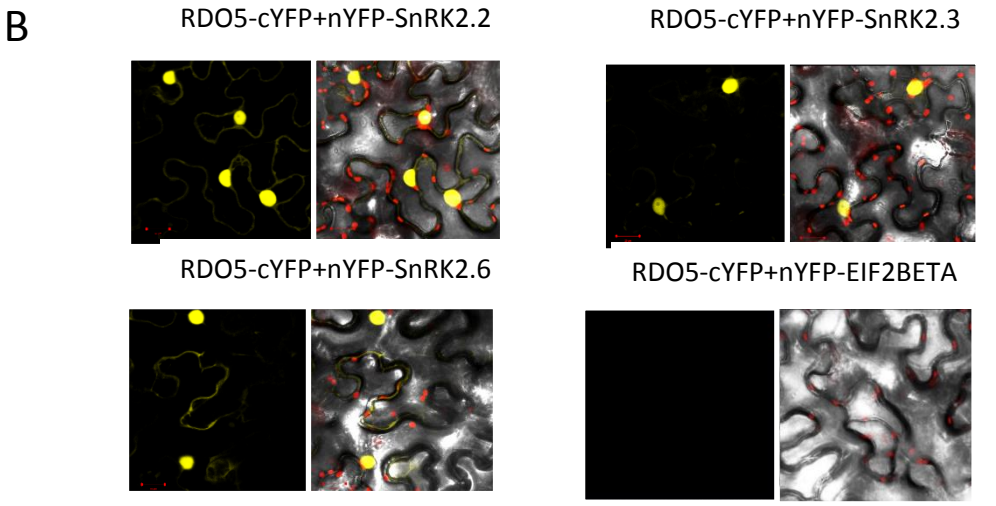


Figure 5. Identification of *rdo5*, *ahg1*, *ahg3* and *dog1* double and triple mutants

Genotyping of *rdo5-2 ahg1-5*, *rdo5-2 ahg3-2*, *rdo5-2 ahg1-5 ahg3-2* and *rdo5-2 dog1-2* mutants. Full length coding sequences of *RDO5*, *AHG1*, *AHG3* were amplified with gene specific forward (F) and reverse (R) primer sets. The T-DNA insertion was confirmed with gene F primer and T-DNA reverse (T-DNA R) primer sets. Genotyping of *dog1-2* mutant was confirmed with restriction enzyme MseI digestion as described (Bentsink et al., 2006; Nakabayashi et al., 2012).

downstream of RDO5 (Figure 4A). In addition, using stratified seeds, the single mutant *rdo5-2* showed a sensitive response to ABA during seed germination, the triple-mutant *rdo5-2 ahg1-5 ahg3-2* showed a similar sensitivity to ABA as the double-mutant *ahg1-5 ahg3-2*, indicating that RDO5 functions upstream of AHG1 and AHG3 for both dormancy and ABA sensitivity (Figure 4B and 4C). It is well known that DOG1 and RDO5 are two positive factors in regulating seed dormancy (Nakabayashi et al., 2012a; Xiang et al., 2014b). Both factors can interact and constitute a complex in seeds (Née et al., 2017b) (Figure 1C). In order to further study the relationship of DOG1 and RDO5, we constructed the double mutant *dog1-2 rdo5-2* (Figure 5). After 3 days of stratification, *rdo5-2* mutant showed slight hypersensitivity to 0.3 µM of ABA (Figure 4B). And the ABA hypersensitive effect of *rdo5-2* was more significant after stratification for 7 days compared with Col-0 (Figure 4C). The *dog1-2* and *dog1-2 rdo5-2* exhibit enhanced ABA hypersensitivity, which germination is more strongly inhibited than *rdo5-2* and Col-0 (Figure 4B and 4C). It was reported that RDO5 interacts with DOG1 (Née et al., 2017c). Taken together, our finding suggests that RDO5 and DOG1 are required and may functions together for the releasing of ABA sensitivity during germination.


A
Input
IP
SnRK2.2-YFP
SnRK2.3-YFP
SnRK2.6-YFP
RDO5-HA
Anti-HA
Anti-YFP


B
RDO5-cYFP+nYFP-SnRK2.2
RDO5-cYFP+nYFP-SnRK2.3
RDO5-cYFP+nYFP-SnRK2.6
RDO5-cYFP+nYFP-EIF2BETA

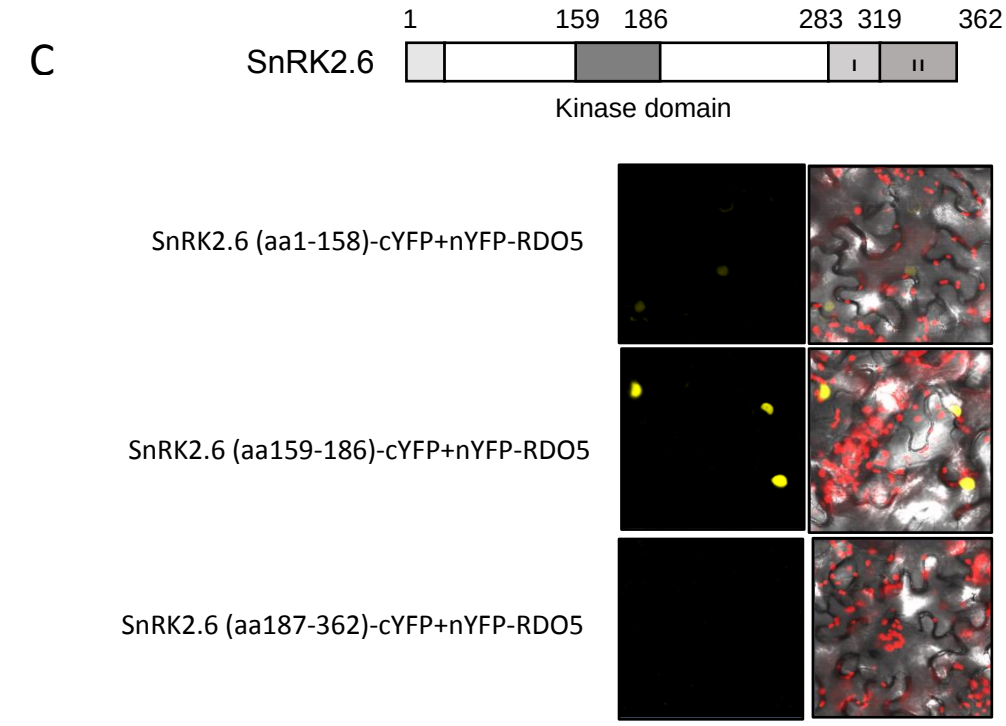

C
SnRK2.6
1
159
186
283
319
362
I
II
Kinase domain
SnRK2.6 (aa1-158)-cYFP+nYFP-RDO5
SnRK2.6 (aa159-186)-cYFP+nYFP-RDO5
SnRK2.6 (aa187-362)-cYFP+nYFP-RDO5

Figure 6. RDO5 interacts with kinase domain of SnRK2s

(**A**) Co-immunoprecipitation of SnRK2.2, SnRK2.3, SnRK2.6, and RDO5. RDO5-HA and SnRK2.2/2.3/2.6-YFP constructs under control of 35S promoter were transiently expressed in Arabidopsis mesophyll protoplast. Co-immunopercipitation was performed by using Anti-GFP Sepharose. The input and immunoprecipitated samples were detected with anti-GFP and anti-HA antibodies. Two independent experiments were performed exhibiting similar results. (**B**) BiFc analysis of interaction between RDO5, SnRK2.2, SnRK2.3 and SnRK2.6. Agrobacterium harboring RDO5-cYFP, nYFP-SnRK2.2, nYFP-SnRK2.3, nYFP-SnRK2.6 and nYFP-EIF2Beta under control of 35S promoter were co-infiltrated into *N. benthamiana* leaves. Fluorescence signal was observed at 2 days post infiltration. Three independent experiments were performed exhibiting similar results. (**C**) BiFc analysis of interaction between RDO5, SnRK2.6 N-terminal (N, aa1-158), kinase domain (KD, aa159-186), and C-terminal (C, aa187-362) regions. Agrobacterium harboring nYFP-RDO5, SnRK2.6N-cYFP, SnRK2.6-KD-cYFP, and SnRK2.6C-cYFP under control of 35S promoter were co-infiltrated into *N. benthamiana* leaves. Fluorescence signal was observed at 2 days post infiltration. Three independent experiments were performed exhibiting similar results.

To determine whether seeds of the double mutant *ahg1-5 ahg3-2* and the triple mutant *rdo5-2 ahg1-5 ahg3-2* stay in a dormant state or have died, we tested the seed viability by imbibing the seeds with 100 μM GA4+7. All seeds germinated, indicating that they are fully viable and maintain a dormant state (Figure 4D).

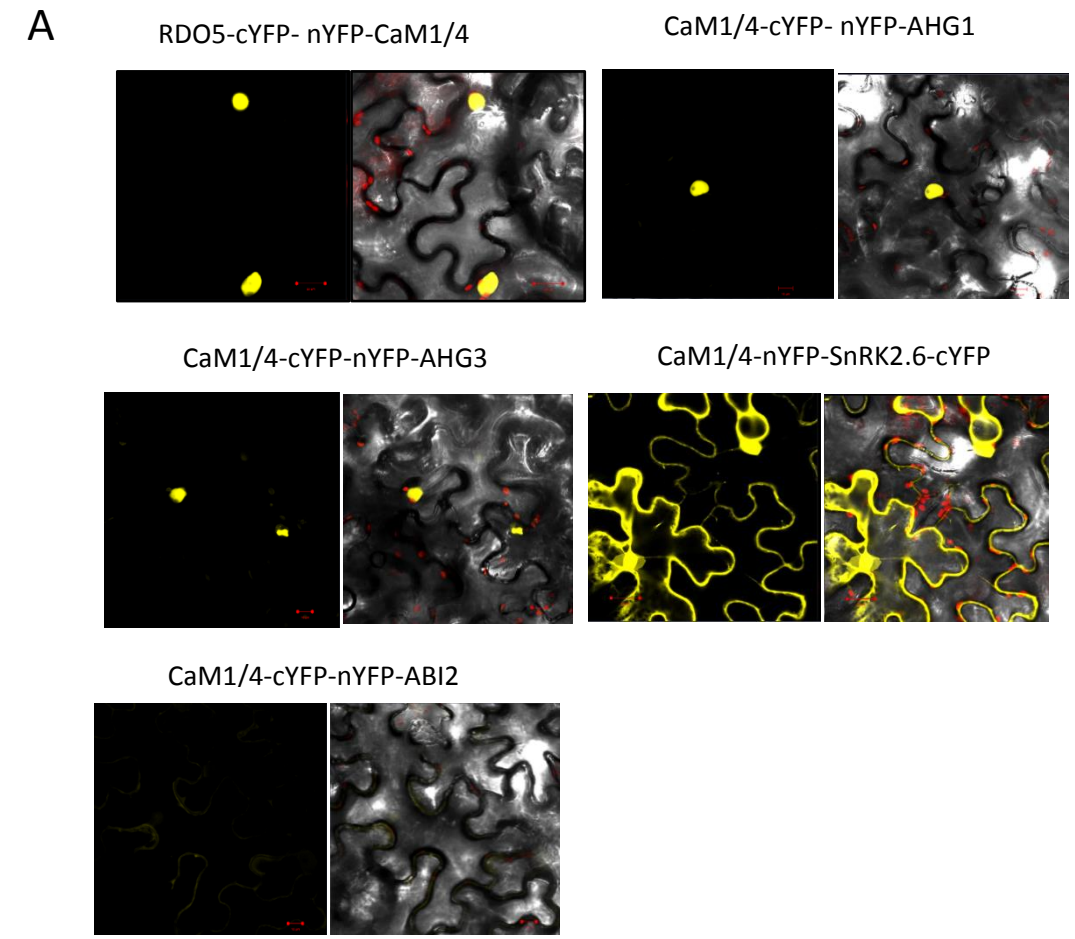
3.4. Physical interaction between RDO5 and SnRK2.6

PP2Cs and SnRK2s family proteins are respectively negative and positive regulators in ABA signaling (Hirayama and Umezawa, 2010). The kinase activity of SnRK2 family proteins is suppressed when it is de-phosphorylated by PP2C family phosphatases (Hirayama and Umezawa, 2010). SnRK2.2, SnRK2.3 and SnRK2.6 belong to the SnRK2s family and have an important function during seed germination (Nakashima et al., 2009). The protein sequences of all SnRK2s contain two important domains, the N-terminal has a highly conserved kinase domain and another domain at the C- terminal can be subdivided into two domains, Domain I and Domain II. Domain I (about 30 amino acids starting from the kinase domain) is required for osmotic stress response. Domain II (about 40 amino acids just after Domain I) is specific to SnRK2.2, SnRK2.3 and SnRK2.6 and is crucial for the ABA response. In addition, Domain II is also required for the protein interaction between ABI1 and SnRK2.6 (Kobayashi et al., 2004; Belin et al., 2006; Yoshida et al., 2006b). Apart from ABI1 and ABI2, Taishi Umezawa showed that AHG1 and AHG3 can directly interact with SnRK2.2, SnRK2.3 and SnRK2.6 (Yoshida et al., 2006b; Umezawa et al., 2009b). Based on these data, I was interested whether RDO5 could interact with SnRK2.2, SnRK2.3 and SnRK2.6. CoiP experiments in *vivo* showed that RDO5 can interact with the three kinases (Figure 6A). BiFc experiments also confirmed that RDO5

can directly interact with SnRK2.2, SnRK2.3 and SnRK2.6 and that the three kinases co-located with RDO5 in the nucleus (Figure 6B). In addition, SnRK2.6 was truncated into three fragments, fragment 1 containing the amino acids from 1 to 158; fragment 2 containing the kinase domain only 28 aminol acids from 159 to 186; fragment 3 containing the amino acids from 187 to 362, which included Domain I and Domain II (Figure 6C). BiFc experiments showed that the kinase domain but not the Domain II (which is required for the protein interaction between ABI1 and SnRK2.6) of SnRK2.6 is important for the interactions between RDO5 and SnRK2s (Figure 6C). Overall, these data suggested that RDO5 can directly interact with SnRK2 family proteins, that RDO5 co-located with these kinases in the nucleus, and that the kinase domain is required for these interactions.

3.5. Physical interactions between CaM1/4 and RDO5, AHG1, AHG3 and SnRK2.6

The calmodulins as calcium sensors play a key role in cellular signaling cascades by regulating numerous substrates (Berridge et al., 2000). CaM1 and CaM4 were identified in RDO5 pull-down and MS experiments to form a complex with RDO5 in seeds (Figure 1). To further clarify the relation between RDO5 and these Calmodulins, we performed a BiFc in epidermis cells of *Nicotiana benthamiana* leaves. BiFc experiments showed that RDO5 can directly interact with CaM1/4 and both factors co-located in the nucleus (Figure 7A). PP2C family proteins are central negative regulators in early ABA signaling pathway (Umezawa et al., 2009b). Calmodulin proteins and AHG1 were identified coincidentally in RDO5 pull-down assays (Figure 1). Because AHG3 belongs to the same clade of PP2Cs as AHG1, we reasoned that CaM1/4 might interact with the two phosphatases AHG1 and AHG3. To clarify the relationships of CaM1/4 and AHG1 and AHG3, we performed a BiFc in epidermis cells of *Nicotiana benthamiana* leaves. BiFc experiments showed that CaM1/4 can directly interact with AHG1 and AHG3 and these factors co-located in the nucleus (Figure 7A). Moreover, we confirmed that CaM1/4 can also interact with SnRK2.6 and both proteins were co-located in the nucleus and cytoplasm (Figure 7A).



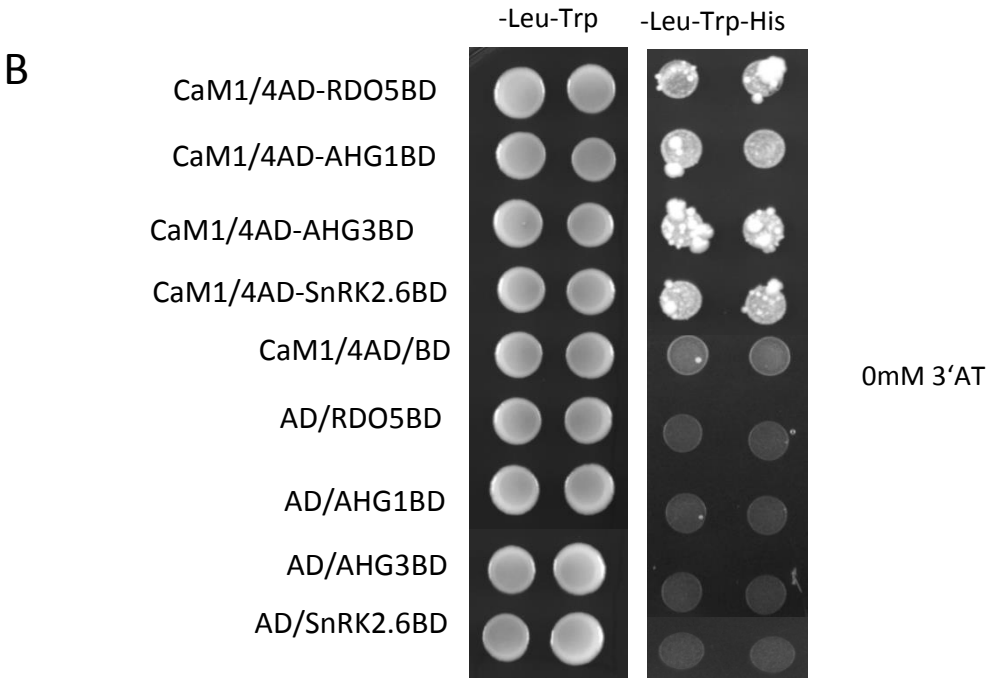


Figure 7. CaM1/4 directly interacts with RDO5, AHG1, AHG3 and SnRK2.6

(**A**) BiFC analysis of interaction between CaM1/4 with RDO5, AHG1, AHG3 and SnRK2.6. Agrobacterium harboring RDO5-cYFP and CaM1/4-nYFP, or CaM1/4-cYFP, nYFP-AHG1, nYFP-AHG3 and nYFP-SnRK2.6 under control of 35S promoter were co-infiltrated into *N. benthamiana* leaves. Fluorescence signal was observed at 2 days post infiltration. nYFP-ABI2 was used as negative control. At least three independent experiments were performed for each interaction. (**B**) Yeast-two hybrid assay for the interaction between CaM1/4, RDO5, AHG1, AHG3 and SnRK2.6. Yeast cells transformed with CaM1/4-GAL4AD, RDO5-GAL4BD, AHG1-GAL4BD, AHG3-GAL4BD, and SnRK- AHG1-GAL4BD were spotted onto YEB (-Leu/-Trp) and YEB (-Leu/-Trp/-His) plates. Yeast growth was detected after 7 days. Three independent experiments were performed exhibiting similar results.

In addition, a yeast two-hybrid experiment was performed to test the physical interactions between CaM1/4 and RDO5, AHG1, AHG3 and SnRK2.6. Co-transformation of CaM1/4 and RDO5, AHG1, AHG3 and SnRK2.6 in yeast competent cells showed that the physical interactions between CaM1/4 and RDO5, AHG1, AHG3 and SnRK2.6 can also occur in yeast (Figure 7B). Overall, all of these data suggested that RDO5 can interact with RDO5, AHG1, AHG3 and SnRK2.6.

3.6. Calmodulins might positively regulate seed dormancy

Calmodulin (CaM) is a universal, calcium receptor that can bind to and regulate numerous different protein targets, affecting many cellular pathways (Popescu et al., 2007). There have been numerous research studies about the roles of CaM1 and CaM4 in the regulation of salt resistance, Age-Dependent Cell Death and freezing tolerance in Arabidopsis (Zhou et al., 2016a). However, the function of calmodulin in the regulation of seed dormancy remain unknown. To understand the role of calcium sensor CaMs in germination and dormancy, we obtained the mutants *cam1* and *cam4*, and generated the *cam1 cam4* (*cam1/4*) double mutant (Figure 8A), which exhibits an absence of CaM1 and CaM4 expression (Figure 8B). The seed germination ratio was further confirmed in the *cam1/4* mutant. In normal conditions, the germination ratio in *cam1 cam4* is similar with Col-0, but after treating the mutant with different concentration ABA, we found that the double mutant *cam1 cam4* showed reduced sensitivity to ABA during seed germination (Figure 9A). To further clarify the function of calmodulins in controlling seed dormancy we subsequently constructed CaM1/4 overexpression transgenic lines (Figure 9B and 9C). Seed germination experiments showed that overexpressed CaM1/4 can increase the level of seed dormancy (Figure 9B). All of these data indicate that CaM1 and CaM4 might positively regulate seed dormancy.

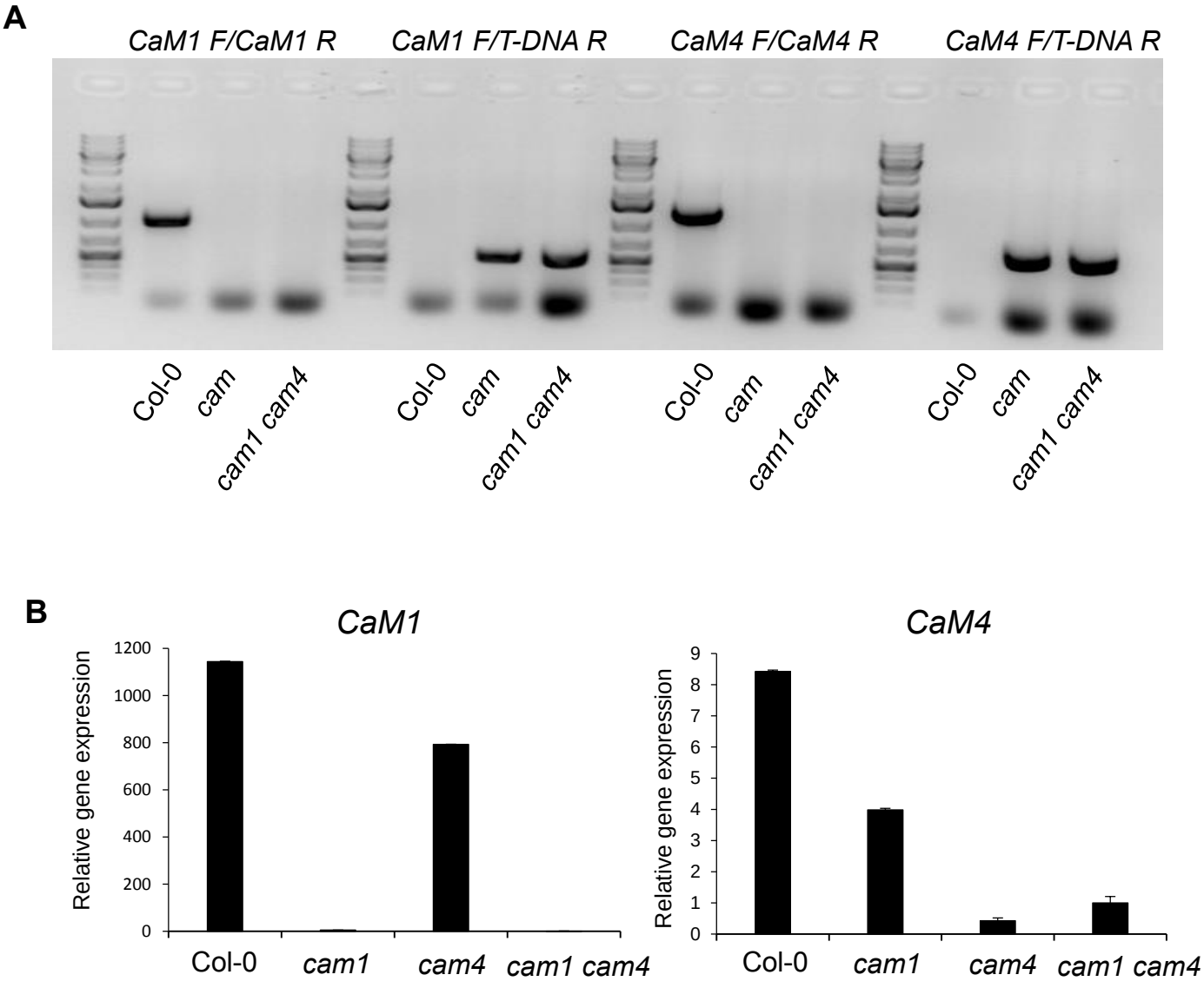


Figure 8. Identification of *CaM1* and *CaM4* mutants

(**A**) Genotyping of *cam1*, *cam4* and *cam1/4* mutants. Full length of *CaM1* and *CaM4* was amplified with gene specific forward (F) and reverse (R) primer sets. The T-DNA insertion was confirmed with gene F primer and T-DNA reverse (T-DNA R) primer sets. (**B**) Relative expression of *CaM1* and *CaM4* was confirmed by qRT-PCR. Gene expression was normalized to the level in *cam1/4* mutant. *ACT8* was set as internal control. Bars represent means and standard errors of two independent experiments. ($p < 0.05$; student t-test).

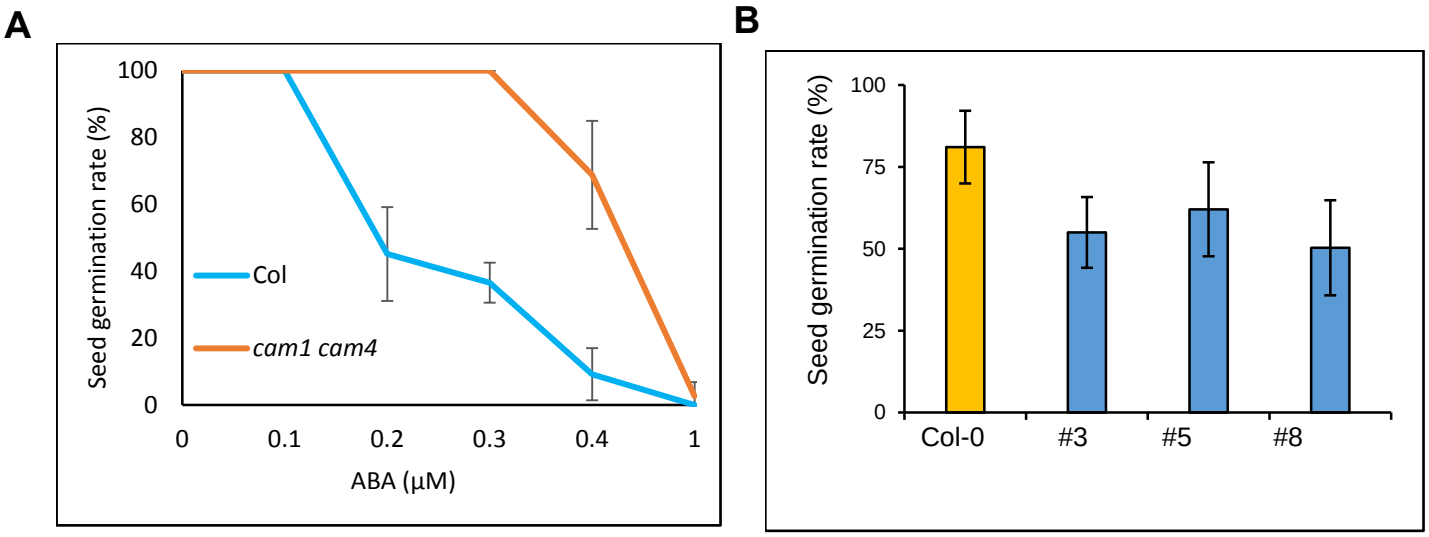


Figure 9. Arabidopsis *CaM1/4* might positively contribute to seed dormancy

(**A**) Effect of ABA on the germination efficiency of *cam1 cam4* mutant. Seeds were after-ripened for 3 weeks and stratified for three days to enhance their germination potential. The seeds were sown on wet filter paper with different concentration of ABA. Germination ratio was counted at 4 days. Bars represent means and standard errors of at least three independent batches of seeds for each genotype. (**B**) Germination ratio of the freshly harvested seeds of Col-0 and *CaM1* overexpression plants (CaM1 OE #3, #5, and #8) were counted at 7 days after germination. (**, $p < 0.01$; student t-test). (**C**) Protein level accumulation in CaM1 OE lines were detected by anti-HA antibody. Equal loading was confirmed by anti-Histone H3 antibody.

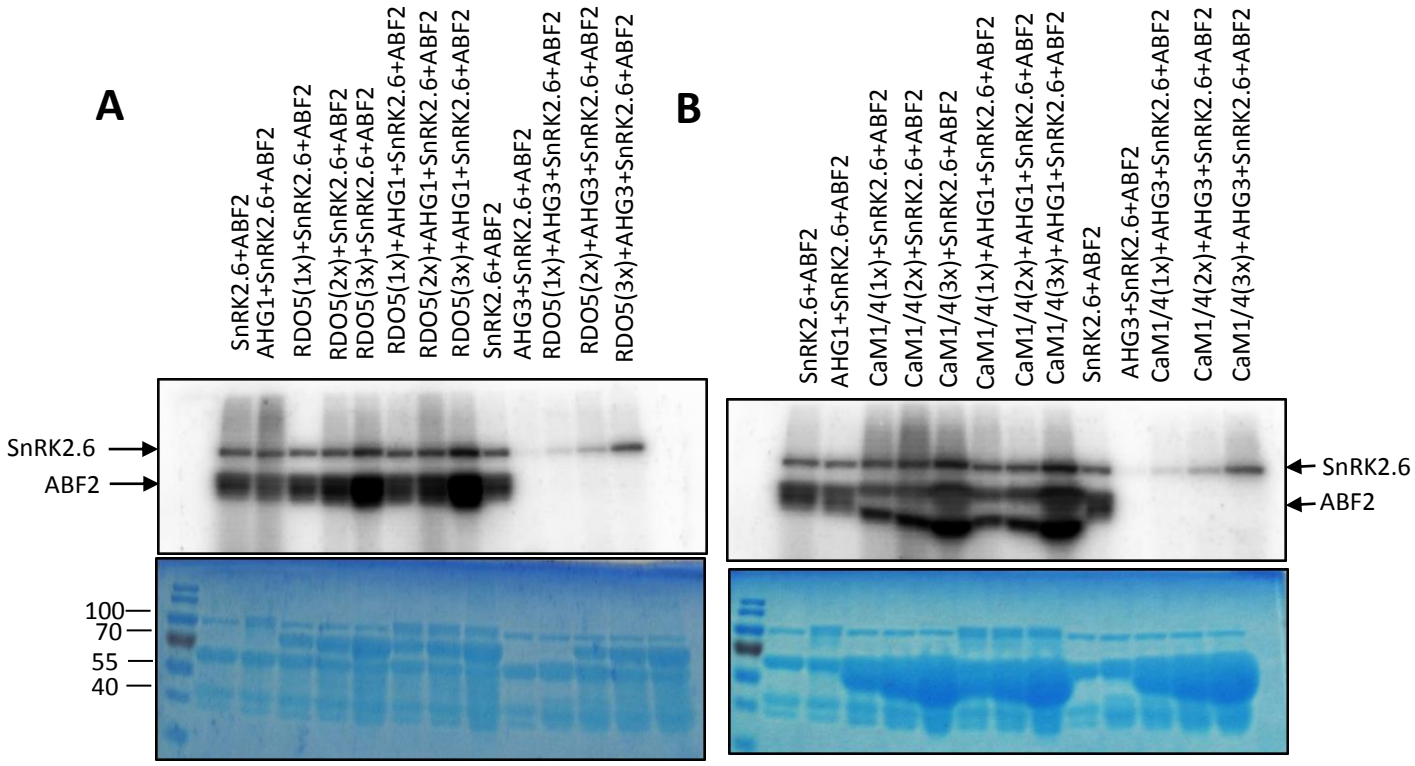


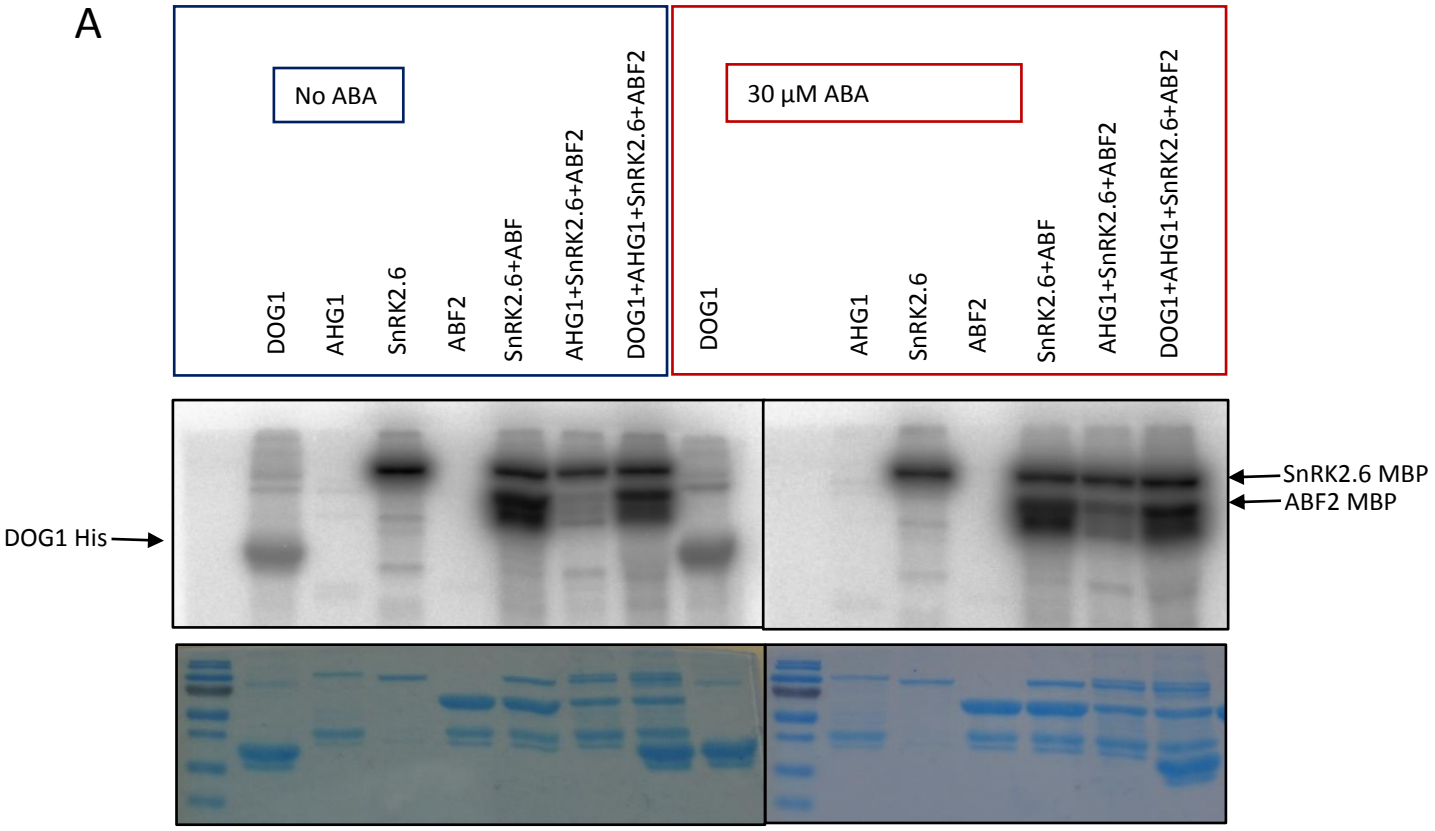
Figure 10. CaM1/4 and RDO5 inhibit AHG1- and AHG3-mediated de-phosphorylation of SnRK2.6

(A) RDO5 increases the kinase activity of SnRK2.6 and prevents the de-phosphorylation of SnRK2.6 and ABF2 from AHG1 and AHG3. SnRK2.6 and ABF2 proteins were incubated for 2 hours, and purified recombinant proteins of RDO5-MBP, AHG1-MBP, and AHG3-GST were added to the mixture. In gel phosphorylation (IGP) assay was applied at 2 hours after incubation. Blotted membrane was stained with CBB for quality control. (B) CaM1/4 increases the kinase activity of SnRK2.6 and prevents the de-phosphorylation of SnRK2.6 and ABF2 from AHG1 and AHG3. SnRK2.6 and ABF2 proteins were incubated for 2 hours, and purified recombinant proteins of CaM1/4-MBP, AHG1-MBP, and AHG3-GST were added to the mixture. In gel phosphorylation (IGP) assay was applied at 2 hours after incubation. Blotted membrane was stained with CBB for quality control.

3.7. RDO5 interacts with kinase domain of SnRK2.6 and protects its kinase activity

It was reported that RDO5 encodes an inactive PP2C protein (Xiang et al., 2014a). And PP2C phosphatase are involved in the ABA signaling regulation by interaction and dephosphorylation of SnRK2 kinases (Soon et al., 2012). Protein interaction experiments above have demonstrated the physical interactions between RDO5 and SnRK2.6 and the two phosphatases AHG1 and AHG3 (Figure 2 and Figure 6). Genetics data suggested that RDO5 functions upstream of the two phosphatases and SnRK2s (Figure 3), which led us to the idea that RDO5 can affect the phosphatase activity of AHG1 and AHG3 or the kinase activity of

SnRK2.6. Recombinant AHG1-MBP and AHG3-GST protein interacted with SnRK2.6 and ABF2 proteins, respectively. Phosphorylation of SnRK2.6 and ABF2 was confirmed by in gel phosphorylation (IGP) assay. Both AHG1 and AHG3 exhibit phosphatase activity, whereas AHG3 exhibits stronger effects in vitro (Figure 10A and 10B). However, when AHG1-MBP and AHG3-GST proteins were added two hours later to the SnRK2.6 and ABF2 reaction mixture, AHG1 could only slightly reduce SnRK2.6 kinase activity but not its phosphorylation. The strong phosphatase activity was still maintained in AHG3 (Figure 10A and 10B). RDO5, known as pseudo-phosphatase, could enhance the phosphorylation of SnRK2.6 in vitro, and protect the phosphorylation of SnRK2.6 in a dosage dependent manner with treatment of AHG1 and AHG3 (Figure 10A). Moreover, appearance of CaM1/4 enhances the phosphorylation and kinase activity of SnRK2.6 in a dosage dependent manner (Figure 10B). Interestingly, CaM1/4 blocks the phosphatase activity of AHG1 and AHG3, and rescues AHG3 mediated SnRK2.6 dephosphorylation but not kinase activity (Figure 10B). All of these data suggested that CaM1/4 and RDO5 may coordinate for the regulation of in seed dormancy via inhibition of AHG1 and AHG3 function.


A
No ABA
30 µM ABA
DOG1
AHG1
SnRK2.6
ABF2
SnRK2.6+ABF
AHG1+SnRK2.6+ABF2
DOG1+AHG1+SnRK2.6+ABF2
DOG1 His
SnRK2.6 MBP
ABF2 MBP

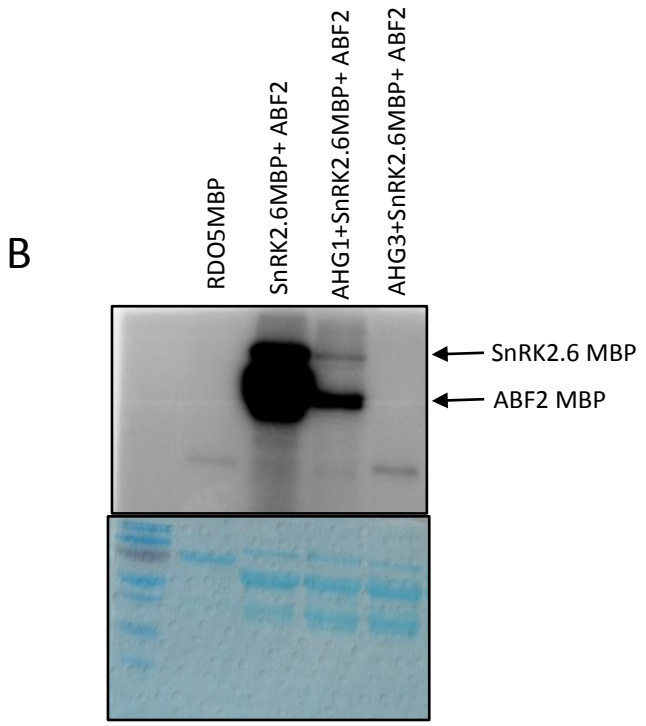

B
RDO5MBP
SnRK2.6MBP+ ABF2
AHG1+SnRK2.6MBP+ ABF2
AHG3+SnRK2.6MBP+ ABF2
SnRK2.6 MBP
ABF2 MBP

Figure 11 . Effect of AHG1, AHG3 and DOG1 on SnRK2.6 activity

(**A**) SnRK2.6 and ABF2 proteins were incubated for 2 hours. IGP assay was applied at 2 hours after incubation. Blotted membrane was stained with CBB for quality control. Same amount of purified recombinant proteins of DOG1-His, AHG1-MBP, and AHG3-GST or AHG1-MBP and AHG3-MBP were incubated with SnRK2.6-ABF2. IGP assay was performed after incubation for 2 hours in the presence and absence of 1 μM of ABA. CBB was used to confirm the protein loading. (**B**) SnRK2.6-MBP, ABF2-MBP, AHG1-MBP, and AHG3-GST proteins were incubated for 2 hours. IGP assay was applied at 2 hours after incubation. Blotted membrane was stained with CBB for quality control.

3.8. DOG1 suppresses AHG1 phosphatase activity in a pathway independent from ABA

Two PP2C family proteins AHG1 and AHG3 are required for the function of DOG1 in the regulation of seed dormancy in Arabidopsis (Née et al., 2017b). Among them, the phosphatase activity of AHG1 can be suppressed by DOG1 by using the synthetic phosphopeptide and this inhibition is independent from ABA signals (Nishimura et al., 2018). Because the importance of the complexes of DOG1, AHG1 and AHG3 in the regulation of seed dormancy, we continued to investigate the relationships between DOG1 and AHG1 and AHG3 by isotope experiments. Incubation of SnRK2.6 and its substrate ABF2 in a kinase buffer for two hours produced a phosphorylated ABF2. The phosphorylated ABF2 subsequently can be dephosphorylated by the two phosphatases of AHG1 and AHG3 (Figure 11A). The auto-phosphorylation of SnRK2.6 can be dephosphorylated by AHG3 under all tested conditions. Interestingly, SnRK2.6 could be dephosphorylated by AHG1 only when we incubated SnRK2.6, ABF2 and AHG1 at the same time (Figure 11B). In contrast, SnRK2.6 could be weakly dephosphorylated by AHG1 when we separated protein phosphorylation and de-phosphorylation into two processes (incubated SnRK2.6 and ABF2 in a kinase buffer for two hours and subsequently added AHG1 into the reaction for another two hours) (Figure 11A). In addition, we found that the activity of AHG1 can be suppressed by DOG1, which is consistent with a previous report (Nishimura et al., 2018). Additionally, we added 30μM ABA into all of the reactions under same conditions which indicated as above (Figure 11A). We found the suppression of AHG1 by DOG1 is completely independent from ABA, which is consistent with a recent study by (Nishimura et al., 2018).

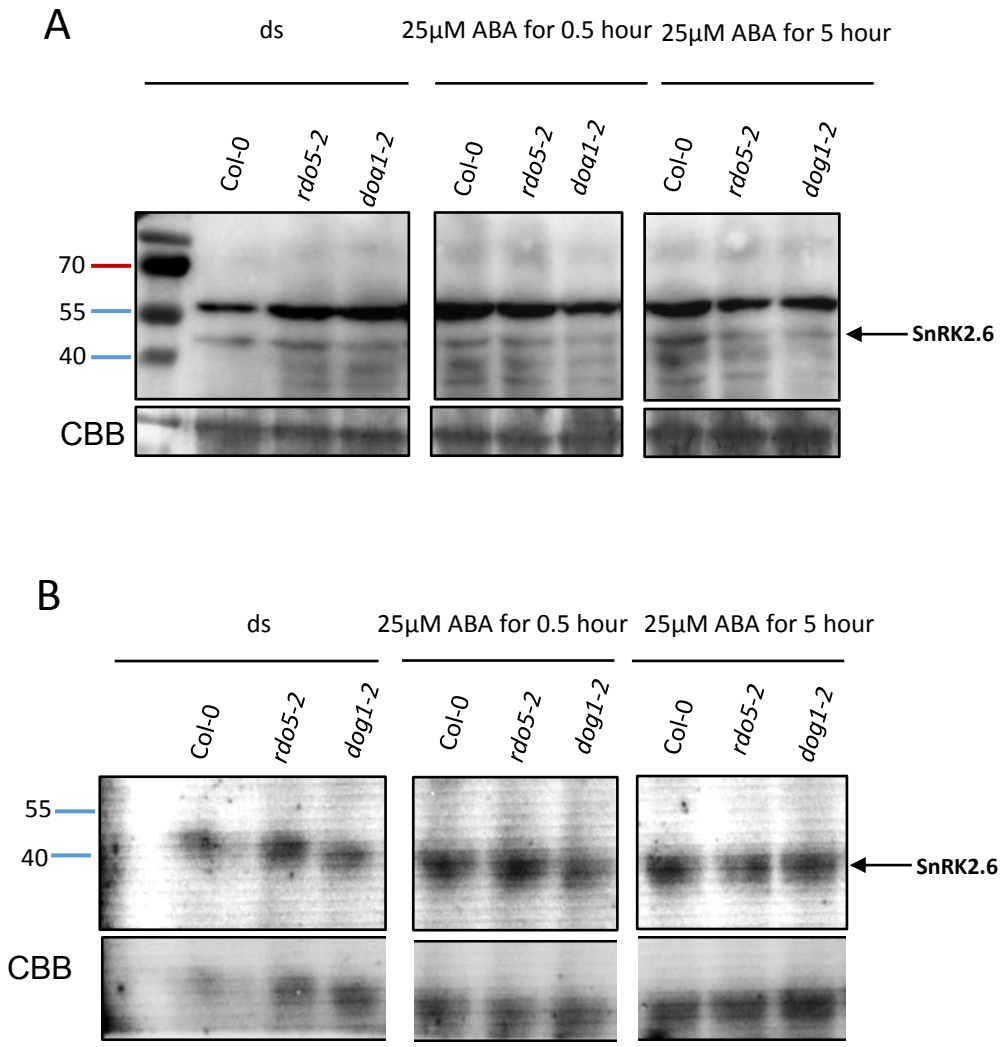


Figure 12 RDO5 and DOG1 promote SnRK2.6 protein accumulation but not its kinase activity in planta

(**A**) Same amount of protein (80 μg) from freshly harvested dry seeds and 25 μM ABA treated seeds of Col-0, *rdo5-2* and *dog1-2* at 0.5 and 5 hours were extracted, and separated on SDS-PAGE. Accumulation of SnRK2.2/2.3/2.6 was detected by Anti-SnRK2.2/2.3/2.6 antibody. (B) Kinase activity of SnRK2.2/2.3/2.6 was detected by in gel kinase assay with ABF2 and ABI5 as substrates. CBB stain was used for equal loading of protein samples. Three independent experiments were performed exhibiting similar results.

3.9. RDO5 and DOG1 affect SnRK2 protein accumulation but not its phosphorylation in seeds.

AHG1 and AHG3 are two PP2C family phosphatases and play an important role in the regulation of seed dormancy and ABA signaling (Née et al., 2017b). In gel kinase experiments have demonstrated that SnRK2 was hyperactivated in mutant seeds of *ahg1–1* and *ahg3–1*,

which further indicate that the important function of AHG1 and AHG3 in controlling seed dormancy acts via regulating the kinase activity of SnRK2 (Umezawa et al., 2009b). Genetics experiments have demonstrated that DOG1 and RDO5 function upstream of the two phosphatases AHG1 and AHG3 and negatively regulate their actions (Figure 4) (Née et al., 2017b). The phosphatase activity of AHG1 but not AHG3 can be suppressed by DOG1 (Figure 11A) (Nishimura et al., 2018). Moreover, we found that the actions of AHG1 and AHG3 can be regulated by RDO5 (Figure 7). To understand the role of RDO5 and DOG1 on the regulation of SnRK2s in seeds, we detected the protein amounts of SnRK2.2/2.3/2.6 in the mutant *rdo5-2*, *dog1-2* and Col by using SnRK2.2/2.3/2.6 antibodies (Agrisera). We found that SnRK2.6 protein was not affected in dry seeds of the mutants *dog1-2* and *rdo5-2*. Interestingly, the protein of SnRK2.6 gradually decreased with the ABA treatment and reach lowest level after 5 hours ABA treatment in the mutants *rdo5-2* and *dog1-2* (Figure 12A). These data suggested important roles of DOG1 and RDO5 in maintaining the protein stability of SnRK2s. To investigate whether the kinase activity of SnRK2s also was affected by RDO5 and DOG1 in vivo, we performed in gel kinase experiments. These showed that the kinase activity of SnRK2s was not affected in mutant seeds of *rdo5-2* and *dog1-2* (Figure 12B). Overall, our data suggested important functions for RDO5 and DOG1 in maintaining the stability of SnRK2 in seeds.

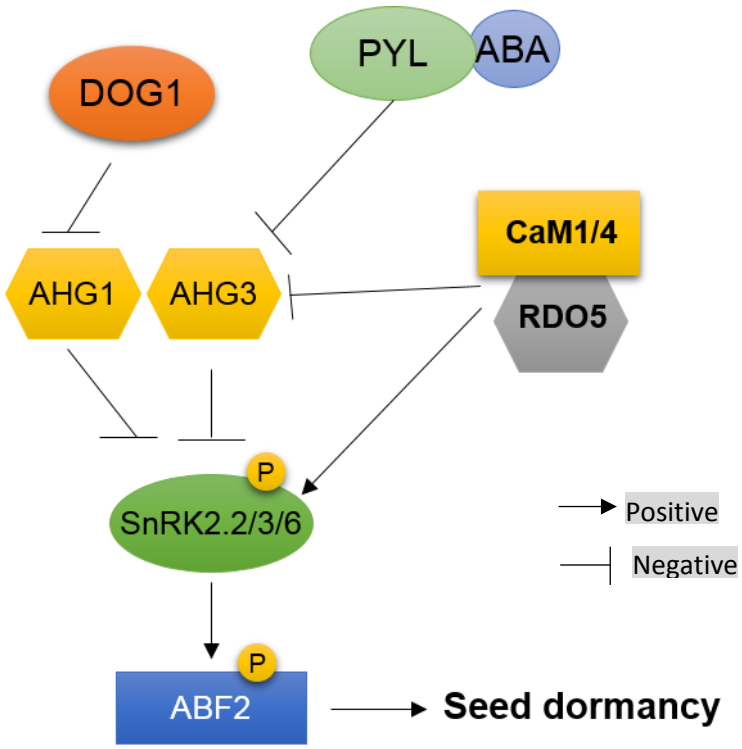


Figure 13. Model for RDO5 in the regulation of seed dormancy

The action of clade A of the type 2C protein phosphatases can be suppressed by RDO5, CaM1/4, DOG1 and ABA. The phosphatase activity of AHG1 can be directly inhibited by DOG1. CaM1/4 interacts with RDO5, and prevents AHG1 and AHG3 mediated SnRK2.2/2.3/2.6 de-phosphorylation.

4. Discussion

RDO5 encodes a member of the PP2C family and plays an important role in the regulation of seed dormancy (Xiang et al., 2014b). Phosphatase activity assays and phosphoproteomics data suggested that RDO5 is a pseudo-phosphatase that still affects protein phosphorylation and de-phosphorylation (Xiang et al., 2016b). In this study, we investigated the molecular function of RDO5 and its interacting protein calmodulin in the regulation of seed dormancy by using molecular, genetic and biochemical methods (Figure 13). Our results, combined with previous publications, demonstrate that (1) RDO5, AHG1 and AHG3 bind to the same SnRK2s; (2) AHG1 and AHG3 are necessary for RDO5 function in seed dormancy; (3) CaM1/4 might positively regulate seed dormancy; (4) CaM1/4 interacts with RDO5, and inhibits AHG1 and AHG3 mediated SnRK2.2/2.3/2.6 de-phosphorylation.

4.1. RDO5 inhibits AHG1 and AHG3 function through substrate competition

AHG1, AHG3 and RDO5 are belonging to the family of PP2C phosphatases. Both AHG1 and AHG3 could directly interact and dephosphorylate SnRK2s, which are the main positive regulators of ABA signaling (Yoshida et al., 2006d; Umezawa et al., 2009a). In the regulation of seed dormancy, AHG1 and AHG3 are functionally redundant. Different from AHG1 and AHG3, RDO5 does not have phosphatase activity, which suggests that it might function as a pseudo-phosphatase (Amiguet‐Vercher et al., 2015b; Xiang et al., 2016a). Phosphoproteomics analysis of the *rdo5* mutant showed a significant alteration in the phosphorylation level of several proteins (Xiang et al., 2016c), suggesting that RDO5 affects protein phosphorylation indirectly. It has also been shown that the pseudo-phosphatase *IBO*, which encodes RDO5, inhibits the phosphorylation of ABI1 in Arabidopsis (Amiguet‐Vercher et al., 2015b). However, the molecular mechanism is unknown. In mammalian cells, the pseudophosphatase STYX directly interacts with its substrate thereby preventing its phosphorylation and degradation (Reiterer et al., 2018). We hypothesized that RDO5 inhibits AHG1 and AHG3 phosphorylation by direct binding to their substrates. Indeed, RDO5 directly interacts with SnRK2.2/2.3/2.6 and protects SnRK2.6 from dephosphorylation by AHG1 and AHG3 (Figure 6 and Figure 10). The C-terminal domain of SnRK2s was shown to be needed for the interaction with PP2C phosphatase (Yoshida et al., 2006a). In contrast, we found that

RDO5 interacts with SnRK2.6 by binding with its kinase domain (Figure 6C). These results suggest that RDO5 inhibits AHG1 and AHG3 activity via substrate binding competition.

4.2. DOG1 and RDO5 work together in their control of seed dormancy.

DOG1 and RDO5 are two key regulators of seed dormancy in Arabidopsis (Nakabayashi et al., 2012a; Xiang et al., 2014b). RDO5 was found in a DOG1 pull-down assay and Y2H and BiFc experiments have confirmed their physical interaction (Née et al., 2017b). Importantly, DOG1 was also pulled down by RDO5 under all tested conditions (Figure 1). These data indicate that RDO5 and DOG1 can form a complex in seeds. However, it is not clear how RDO5 and DOG1 work together to regulate dormancy and what is the genetic relation between them. *RDO5* encodes a PP2C family protein and a phosphatase activity experiment showed that RDO5 is a pseudo-phosphatase (Xiang et al., 2014b; Amiguet-Vercher et al., 2015a; Xiang et al., 2016b). Although RDO5 and DOG1 do not influence the RNA and protein accumulation of each other, they physically interact (Xiang et al., 2014c; Née et al., 2017b). And both mutants exhibit a complete loss of seed dormancy, suggesting that RDO5 and DOG1 function together in the regulation of seed dormancy (Xiang et al., 2014c; Née et al., 2017b). Moreover, both AHG1 and AHG3 are necessary for the function of DOG1 and RDO5 in seed germination (Figure 4A) (Nee et al., 2017), illustrating that DOG1 and RDO5 signaling are converted at AHG1 and AHG3. Interestingly, the *dog1 rdo5* double mutant showed similar ABA sensitivity with *dog1*, and *rdo5* is less sensitive (Figure 4C), indicating that RDO5 may act upstream of DOG1 in seed dormancy. However, we cannot exclude the possibility that DOG1 is a master regulator for seed dormancy that functions through RDO5 and its homologs.

4.3. The mutant *dog1-2* shows a sensitive response to ABA

Seed dormancy is a complicated process, which is affected by numerous regulators such as plant hormones, dormancy related proteins and many environmental factors (Donohue et al., 2010). Freshly harvested seeds have a relatively high dormancy level that is gradually released during subsequent storage or low temperature stratification (Née et al., 2017a). A previous work showed that the seed germination rate of wild type Col-0 can reach around 60% with 3μM ABA treatment after 3 days cold stratification, while increasing the stratification time to 7 days increased the germination rate to 100%, which indicates that the sensitivity of seeds to ABA is decreased with the increase of cold stratification (Chen et al., 2013; Lee et al., 2015). DOG1 has an important function in the regulation of dormancy. Interestingly, a lower seed

maturation temperature can increase the protein levels of DOG1 and subsequently lead to higher dormancy levels (Nakabayashi et al., 2012a). The balance between the ABA and GA pathways determines the dormancy level and germination potential of seeds (Holdsworth et al., 2008). The mutant seeds of *dog1* were after-ripened for 1 year and stratified for 3 days to check their germination potential at different concentrations ABA. Seed germination experiments showed that the *dog1-2* mutant showed a reduced sensitivity to ABA (Bentsink et al., 2006b; Née et al., 2017b). However, a recent study showed that the mutant seeds of *dog1-2* have nearly wild-type sensitivity to applied ABA after 3 days stratification (Nishimura et al., 2018). Consistent with these studies, our seed germination experiments showed that the mutant seeds of *dog1-2* also showed no obvious response to ABA under 3 days cold stratification (Figure 4B). Interestingly, the mutant seeds of *dog1-2* showed a sensitive response to ABA with increasing stratification times (Figure 4C). Overall, these data showed that the dormancy level and the sensitivity of seeds to ABA might be greatly affected by the seed storage time and the seed stratification time.

4.4. CaM1/4 might positively regulate seed dormancy

Calmodulin is the primary calcium sensor, and is involved in diverse cellular signal transduction processes in plants (Perochon et al., 2011). The Arabidopsis genome harbours seven CaMs, which only encode four different CaM proteins (McCormack et al., 2005b). This gene duplication during evolution indicates the importance of CaMs. Calmodulin proteins have important roles in response to a rise in the cytoplasmic concentration of Ca2+ in many physiological processes in plants and animals (Clapham, 2007). Considering the many processes in which they are involved, their diverse subcellular localization patterns, and their assorted responses to various environmental stimuli, each CaM isoform may have a specific function (Steinhorst and Kudla, 2014). For example, AtCaM3 is involved in the induction of cold- and heat-responsive genes (Townley and Knight, 2002; Steinhorst and Kudla, 2014), while AtCaM7 is involved in the induction of light-responsive genes (Kushwaha et al., 2008). Arabidopsis CaM1 and CaM4 are isoforms which encode the same protein sequence (McCormack et al., 2005b). CaM1 and CaM4 exhibit functional redundancy, but still have their specificity under different conditions. For instance, the c*am1 cam4* double mutant exhibits an enhanced production of nitric oxide, which increases salt stress resistance. This phenotype is not observed in the *cam1* and *cam4* single mutants, illustrating the functional redundancy of CaM1 and CaM4 in salt stress resistance (Zhou et al., 2016b). CaM1 is also involved in ABA mediated ROS production in Arabidopsis. Absence of NADPH Oxidases *RbohD* and *RbohF*

negatively contribute to the expression of *CaM1* (Dai et al., 2018), suggesting a feedback loop of calcium signaling and ROS production via *CaM1*. Overexpression of *CaM1* enhances *RbohF* expression and ROS production, which leads to an early senescence of the plant. Moreover, it was also reported that CaM4 contributes to ROS accumulation and plant senescence via direct interaction with RbohF (Koo et al., 2017b). Since CaM1 and CaM4 share their amino acid sequence, it is difficult to distinguish their role in overexpression plants. Interestingly, the *cam4* mutant confers increased freezing tolerance, but *cam1* RNAi lines are comparable to wild type plant (Chu et al., 2018a). Our results showed that the mutant *cam1 cam4* showed a less sensitive response to ABA during seed germination (Figure 9A). Moreover, overexpression of CaM1/4 in Col wild-type caused enhanced dormancy (Figure 9B). These data suggested that CaM1 and CaM4 might positively regulate seed dormancy.

4.5. CaM1/4 inhibits AHG1 and AHG3 function through substrate competition

CaM1/4 probably functions in the regulation of seed dormancy. Freshly harvested seeds from the double mutant *cam1 cam4* showed less sensitivity to ABA during seed germination, which is similar to the phenotype of the *snrk2.2/2.3/2.6* mutant (Figure 9A) (Fujita et al., 2009). This suggests that CaM1/4 might positively regulate the activity of SnRK2.2/2.3/2.6. In addition, compared to wild type plant seeds, overexpression of CaM1/4 in Col results in enhanced dormancy (Figure 9B), which suggests that CaM1/4 might positively regulate seed dormancy. Protein interaction experiments showed that CaM1/4 can directly interact with SnRK2s (SnRK2.2, SnRK2.3 and SnRK2.6) and PP2Cs (AHG1 and AHG3) (Figure 7). In addition, CaM1/4 was identified in RDO5 pull-down experiments and Y2H and BiFC experiments showed that both factors can directly interact with each other (Figure 1 and Figure 7). Isotope experiment showed that CaM1/4 enhances the phosphorylation level and kinase activity of SnRK2.6 in a dosage dependent manner (Figure 10B). Interestingly, CaM1/4 blocks the phosphatase activity of AHG1 and AHG3, and rescues AHG3 mediated SnRK2.6 de-phosphorylation but not kinase activity (Figure 10B). All of these results suggest that, similar to the function of RDO5, CaM1/4 inhibits AHG1 and AHG3 activity via substrate binding competition. CaM1/4 and RDO5 may have a coordinative role in the regulation of seed dormancy via inhibition of AHG1 and AHG3 function.

4.6. Future directions to study the molecular mechanisms of RDO5, DOG1, AHG1 and CaM1/4 in the regulation of seed dormancy

4.6.1. Understanding the molecular mechanisms of RDO5 and DOG1

RDO5 and DOG1 have been confirmed as core factors in the regulation of seed dormancy in Arabidopsis. Mass spectrum experiments showed that RDO5 and DOG1 are always identified together (Figure 1) (Née et al., 2017b). Protein interaction experiments showed that RDO5 can directly interact with DOG1 (Née et al., 2017b), which indicates that both proteins can form a complex in seeds. Our current data showed that RDO5 negatively regulates the actions of AHG1 and AHG3 probably through inhibiting dephosphorylation of SnRKs by binding (Figure 10A). It is known that the phosphatase activity of AHG1 but not AHG3 can be suppressed by DOG1(Figure 11A)(Nishimura et al., 2018). However, we are wondering whether DOG1 could also prevent the dephosphorylation of SnRK2s by the phosphatases AHG1 and AHG3. In addition, previous study showed that DOG1 gradually loses its activity during storage of seeds. We are interested to understand how the DOG1 protein loses its activity during storage of seeds and whether the protein RDO5 also has a similar behavior as DOG1 during storage of seeds.

4.6.2. Understanding how the calmodulin proteins work together with RDO5 to regulate seed dormancy

CaM1 and Cam4 were identified in RDO5 but not in DOG1 pull-down experiments (Figure 1) (Née et al., 2017b), which indicates that the function of CaM1 and CaM4 might be specific for RDO5. Y2H and BiFC have confirmed the physical interactions between RDO5 and CaM1/4 (Figure 7), which suggests that RDO5 can form a complex with CaM1/4 in seeds. Isotope experiments further showed that RDO5 and CaM4 both inhibit AHG1 and AHG3 function (Figure 12). Moreover, the mutant *cam1 cam4* showed reduced sensitivity to ABA (Figure 9A), and overexpression of CaM1/4 can increase seed dormancy (Figure 9B), which indicates that CaM1/4 might positively regulate seed dormancy. Moreover, we have confirmed the protein interactions among RDO5, CaM1/4 and AHG1, which suggests that these three factors can form a complex in seed (Figure 2 and Figure 7). The protein phosphatase AHG1 but not AHG3 can be directly suppressed by DOG1 and this suppression is independent from ABA (Figure 11A) (Nishimura et al., 2018), which raises the question whether the phosphatase activity of AHG1 could be directly suppressed by RDO5 and CaM1/4. Although our current data do not support this hypothesis (Figure 10), we still cannot exclude the possibility that RDO5 and CaM1/4 might directly suppress the phosphatase activity of AHG1 and AHG3.

Calmodulins are calcium binding proteins, and therefore calcium (signalling) probably plays a role in their function. An interesting question would therefore be how calcium signaling would fit into the regulation of dormancy and how calcium signaling would fit into the regulation of dephosphorylation.

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