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A Study on the Genes Related with Seed Dormancy in *Arabidopsis thaliana*

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ABSTRACT

Seed dormancy is a phenomenon that seed postpones its growth until environment is suitable for its survival. Some genetic or environmental factors that affect seed dormancy have been identified but the mechanism of controlling seed dormancy is still elusive. Recently, some epigenetic regulators of seed dormancy have been reported. *AtJmj4*, a gene encoding H3K4 demethylase in Arabidopsis, is expressed highly in seed of freshly-harvested (FH) state which possesses the highest seed dormancy level. This suggests that *AtJmj4* might play a role in the regulation of seed dormancy. Meanwhile, *OsSDR4* is well known as a seed dormancy regulator in rice. Several homologs of *OsSDR4* in Arabidopsis have been discovered depending on their amino acid sequence homologies; *AtSDR4* is the closest homolog of *OsSDR4*. The lack of *AtSDR4* caused enhanced seed dormancy, which is the opposite phenotype of *ossdr4* mutation, which causes a reduced seed dormancy. The function of other homologs, *AtSDR4H1* and *AtSDR4H2*, in seed dormancy has also not been reported. In this study, *AtJmj4* and *AtSDR4* were investigated at the aspect of seed dormancy regulation. The *atjmj4-1* mutation did not alter the seed dormancy phenotype in wild-type (WT) Columbia (Col) background or Col^{DOG1_Cvi} backgrounds at 22 ℃. However, *atjmj4-1* seeds showed reduced

dormancy than WT seeds at 12 ℃. In the dormancy test using *atsdr4-1*, *atsdr4h1-1*, *atsdr4h2-1*, and their multiple mutants, it was suggested that *atsdr4-1* but not *atsdr4h1-1* or *atsdr4h2-1* mutation enhances the seed dormancy. Gene expression analysis revealed that the genes involved in ABA synthesis and signaling, as well as *DOG1*, the representative seed dormancy regulator, are upregulated in *atsdr4-1* FH seeds than in WT seeds. To test if any phenotypes related to germination also exist, red light-dependent germination tests were performed and none of *atjmj4-1, atsdr4-1, atsdr4h1-1* and *atsdr4h2-1* showed substantially altered germination phenotypes, although *atsdr4-1* showed retarded radicle-emergence phenotype. Germination tests with exogenously supplied ABA suggested that the slow radicle extrusion phenotype of *atsdr4-1* is probably due to the ABA hypersensitivity. Overall, these results indicate that *AtJmj4* is a minor positive regulator of seed dormancy at low temperature and *AtSDR4* is a major negative regulator of seed dormancy.

Keywords: Seed dormancy, freshly-harvested seed, *AtJmj4*, *AtSDR4*, *DOG1*, seed germination, abscisic acid

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ABBREVIATIONS

WT: wild type

FH: freshly harvested

AR: after ripened

GUS: β-glucuronidase

DOG1: *DELAY OF GERMINATION 1*

ABA: abscisic acid

GA: gibberellin

H3K4: Histone H3 lysine 4

qRT-PCR: quantitative real-time polymerase chain reaction

I. INTRODUCTION

Seed dormancy is a unique feature that only exists in spermatophyte. Seed dormancy enables seed to 'pause' their growth until the surrounding environment is suitable and begin their next generation (Bentsinka and Koornneef, 2008). This incredible feature might have been one of the driving forces for angiosperm to thrive on the earth with enormous species number covering over 90% of land plant species and one fifth of whole species in the world (http://www.catalogueoflife.org/col/browse/ tree; Catalogue of Life, 2017). Seed dormancy is very complicated trait resulted from both genetic and environmental factors (Bewley, 1997). Seed dormancy varies among species; the degree ranges from a few days to years, and the mode and depth of seed dormancy are classified into five and three classes, respectively (Baskin and Baskin, 2004). Proper seed dormancy level is necessary for wild plants not to germinate in inappropriate environment leading death or postpone germination forever, but also for domesticated crops to avoid pre-sprouting and germinate equally in proper time. Despite of the importance of seed dormancy, the mechanism of controlling seed dormancy is not well defined.

From a quantitative trait locus (QTL) analysis a gene was identified: *DELAY OF GERMINATION 1* (*DOG1*) (Bentsink et al., 2006). It is regarded as a key gene of embryonic seed dormancy in Arabidopsis; *DOG1* is expressed exclusively in seed, and the protein level of DOG1 during seed maturation correlates with the seed dormancy level (Nakabayashi et al., 2012). *dog1* mutant has no seed dormancy. Even though *DOG1* is regarded as a master gene of seed dormancy in Arabidopsis, the working mechanism of *DOG1* is still elusive. Not only *DOG1*, the genes involving seed development—*ABA INSENSITIVE (ABI3), FUSCA3 (FUS3)*, *LEAFY COTYLEDON(LEC)* (Raz et al., 2001)—or the histone modifier genes—*KRYPTONIGHT (KRP), HISTONE MONO-UBIQUITINATION 1* and *2 (HUB1, HUB2)* (Graeber et al., 2012)—are also necessary to control seed dormancy. Abscisic acid (ABA) and gibberellins (GA) are two known hormones that positively or negatively regulate seed dormancy, respectively. The lack of *ABA DEFICIENT 1 (ABA1),* ABA synthesis gene, shows extremely reduced seed dormancy, and the mutation of *GA REQUIRING 1 (GA1)*, a GA synthesis gene, results in the enhanced seed dormancy (Kucera et al., 2005). The genes required to ABA or GA signaling also influence seed dormancy. Maintenance or breaking of seed dormancy should be controlled precisely, As mentioned here,

many epigenetic regulators are involved in regulating seed dormancy. This suggests that other epigenetic regulators might have the possibility to control seed dormancy as well.

AtJmj4 is a H3K4 demethylase in Arabidopsis, which is one of the Jumonji (Jmj) C domain-containing protein that known to be able to demethylate mono-, di-, or trimethylated lysine residues of histone proteins (Qian et al., 2015). Arabidopsis has 21 genes encoding JmjC domain-containing proteins, grouped into 5 clades (Hong et al., 2009). *AtJmj4* is a member of Clade II, including *AtJmj 5,6,7,8* and *9*, and has single copy of JmjN, JmjC, C5HC2 ZnF, and FYRN/FYRC domain like most of genes in Clade II do. Since JmjC domain-containing proteins possess wider substrate spectrum than other types of histone demethylases (Shi and Whetstine, 2007), it is expected that they may play important roles in complicated features of Arabidopsis such as developmental transitions. The plant undergoes a series of the developmental transitions: germination, juvenile-to-adult transition, flower transition, meiosis and fertilization (Huijser and Schmid, 2011). It has known that AtJmj4 controls floral transition by repressing *FLOWERING LOCUS T (FT)* chromatin and preventing early flowering (Jeong et al., 2009). As controlling flowering transition, it is

suggested that AtJmj4 may regulate another feature related to one of those transitions: seed dormancy.

Meanwhile, in rice, the QTL study of seed dormancy revealed *OsSDR4*: a major regulator of seed dormancy (Lin et al., 1998) (Sugimoto et al., 2010). *OsSDR4* is expressed only in seeds and *ossdr4* mutant has almost no seed dormancy. *OsSDR4* acts the downstream of *OsVP1*, the homolog of *ABI3* of Arabidopsis. It seems that *OsSDR4* positively controls *OsDOG1*-like genes in rice, suggesting that those genes positively regulate seed dormancy together (Sugimoto et al., 2010). However, nothing is clear about the mechanism of *OsSDR4* in regulating seed dormancy since the domains or the functions of *OsSDR4* have not been uncovered. Various plant species possesses homologs of *OsSDR4* and their expression and putative functions also vary among the species. Among them, several species such as *A. thaliana, G. max* and *Z. mays* show exclusive expression of the each homolog of *OsSDR4* in seed only (Subburaj et al., 2016). It could be suggested that *OsSDR4* homologs in these species may play an important role related to seed dormancy and development.

Arabidopsis has several *OsSDR4* homologs. The closest one to *OsSDR4* is At1g27461, so-called *AtSDR4* here. Two of other *OsSDR4* homologs in Arabidopsis were selected to study here: At3g48510

(*AtSDR4H1*) and At5g63350 (*AtSDR4H2*) (Choi, 2012). The expression of *AtSDR4* and *AtSDR4H1* is only observed in seed (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter and Vinegar, 2007) and especially high in FH seed. *AtSDR4H2* is the closest homolog to *AtSDR4H1,* and its expression pattern has not been studied. Interestingly, the previous study revealed that *atsdr4-1* mutant has stronger seed dormancy than wild-type, which is the opposite phenomenon in rice. The mutant study of the other two homologs have not been done deeply. Considering the expression pattern and the interesting mutant phenotype of *AtSDR4*, further study should be proceeded to reveal the features of *AtSDR4, AtSDR4H1* and *AtSDR4H2* regarding the seed dormancy in Arabidopsis.

In this study, dormancy tests and germination tests were performed using *atjmj4-1, atsdr4-1, atsdr4h1-1, atsdr4h2-1* and their multiple mutants. From the dormancy test using FH seed of WT, *atjmj4-1* and *atjmj4/5/6/7/8/9*, it was revealed that the reduced dormancy phenotype of two mutants is dependent to the mutation of *AtJmj4*. Furthermore, the reduction of seed dormancy was only observed in Col background at 12 ℃, the temperature enhancing seed dormancy, but not in Col^{DOG1_Cvi} background at 22 ℃, the genetic background strengthening seed dormancy. This suggests that *AtJmj4* is

a minor, positive regulator of seed dormancy at low temperature. However, many of known dormancy-related genes showed no significant differences of mRNA level between WT and *atjmj4-1* FH seeds, suggesting *AtJmj4* may contribute seed dormancy through unknown pathway. The germination tests revealed that *AtJmj4* and *AtJmj5, 6, 7, 8. 9* had no contribution to germination. The dormancy test using FH seed of WT, *atsdr4-1, atsdr4h1-1, atsdr4h2-1* and their multiple mutants showed that *atsdr4-1* has strong seed dormancy phenotype, while *atsdr4h1-1, atsdr4h2-1* and *atsdr4h1-1 atsdr4h2-1* were not. *atsdr4-1 atsdr4h1-1 atsdr4h2-1* triple mutant showed the similar degree of seed dormancy to *atsdr4-1*. Gene expression analysis data suggested that the increased seed dormancy of *atsdr4-1* might be due to the increased ABA synthesis and ABA signaling. Red light-dependent germination test and ABA-dependent germination test showed that *atsdr4-1* has the retarded radicle extrusion possibly due to its hypersensitivity to ABA. In summary, it is proposed that *AtJmj4* is a minor factor in positively regulating seed dormancy at low temperature and *AtSDR4* is a major factor to control seed dormancy negatively through ABA-related novel pathway.

II. MATERIALS AND METHODS

2.1. Plant Materials and Growth Conditions

Columbia ecotype (Col-0) was used as wild-type (WT) in every experiment. All of the mutants used were T-DNA mutants obtained from Salk collection. The Salk line numbers of *atjmj4-1, atjmj5, 6, 7, 8, 9, atsdr4-1, atsdr4h2-1* are here: Salk_135712, CS463587, Salk_029630, Salk_089978, Salk_073422, Salk_025269, Salk_022729 and Salk_130501, respectively. *atsdr4h1-1* possesses two T-DNA insertion of which Salk line number is Salk_074718 and Salk_128578. *atjmj4/5/6/7/8/9* obtained from multiple cross of the mutants was available in our laboratory seed stock. *AtJmj4* overexpression line (*AtJmj4*^{OE}) and *pAtJmj4::GUS* seed were also already prepared. *pAtSDR4::GUS* construct in c58C1 was available and transformed into WT by floral dipping method. The T0 seeds were sown and selected on the petri dish with Murashige and Skoog (MS) media containing GA and kanamycin. T3 homozygous seed was used for GUS staining. All the plants have been grown at 22° C in the long-day (day 16 h, night 8 h) condition unless any special growth condition is mentioned.

2.2. GUS Staining of FH Seed

The intact seed and the uncoated embryo were used in GUS staining. For staining of embryo, testa and endosperm was teared off and removed carefully using micro-dissecting forceps. Whole seeds, embryos and isolated endosperms were soaked with the chilled 90% acetone to be fixed for 20 min. The samples were transferred to the tubes with ice-cold GUS-staining solution. For the staining of whole seed, vacuum was used for 3.5h. The tubes were wrapped with aluminum foil not to be exposed to the light and incubated at 37 ℃ for 30 min to a couple of hours. After staining, the samples were washed several times with 70% ethanol for de-staining. Under the bright field microscope, the samples were set onto the slide glass and photos were taken.

2.3. Dormancy Test

Plants were grown under long-day condition (16 h of day, 8 h of night) at 22 ℃ (or 12 ℃ for some dormancy tests). The plants used in a single set of dormancy test were grown in the same batch to minimize variances from local environment. The siliques were harvested at freshly-harvested stage when the color of silique is light yellow green.

Harvested silique were collected into 1.7 ml (for less siliques) or 2 ml tube (for many silique) and the tubes were sealed by 3M tape. The siliques were dried at the same growth room for several days with proper intervals. After drying period, seed were isolated from siliques and surface-sterilized by voltexing with 70% ethanol added with 0.08% Triton X-100 for several times. The seed were rinsed with 70% ethanol followed by washing with 95% ethanol and dried on the filter paper in Clean Bench. Dried seed were sown on the petri dish with half-strength MS media with no sugar and the dish was sealed with 3M tape. The dish was placed under constant white light (about 120 $\mu mol/m^2s$) at 22 ℃ for 7 days and germination rate was counted. A seed with radicle emergence was regarded as a germinated seed.

2.4. Germination Test

For red light-dependent germination test, AR seed of plants grown under long-day condition at 22 ℃ were surface-sterilized and sown on the half-strength MS media plate with no sugar, as mentioned in dormancy test method section. Five plates were prepared as each plate would be counted day-by-day (two plates for day 4). The plates were incubated in dark chamber for an hour and irradiated with 5min of

far-red pulse (2 μmol/m²s). 4 plates were lighted with red pulse, while one plate were not, to be used as far-red control plate. The plates were individually wrapped with aluminum foil and incubated in dark chamber for several days. The control plated was opened at day 4 to check the proper treatment of far-red light. For ABA-dependent germination test, constant white light was used. Surface-sterilized AR seeds were sown on the half-strength MS media plate containing 0, 1, 2, 5, 10 μM ABA. ABA was used as 10 mM ABA stock solution whose solvent is 20% EtOH. The plates were wrapped with aluminum foil, and after 3 days of stratification, placed under the constant white light for up to 24 days with 4-day interval. 120 to 200 seed were used each set and every seed batch was after-ripened at least a month. The germination rate was counted depending on the two different standards: testa rupture or radicle extrusion.

2.5. Preparation of cDNA of FH Seed

FH silique were collected and opened by rubbing by fingers on copy papers. Siliques and seeds were handled with gloved hands. The seed was collected into 1.7 ml tube and wash briefly with ddH_2O. The remained water was removed by pipetting after spin-down. The tube

was immediately frozen by liquid nitrogen and stored at −80 ℃ for several days to months. The procedure to extract total seed RNA was referred from a publication (Meng and Feldman, 2010). First several steps were done as mentioned in the paper and the latter procedure was replaced with the protocol using QIAGEN RNeasy Plant Mini Kit. The elution volume was 32 μl. RT−PCR was performed to yield 3 μg of cDNA. 2 μl of oligo dT primer (18−mer), 8 μl of 10 mM dNTPs (2.5 mM for each) (iNtRON BIOTECHNOLOGY) and DEPC−treated water were added to RNA to make 30 μl of mixture. The mixture was pre−heated for 7 min at 65 ℃, and 1 μl of Reverse Transcriptase, 1 μl of RNase Inhibitor and 8 μl of 5X Reaction Buffer for M−MuLV RT (all three from Fermentas) was added to the mixture and PCR was performed.

2.6. RT−PCR

For RT−PCR reagent, *i−Taq*™ DNA Polymerase, 10x PCR buffer with 20 mM $MgCl_2$, 10 mM dNTPs (2.5 mM each) from iNtRON BIOTECHNOLOGY were used. The reaction volume was 12.5 μl and the PCR product was loaded on 3% agarose gel containing EtBr. Primers used for qRT−PCR are listed in Table 1.

2.7. qRT-PCR

qRT-PCR was performed by Applied Biosystems 7300 real-time PCR system. For qRT-PCR reagent, SYBR® Fast qPCR Mix (Takara) was used. The reaction volume was 20 μl and the samples were normalized by *UBQ11* expression level. Ct Value with threshold value of 0.1 was used. Primers used for qRT-PCR are listed in Table 2.

Table 1. Primers used for RT-PCR

Gene	Type	Sequence (5' to 3')
UBQ10	Forward	GATCTTTGCCGGAAAACAATTGGAGGATGGT
	Reverse	CGACTTGTCATTAGAAAGAAAGAGATAACAGG
UBQ11	Forward	GATCTTCGCCGGAAAGCAACTT
	Reverse	CCACGGAGACGGAGGACC
DOG1	Forward	AAGAAAGTCTCAAGCCTAC
	Reverse	CGAGGATCTTCGCTAAAG
AtJmj4	Forward	GCTTGACCCAACAAACCTAACC
	Reverse	TCTCACCACACAGAAGTCCATGC
GAI†	Forward	AGCGTCATGAAACGTTGAGTCAGTG
	Reverse	TGCCAACCCAACATGAGACAGC
GA3ox1	Forward	GGTCTAGCAGCTCATACCGACT
	Reverse	CAACACGCTTTTAAACAATCCA
GA3ox2	Forward	CGCATCCCATTCACATCCCACTC
	Reverse	GATAACTGCTTGGGTTCCTTGAAAGTCTG
NCED6†	Forward	ACCGGGTCGGATATAAATTGGGTTG
	Reverse	CCCGGGTTGGTTCTCCTGATTC
CYP707A1	Forward	GAGAAAGGTTACAATTCGATGCCAGTGAATC
	Reverse	CGTTGGGATTCTCGGCTAGGTACTTGAG
CYP707A2	Forward	GGAGAGAAGAAGGAGGACTATTGGGAGTAC
	Reverse	GGGTACAGGGAAAGGACCATACTGTAT
Em6	Forward	CAGATGGGACGCAAAGGTGGTCTTA
	Reverse	GGTCTTGGTCCTGAATTTGGATTCG
RAB18	Forward	CGTCTTACCAGAACCGTCCAGG

	Reverse	TCCGTATCCTTGGCCACCTG
RD29A	Forward	TCAACACACACCAGCAGCAC
	Reverse	TCGATCACTTCAGGTTCTAGCTCG
RD29B	Forward	AGCAAGCAGAAGAACCAATCAG
	Reverse	CGAGAGGATAATGAGTCGGTG
ABI3	Forward	AAGCTGAGACACACTTGCCG
	Reverse	CCAAAACCTGTAGCGCATGT
ABI5	Forward	GGTGAGACTGCGGCTAGACA
	Reverse	GTTTTGGTTCGGGTTTGGAT
ABA1†	Forward	GATGCAGCCAAATATGGGTCAAGG
	Reverse	GCCATTGCATGGATAATAGCGACTC

†This primer is referred (Cho et al., 2012).

Table 2. Primers used for qRT-PCR

Gene	Type	Sequence (5' to 3')
UBQ11	Forward	GATCTTCGCCGGAAAGCAACTT
	Reverse	CCACGGAGACGGAGGACC
DOG1	Forward	AAGAAAGTCTCAAGCCTAC
	Reverse	CGAGGATCTTCGCTAAAG
GAI†	Forward	AGCGTCATGAAACGTTGAGTCAGTG
	Reverse	TGCCAACCCAACATGAGACAGC
ABI3	Forward	AAGCTGAGACACACTTGCCG
	Reverse	CCAAAACCTGTAGCGCATGT
Em6	Forward	CAGATGGGACGCAAAGGTGGTCTTA
	Reverse	GGTCTTGGTCCTGAATTTGGATTCG
RD29A	Forward	TCAACACACACCAGCAGCAC
	Reverse	TCGATCACTTCAGGTTCTAGCTCG
ABA1†	Forward	GATGCAGCCAAATATGGGTCAAGG
	Reverse	GCCATTGCATGGATAATAGCGACTC
NCED6†	Forward	ACCGGGTCGGATATAAATTGGGTTG
	Reverse	CCCGGGTTGGTTCTCCTGATTC
CYP707A2†	Forward	ATGGGGTTGCCTTACATCGGAGA
	Reverse	TGGCTTGAACAAGTGAGCTTTGCT

†This primer is referred (Cho et al., 2012).

III. RESULTS

3.1 The Expression of *AtJmj4* in FH Seed

The expression of *AtJmj4* is observed in the most of Arabidopsis tissues. (Hong et al., 2009) *AtJmj4* expression was higher in FH seed than other tissues, especially at seed maturation stages. (**Figure 1A**) It could be expected that AtJmj4 may play a role related to seed development or seed dormancy. *pAtJmj4::GUS* seed was stained with GUS solution to study spatial expression of *AtJmj4* in freshly-harvested (FH) seed (**Figure 1B**). The embryo was globally stained and the strong staining was observed at the tip of radicle. Thus, it could be said *AtJmj4* is expressed in the whole parts of the embryo of FH seed.

3.2. Dormancy and Germination Phenotype of *atjmj4-1* and *atjmj4/5/6/7/8/9*

To examine whether *AtJmj4* contributes to seed dormancy phenotype, dormancy test was performed using FH seed of WT and *atjmj4-1*. *atjmj4/5/6/7/8/9* mutant was also used because of the putative redundancy among Clade II JmjC domain-containing proteins. As a result, there were no differences of seed dormancy phenotype between

WT and *atjmj4-1* (Figure 2A), or WT and *atjmj4/5/6/7/8/9* (Figure 2B).

A probability was suggested: the difference between WT and each mutant is too small, so the difference could be seen with the wider window of seed dormancy. Since it has been known that the seeds matured in the cold environment possess higher seed dormancy than in the warm environment(Chiang et al., 2011), dormancy tests were done using seeds matured at 12 ℃. FH siliques were collected and dried next to the mother plants, and the dormancy test was done at 22 ℃ in the constant white light chamber. It was observed that seed dormancy broke down faster in *atjmj4-1* and *atjmj4/5/6/7/8/9* than WT (Figure 3A). Furthermore, there was no significant difference of the germination rate between *atjmj4-1* and *atjmj4/5/6/7/8/9*. This suggests the lack of *AtJmj4* results in the weakness of seed dormancy, but the absence of other Clade II JmjC domain-containing protein-coding genes do not at 12 ℃. Since the normal growth condition of Arabidopsis is 22 ℃, another system at 22 ℃ was used to verify the effect of *AtJmj4* in seed dormancy. *DOG1* gene of Cape Verde Island (Cvi) ecotype has been well known for its strong seed dormancy (Alonso-Blanco et al., 2003). *atjmj4-1* mutation was introduced in Col^{DOG1_Cvi}, which is the near isogenic line possessing *DOG1* of Cvi in

Col background. Dormancy tests revealed that there was no significant difference between Col^{DOG1_Cvi} and *atjmj4-1* in Col^{DOG1_Cvi} in seed dormancy (**Figure 3B~E**). It suggests that the lack of *AtJmj4* leads the reduced seed dormancy only at 12 ℃, but not at 22 ℃. Since *atjmj4-1* revealed the reduced seed dormancy phenotype only at 12 ℃, the gene expression pattern of Col and *atjmj4-1* grown at 12 ℃ was investigated by RT-PCR (**Figure 3F**). It has known that seed dormancy is regulated by *DOG1* gene and plant hormones, gibberellic acid and abscisic acid (Finch-Savage and Leubner-Metzger, 2006) (Nakabayashi et al., 2012). However, there was no significant differences in the expression of *DOG1* and several genes related to ABA and GA between WT and *atjmj4-1* FH seed. Thus, it suggests that *AtJmj4* does not regulation the expression of *DOG1* and ABA- or GA-related genes.

Germination is the following step of dormancy breaking and both phases are controlled by ABA and GA (Finch-Savage and Leubner-Metzger, 2006). In Arabidopsis, phyB is a major regulator of seed germination, which senses red light and mediates further signaling required for seed germination (Shinomura et al., 1994). In order to confirm that the dormancy phenotype of *atjmj4-1* is not due to its germination phenotype, red light-dependent germination tests were performed (**Figure 4A~C**). As a result, there were no significant

differences of germination rate among *atjmj4-1*, *atjmj4/5/6/7/8/9* and *AtJmj4*^{OE}. Therefore, it could be said that *AtJmj4* does not regulates seed germination.

3.3. The Expression of *AtSDR4* in FH Seed

It has known that *AtSDR4* is only expressed in seed tissue (Choi, 2012) like *OsSDR4* (**Figure 5A**). The spatial expression pattern of *pAtSDR4::GUS* was visualized via GUS staining (**Figure 5B~5D**). In the embryo of FH seed, the expression of the construct was observed globally: the root tip and shoot apical meristem region are strongly stained (**Figure 5B**). While the testa was not stained, it seems that the endosperm layer also expresses *AtSDR4* (**Figure 5C, 5D**). This suggests that *AtSDR4* is expressed globally in the embryo, as well as the endosperm, of FH seed.

3.4. Dormancy and Germination Phenotype of *atsdr4-1*, *atsdr4h1-1* and *atsdr4h2-1*

Dormancy test was carried out to investigate dormancy phenotype of *atsdr4-1, atsdr4h1-1* and *atsdr4h2-1*. Compared to WT, *atsdr4-1* FH

seed had significantly stronger seed dormancy (Figure 6A). This is noticeable because loss-of-function mutant of *OsSDR4* has extremely reduced seed dormancy. FH seed of *atsdr4h1-1* and *atsdr4h2-1* did not showed significant dormancy phenotype compared to WT (Figure 6B).

To test any redundant role among *AtSDR4*, *AtSDR4H1* and *AtSDR4H2* in seed dormancy, multiple mutant lines were used for the dormancy test. *atsdr4h1-1 atsdr4h2-1* double mutant and each single mutants have similar dormancy phenotype (Figure 6C). *atsdr4-1 atsdr4h1-1 atsdr4h2-1* triple mutant showed strong seed dormancy; the dormancy phenotype of triple mutant appears to resemble *atsdr4-1*. It suggests that *AtSDR4*, but not *AtSDR4H1* and *AtSDR4H2*, might play an important role in negatively regulating seed dormancy.

To find any phenotype related to germination, after-ripened seed of WT, *atsdr4-1*, *atsdr4h1-1* and *atsdr4h2-1* were tested. First, red light-dependent germination tests were performed to verify the ability of red light perception of the mutants (Figure 7A, 7C~7E). As the seed receive the germination signals, the testa is ruptured first, and the radicle grows out tearing the endosperm layer (Figure 7B). The seeds with ruptured testa were counted, which are considered to receive red light properly and start to germinate. The germination rate of

atsdr4-1 at day 2 was slightly lower than other genotypes and *atsdr4h2-1* showed marginally faster germination at day 1 (**Figure 7C**). However, generally, there was no significant germination phenotype in *atsdr4h1-1* and *atsdr4h2-1*. Meanwhile, a significant delay of radicle extrusion of *atsdr4-1* was observed (**Figure 7D**). At day 2, less than half of *atsdr4-1* seed showed radicle extrusion while almost every seeds of other genotypes already done. All of the mutants germinated only under red light, not under far-red light (**Figure 7E**). This means that red-light perception is normal but seedling establishment is delayed in *atsdr4-1*. Similar result was observed from germination test under white light (**Figure 7F**). Since the germination rate at day 4 is about 100%, it could be said that the germination phenotype does not mask the dormancy phenotype of *atsdr4-1*. Taken together, it could be said that *atsdr4-1*, *atsdr4h1-1* and *atsdr4h2-1* have no remarkable red light-dependent germination phenotype and *atsdr4-1* has slow radicle extrusion phenotype.

Since *atsdr4-1* showed delayed radicle extrusion, higher activation of ABA signaling or larger amount of ABA in *atsdr4-1* than WT could be suggested. It has known that ABA inhibits endosperm rupture leading delayed germination in Arabidopsis. To test whether *atsdr4-1* is hypersensitive to ABA, germination test on ABA media was

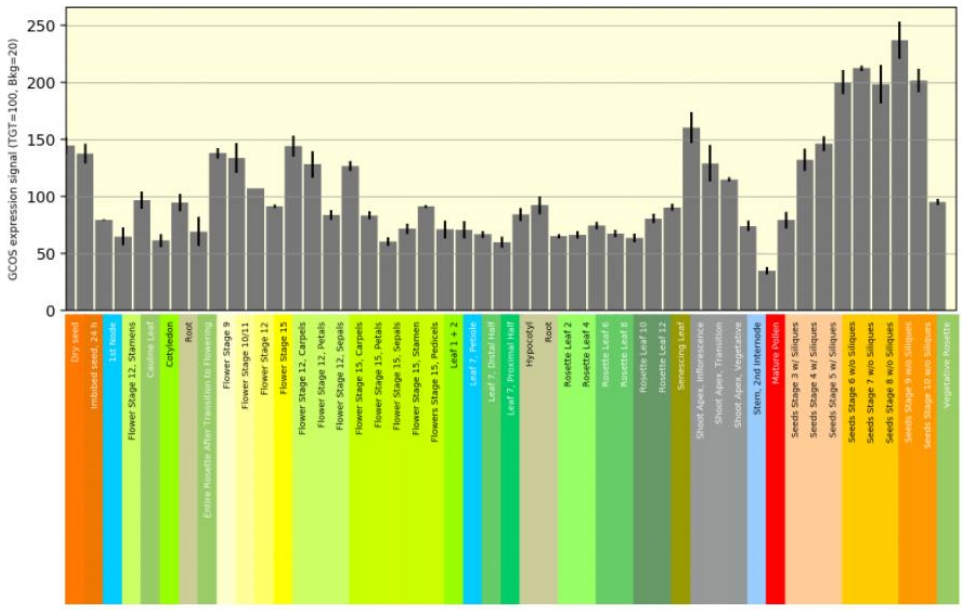
performed. To discard the effect of endogenous ABA, seeds were after-ripened over a month and underwent stratification for 3 days. Although *atsdr4-1* seed were available to fully germinate on 1/2 MS media (**Figure 8E**), germination rates of *atsdr4-1* seed were lower than WT in ABA media (**Figure 8A~8D**). Thus, it suggests that *atsdr4-1* AR seed has retarded germination phenotype on ABA-containing media, possibly due to the ABA hypersensitivity or the endogenous ABA.

3.5. Gene Expression Pattern in *atsdr4-1* FH Seed

To compare gene expression pattern related to seed dormancy between WT and *atsdr4-1* FH seed, *DOG1* and ABA or GA-related genes were selected for RT-PCR. Many ABA-related genes rather than GA-related genes were chosen because the phenotype tests revealed that *atsdr4-1* has strong dormancy phenotype which is more affected by ABA, and weak germination phenotype which is mostly controlled by GA (Rodríguez-Gacio et al., 2009) (Finch-Savage and Leubner-Metzger, 2006). *DOG1*, which is a marker gene of seed dormancy was upregulated in *atsdr4-1* FH seed (**Figure 9A**). *ABI3*, the ABA signaling gene, and *NCED6*, the ABA synthesis genes, expressions were increased in *atsdr4-1*. The expression levels of those genes were measured by

qRT-PCR (**Figure 9B**). *DOG1*, *ABI3* and *NCED6* showed elevated expression in *atsdr4-1*. The increase of *ABI3* is interesting because it looks like *AtSDR4* is upstream of *ABI3* in Arabidopsis, while in rice *OsSDR4* is downstream of *OsVp1*, the homolog of *ABI3*. The expression of *RD29A, Em6* and *GAI* did not changed significantly. *RD29A* and *Em6* is positively regulated by ABA (Chen et al., 2014) and *GAI* is a DELLA protein-encoding gene which prevents testa rupture (Piskurewicz et al., 2009). *CYP707A2* showed no difference; it seems that ABA catabolism, which is the downstream of ABA signaling (Yamaguchi-Shinozaki, 1994), was not affected as well. Taken together, the results suggest that the elevated level of ABA synthesis and signaling contributes to strong seed dormancy of *atsdr4-1*.

A


GCOS expression signal (TGT=100, Bkg=20)
250
200
150
100
50
0
Dry seed
Imbibed seed, 24 h
1st Node
Flower Stage 12, Stamens
Cauline Leaf
Cotyledon
Root
Entire Rosette After Transition to Flowering
Flower Stage 9
Flower Stage 10/11
Flower Stage 12
Flower Stage 15
Flower Stage 12, Carpels
Flower Stage 12, Petals
Flower Stage 12, Sepals
Flower Stage 15, Carpels
Flower Stage 15, Petals
Flower Stage 15, Sepals
Flower Stage 15, Stamen
Flowers Stage 15, Pedicels
Leaf 1 + 2
Leaf 7, Petiole
Leaf 7, Distal Half
Leaf 7, Proximal Half
Hypocotyl
Root
Rosette Leaf 2
Rosette Leaf 4
Rosette Leaf 6
Rosette Leaf 8
Rosette Leaf 10
Rosette Leaf 12
Senescing Leaf
Shoot Apex, Inflorescence
Shoot Apex, Transition
Shoot Apex, Vegetative
Stem, 2nd Internode
Mature Pollen
Seeds Stage 3 w/ Siliques
Seeds Stage 4 w/ Siliques
Seeds Stage 5 w/ Siliques
Seeds Stage 6 w/o Siliques
Seeds Stage 7 w/o Siliques
Seeds Stage 8 w/o Siliques
Seeds Stage 9 w/o Siliques
Seeds Stage 10 w/o Siliques
Vegetative Rosette

B

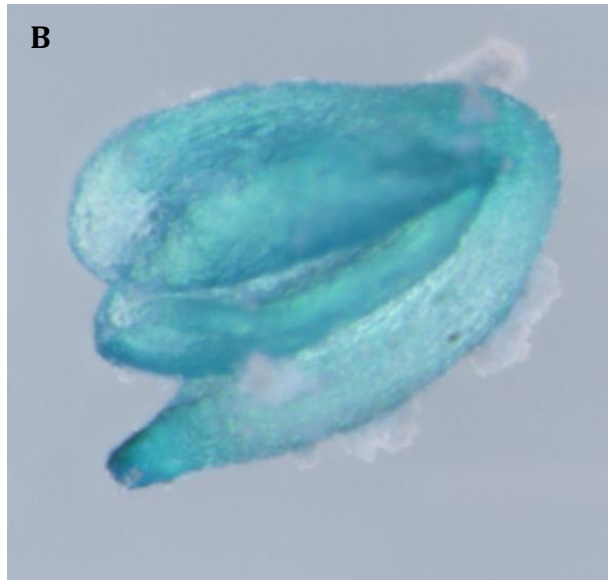
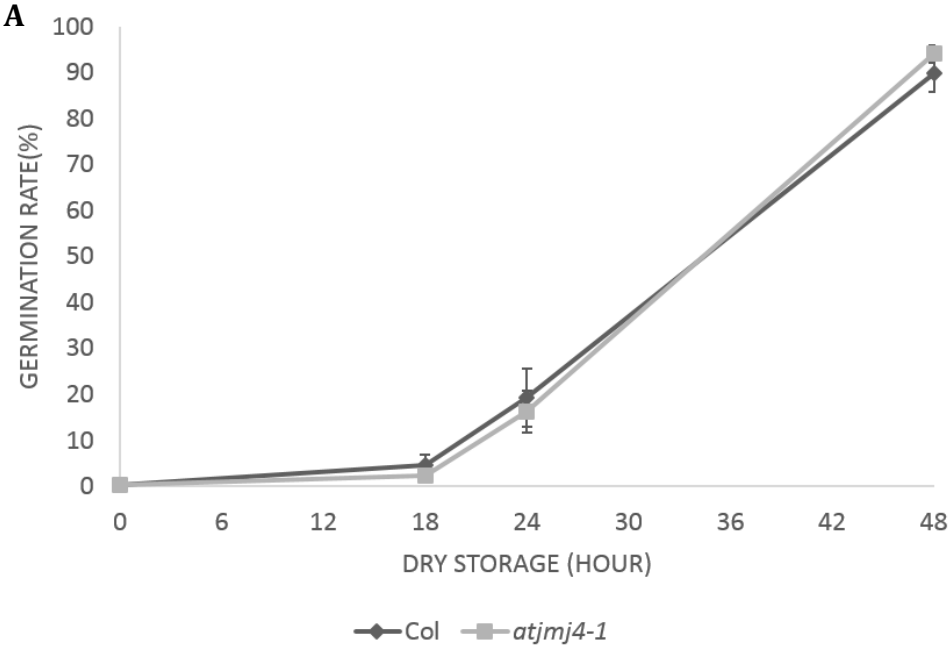


Figure 1. Expression of *AtJmj4* in FH seed

(A) The expression pattern of *AtJmj4* at various tissues or developmental phases in Arabidopsis. (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter and Vinegar, 2007). (B) The embryo of FH seed expressing *pAtJmj4::GUS*. Seed coat of FH seed of *pAtJmj4::GUS* was removed to obtain the intact embryo.

A


GERMINATION RATE(%)
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
DRY STORAGE (HOUR)
Col
atjmj4-1

B

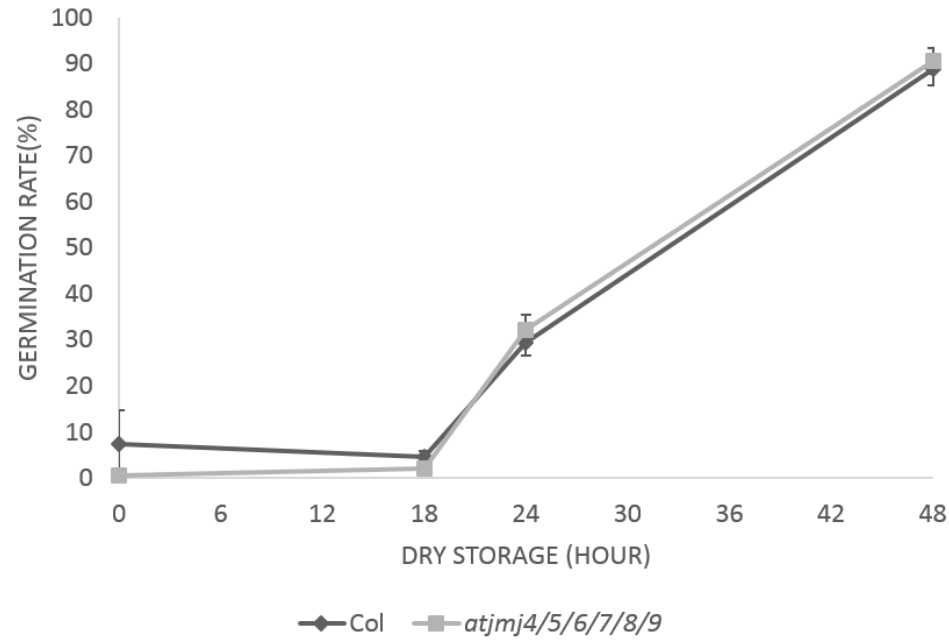
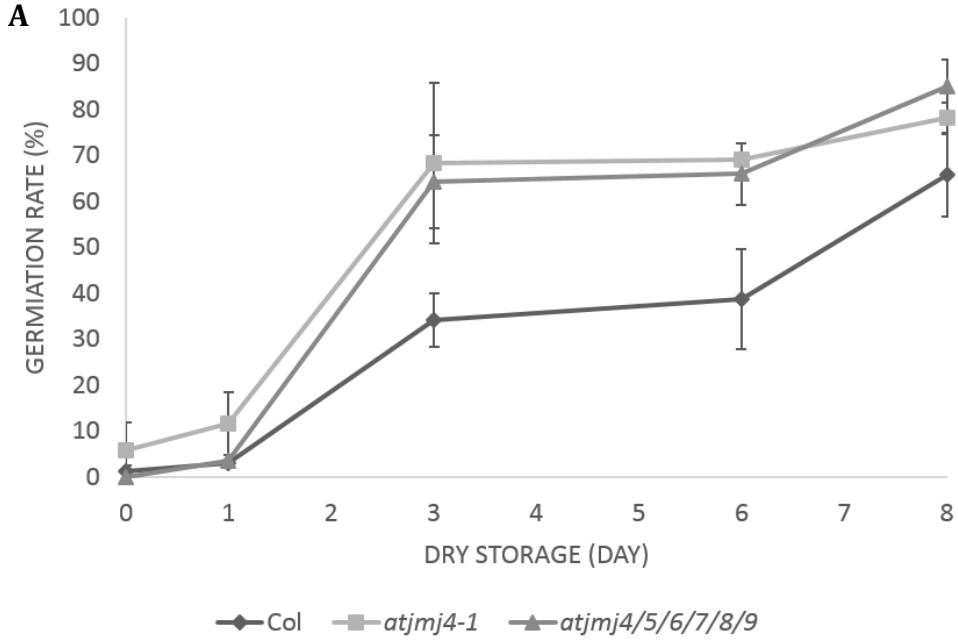
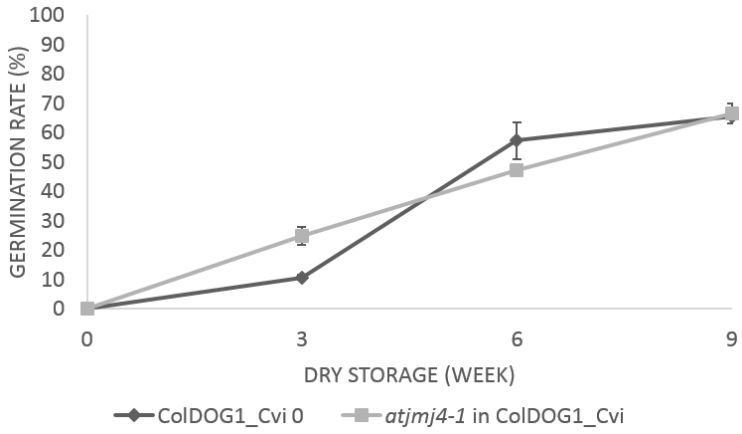

GERMINATION RATE(%)
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
DRY STORAGE (HOUR)
Col
atjmj4/5/6/7/8/9

Figure 2. Dormancy phenotype of *atjmj4-1* and *atjmj4/5/6/7/8/9*

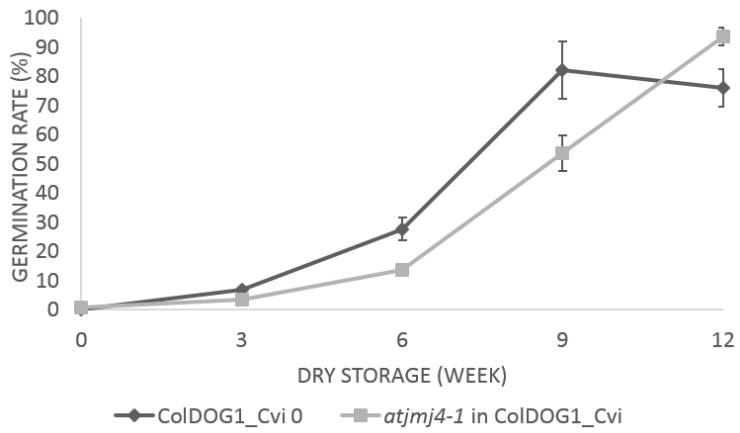
Dormancy test was performed using FH seed of (A) WT and *atjmj4-1*, or (B) WT and *atjmj4/5/6/7/8/9*. FH siliques dried for 0, 18, 24 or 48 hours. Mean values and standard errors of three biological repeats are shown. Each biological repeat contains 80 to 200 seed for each genotype.


A
100
90
80
70
60
50
40
30
20
10
0
GERMIATION RATE (%)
0
1
2
3
4
5
6
7
8
DRY STORAGE (DAY)
Col
atjmj4-1
atjmj4/5/6/7/8/9

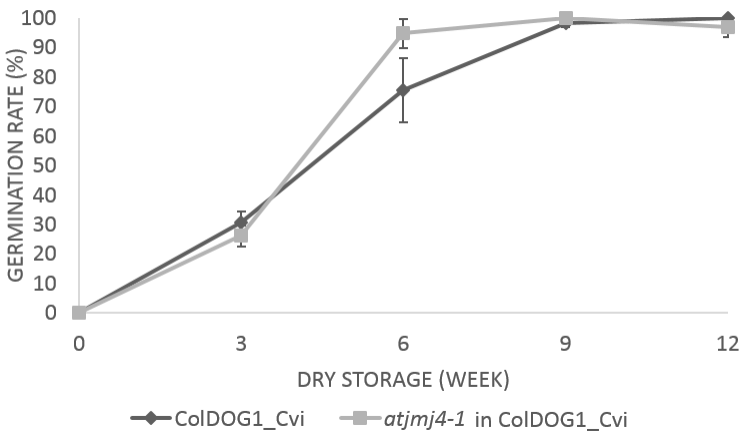
B


GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
DRY STORAGE (WEEK)
ColDOG1_Cvi 0
atjmj4-1 in ColDOG1_Cvi

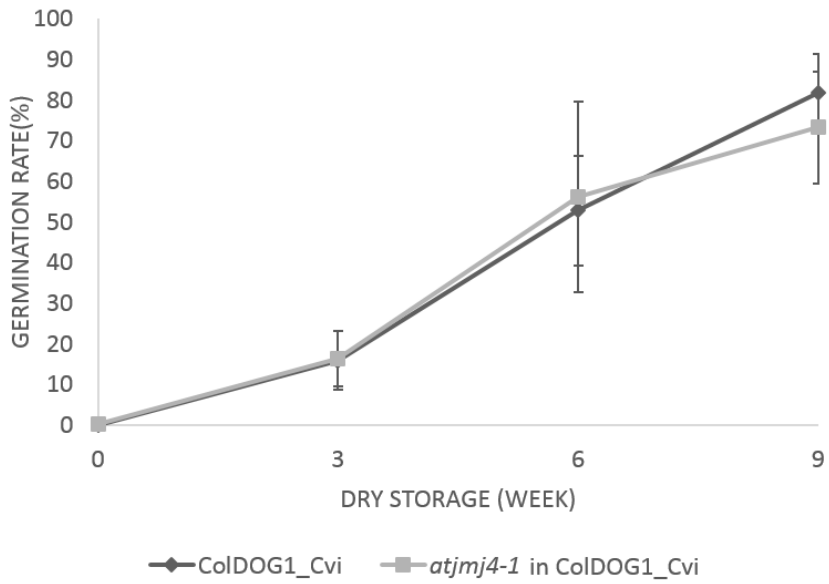
C


GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
DRY STORAGE (WEEK)
ColDOG1_Cvi 0
atjmj4-1 in ColDOG1_Cvi

D


GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
DRY STORAGE (WEEK)
ColDOG1_Cvi
atjmj4-1 in ColDOG1_Cvi

E


100
90
80
70
60
50
40
30
20
10
0
GERMINATION RATE(%)
0
3
6
9
DRY STORAGE (WEEK)
ColDOG1_Cvi
atjmj4-1 in ColDOG1_Cvi

F

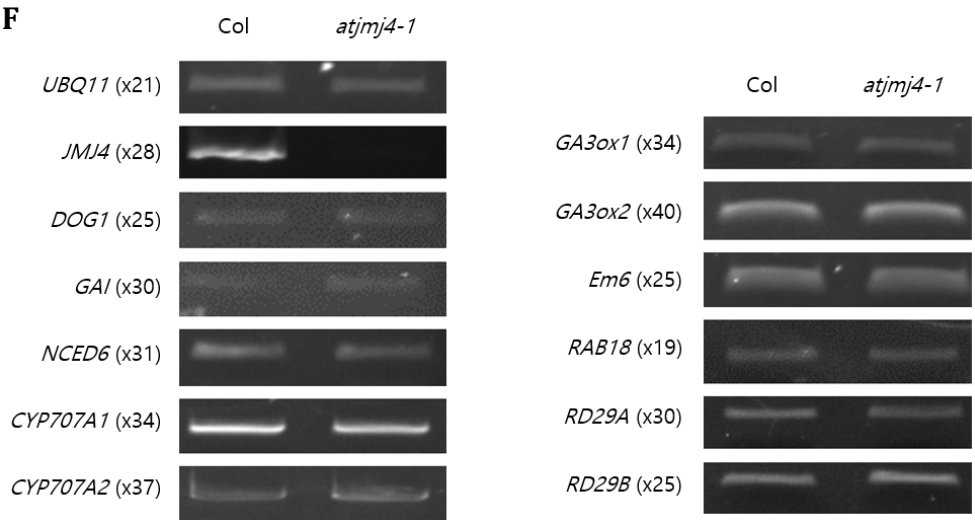
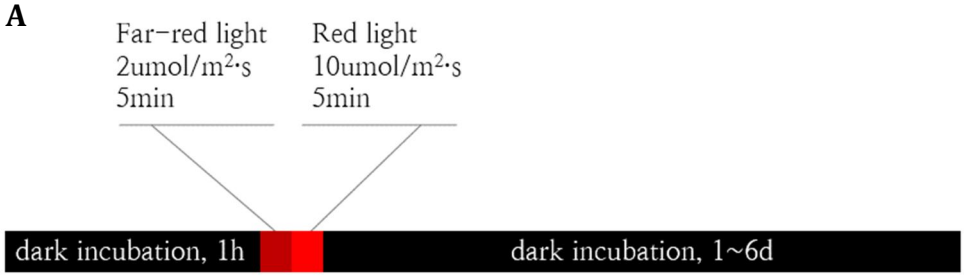

Col
atjmj4-1
UBQ11 (x21)
JMJ4 (x28)
DOG1 (x25)
GAI (x30)
NCED6 (x31)
CYP707A1 (x34)
CYP707A2 (x37)
Col
atjmj4-1
GA3ox1 (x34)
GA3ox2 (x40)
Em6 (x25)
RAB18 (x19)
RD29A (x30)
RD29B (x25)

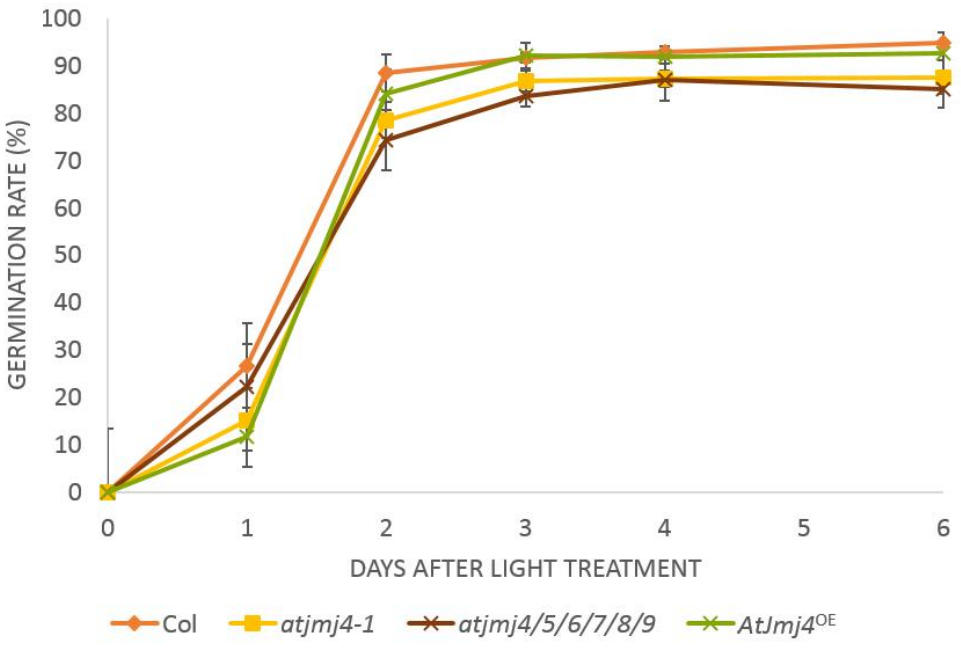
Figure 3. Dormancy phenotype of *atjmj4-1* in high dormancy background

(A) Dormancy test was performed using FH seed of WT, *atjmj4-1* and *atjmj4/5/6/7/8/9* grown at 12° C. FH siliques dried for 0, 1, 3, 6 and 8 days. (B~D) The dormancy test of FH seed of Col^{DOG1_Cvi} and *atjmj4-1* in Col^{DOG1_Cvi}. FH siliques dried for 0, 3, 6, 9 (and 12) weeks. Each graph indicates individual biological repeat and the error bars mean the standard deviations of three technical repeats. (E) The merged graph of (B~D). Each point indicates the mean value of three biological repeats and the standard errors are shown as the error bars. 120 to 300 seed were totally used in three biological repeats for each genotype. (F) RT-PCR was performed to investigate the gene expression patterns of *DOG1* and ABA or GA-related genes in WT and *atjmj4-1* FH seed.

A


Far-red light
2umol/m²·s
5min
Red light
10umol/m²·s
5min
dark incubation, 1h
dark incubation, 1~6d

B


GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
DAYS AFTER LIGHT TREATMENT
Col
atjmj4-1
atjmj4/5/6/7/8/9
AtJmj4OE

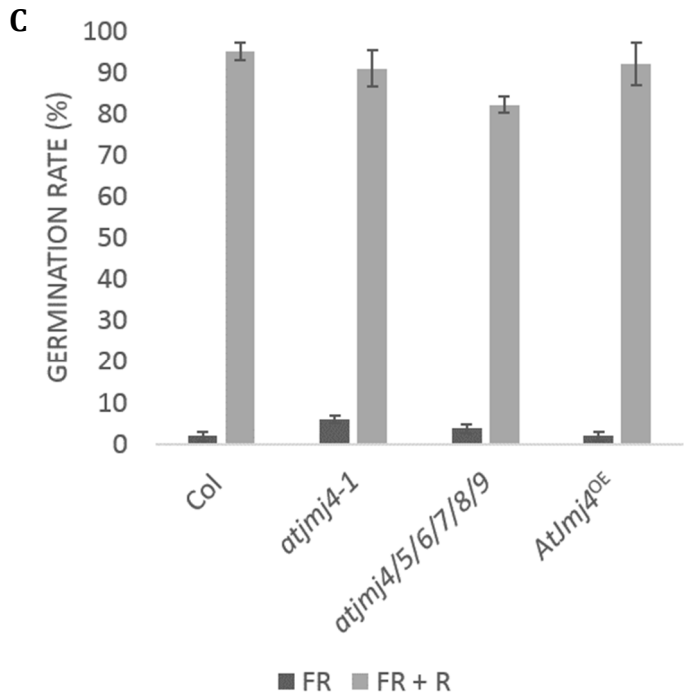
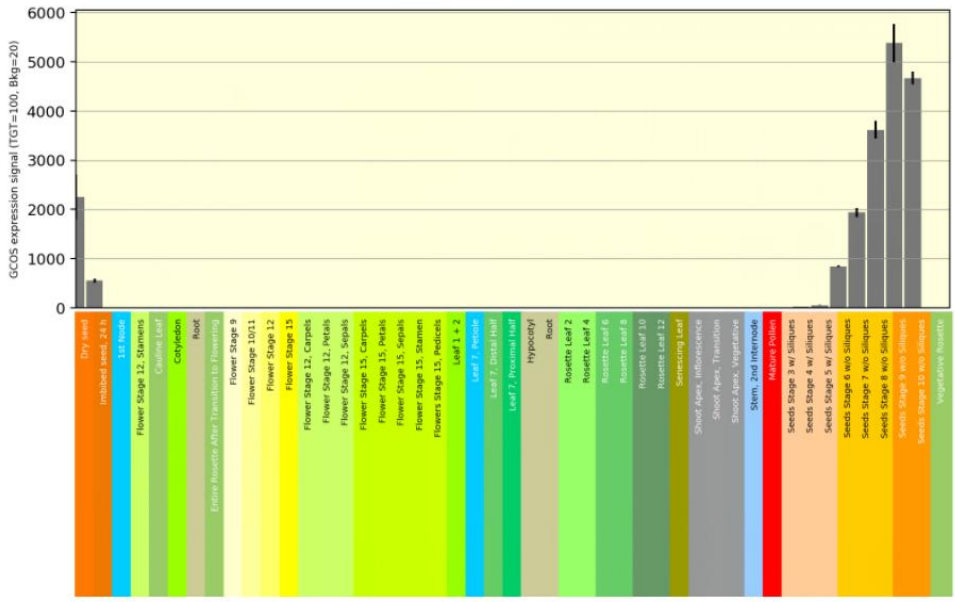


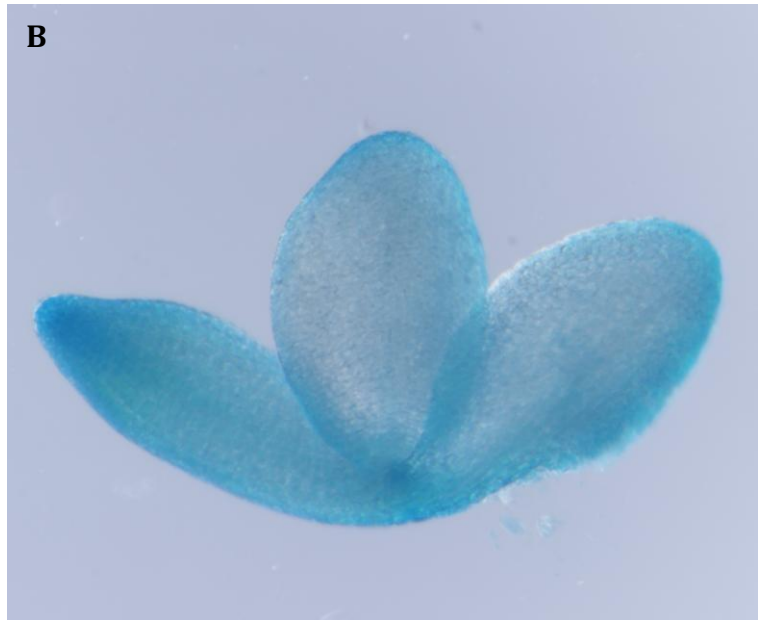
Figure 4. Germination phenotype of *atjmj4-1*, *atjmj4/5/6/7/8/9* and *AtJmj4*^{OE}

Germination test was performed using AR seed of Col, *atjmj4-1*, *atjmj4/5/6/7/8/9* and *AtJmj4*^{OE}. (B) Red light-dependent germination test was done as a skim of (A). Seeds with ruptured testa were counted as germinating seeds. (C) Germination rate was counted 4 days after treatment of far-red light only or far-red light following red light. The mean values and the standard errors of biological three replicates are shown. Each biological repeat contains 100 to 300 seeds for each genotype.

A


GCOS expression signal (TGT=100, Bkg=20)
6000
5000
4000
3000
2000
1000
0
Dry Seed
Imbibed seed, 24 h
1st Node
Flower Stage 12, Stamens
Cauline Leaf
Cotyledon
Root
Entire Rosette After Transition to Flowering
Flower Stage 9
Flower Stage 10/11
Flower Stage 12
Flower Stage 15
Flower Stage 12, Carpels
Flower Stage 12, Petals
Flower Stage 12, Sepals
Flower Stage 15, Carpels
Flower Stage 15, Petals
Flower Stage 15, Sepals
Flower Stage 15, Stamen
Flowers Stage 15, Pedicels
Leaf 1 + 2
Leaf 7, Petiole
Leaf 7, Distal Half
Leaf 7, Proximal Half
Hypocotyl
Root
Rosette Leaf 2
Rosette Leaf 4
Rosette Leaf 6
Rosette Leaf 8
Rosette Leaf 10
Rosette Leaf 12
Senescing Leaf
Shoot Apex, Inflorescence
Shoot Apex, Transition
Shoot Apex, Vegetative
Stem, 2nd Internode
Mature Pollen
Seeds Stage 3 w/ Siliques
Seeds Stage 4 w/ Siliques
Seeds Stage 5 w/ Siliques
Seeds Stage 6 w/o Siliques
Seeds Stage 7 w/o Siliques
Seeds Stage 8 w/o Siliques
Seeds Stage 9 w/o Siliques
Seeds Stage 10 w/o Siliques
Vegetative Rosette

B



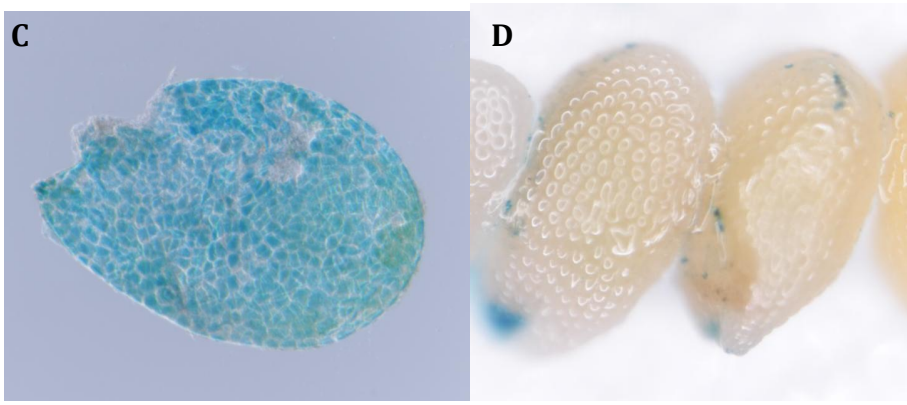
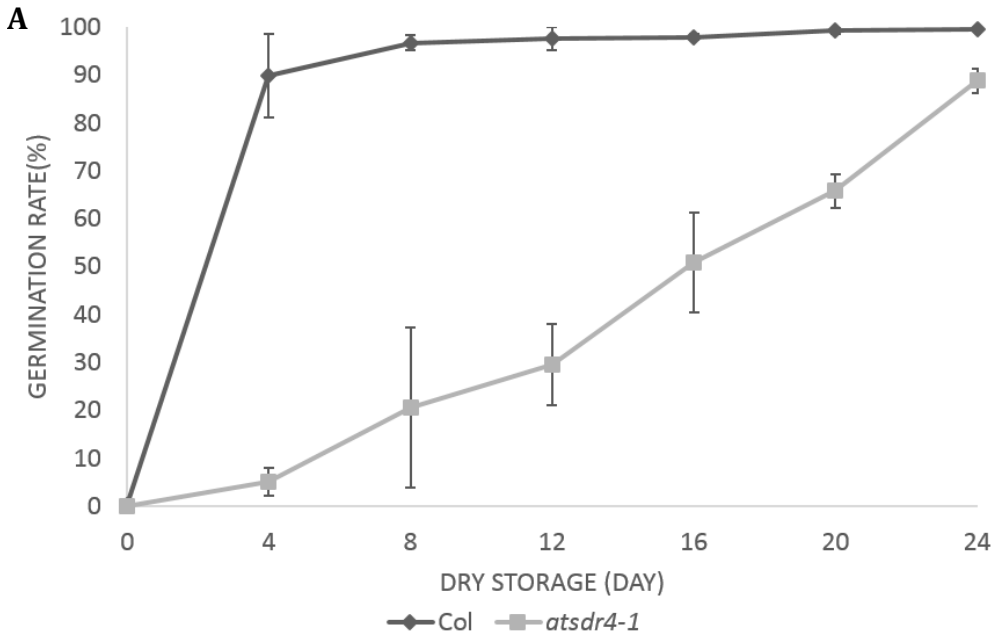
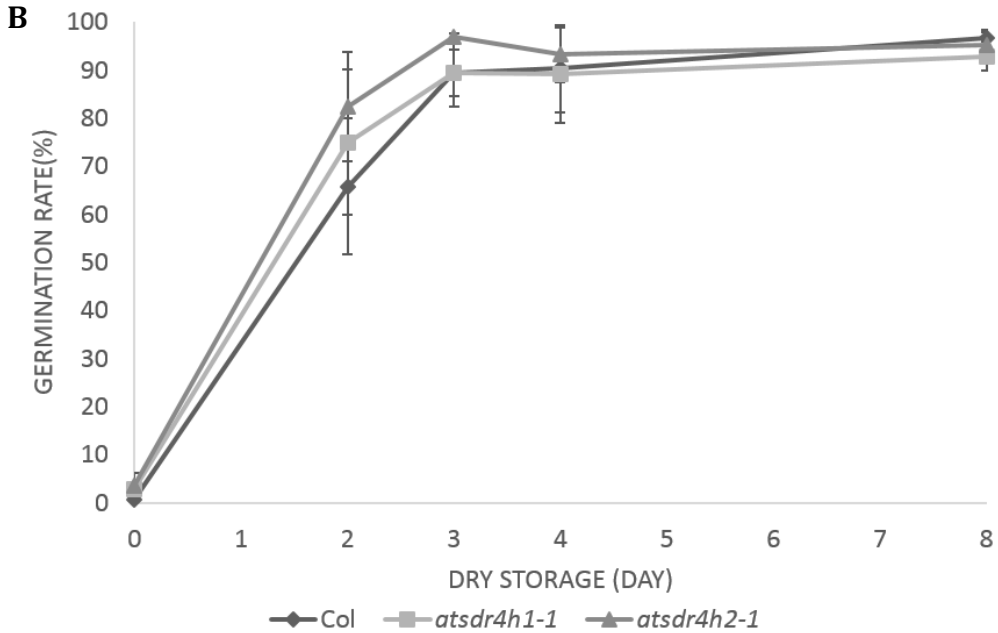


Figure 5. Expression of *pAtSDR4::GUS* in FH seed

(A) The expression pattern of *AtSDR4* at various tissues or developmental phases in Arabidopsis. (Winter and Vinegar, 2007). (B~D) GUS staining of FH seed of *pAtSDR4::GUS*. (B) The embryo separated from the seed coat. (C) The endosperm. (D) The whole seed.


A
100
90
80
70
60
50
40
30
20
10
0
GERMINATION RATE(%)
0
4
8
12
16
20
24
DRY STORAGE (DAY)
Col
atsdr4-1


B
100
90
80
70
60
50
40
30
20
10
0
GERMINATION RATE(%)
0
1
2
3
4
5
6
7
8
DRY STORAGE (DAY)
Col
atsdr4h1-1
atsdr4h2-1

C

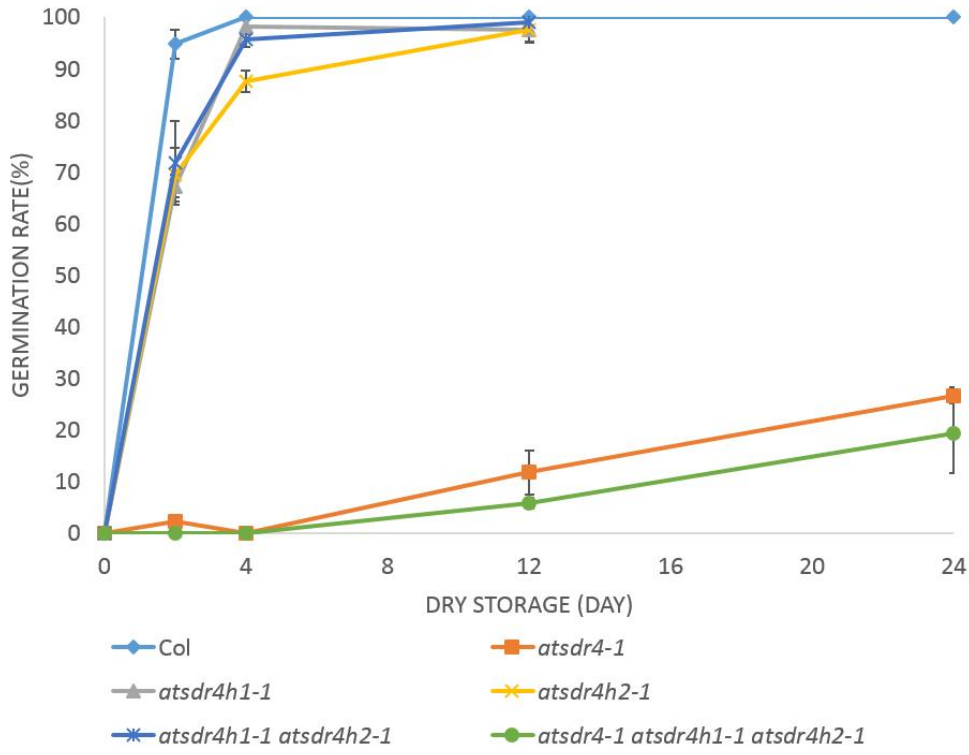
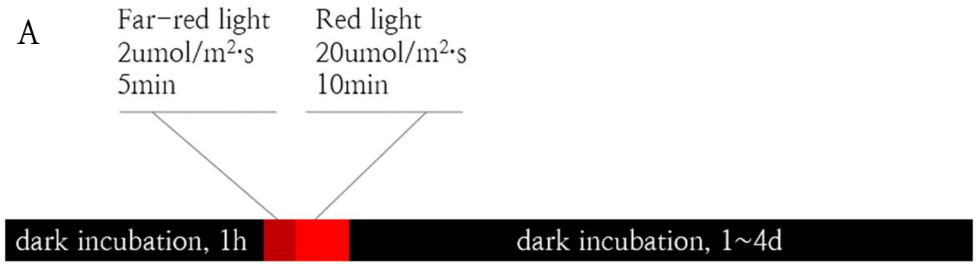

GERMINATION RATE(%)
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
DRY STORAGE (DAY)
Col
atsdr4-1
atsdr4h1-1
atsdr4h2-1
atsdr4h1-1 atsdr4h2-1
atsdr4-1 atsdr4h1-1 atsdr4h2-1

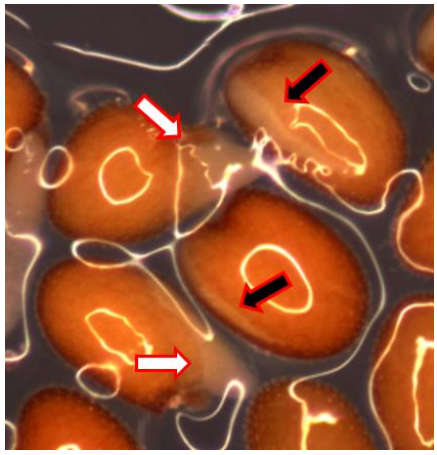
Figure 6. Dormancy phenotype of *atsdr4-1, atsdr4h1-1* and *atsdr4h2-1*

Dormancy test was performed using freshly-harvested seed of (A) Col and *atsdr4-1*. FH siliques dried for 0 day to 24 days with 4-day interval. (B) FH siliques of Col, *atsdr4h1-1* and *atsdr4h2-1* dried for 0, 2, 3, 4, and 8 days. Mean values and standard errors of biological three repeats are shown. (C) Dormancy phenotype of multiple mutants. FH siliques were dried for 0, 2, 4, 12 and 16 days. Error bars represent the standard deviation of three technological repeats. Each biological repeat contains 70 to 150 seeds for each genotype.

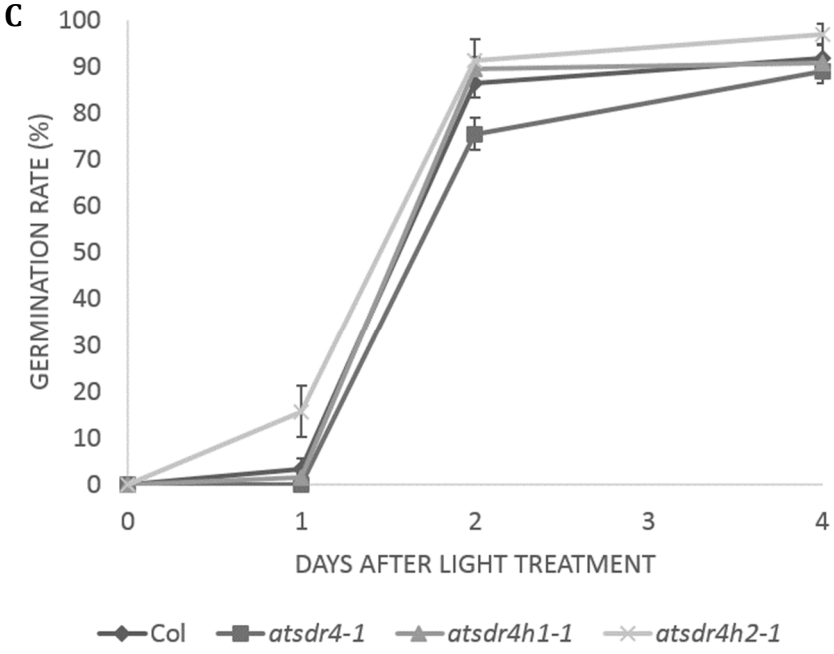
A

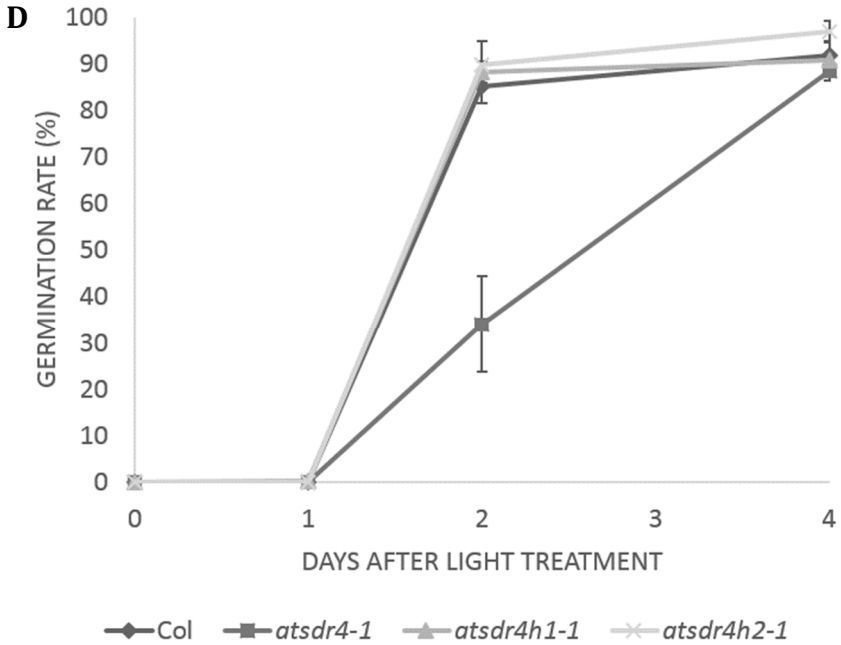

Far-red light
2umol/m²·s
5min
Red light
20umol/m²·s
10min
dark incubation, 1h
dark incubation, 1~4d

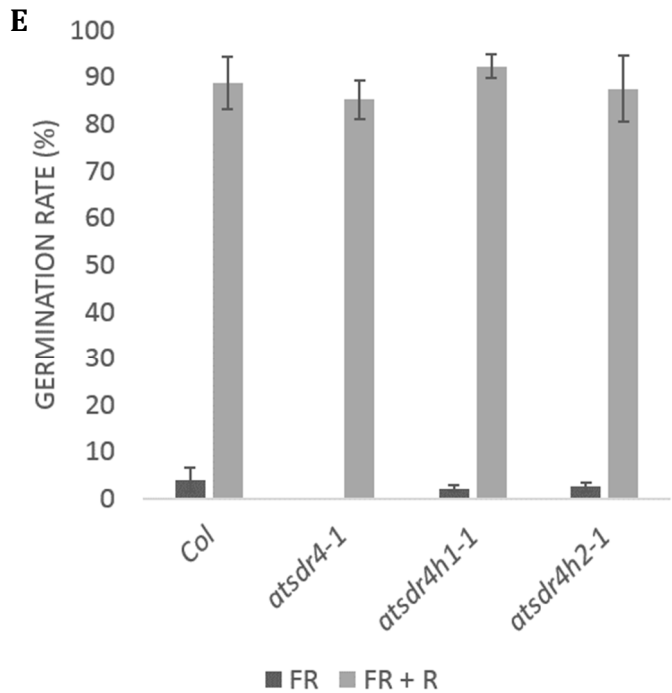
B



C


GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
DAYS AFTER LIGHT TREATMENT
Col
atsdr4-1
atsdr4h1-1
atsdr4h2-1


D
GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
DAYS AFTER LIGHT TREATMENT
Col
atsdr4-1
atsdr4h1-1
atsdr4h2-1


E
GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
Col
atsdr4-1
atsdr4h1-1
atsdr4h2-1
FR
FR + R

F

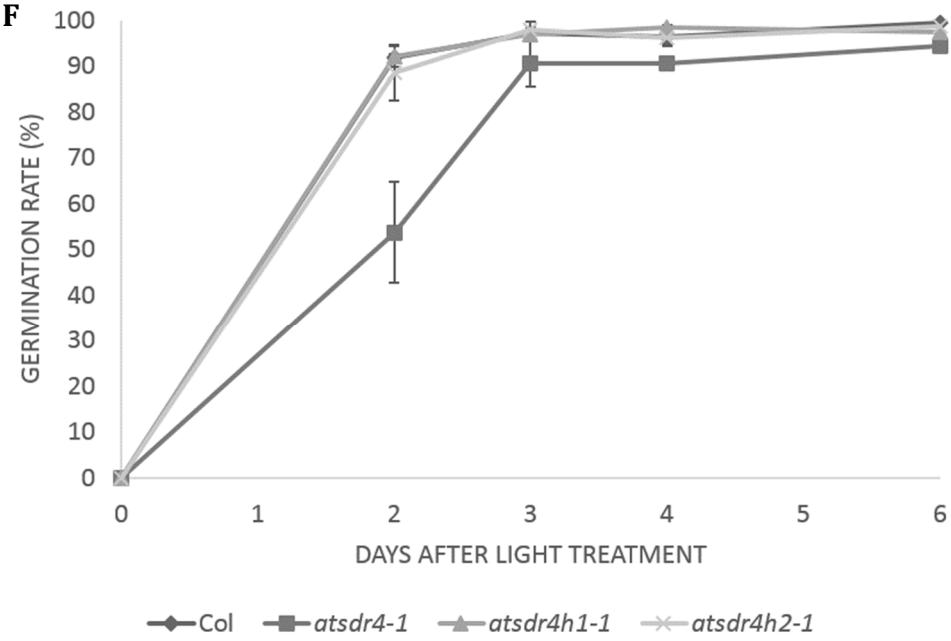

GERMINATION RATE (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
DAYS AFTER LIGHT TREATMENT
Col
atsdr4-1
atsdr4h1-1
atsdr4h2-1

Figure 7. Light-dependent germination phenotype of *atsdr4-1, atsdr4h1-1* and *atsdr4h2-1*

Germination test was performed using after-ripened (AR) seed of WT, *atsdr4-1, atsdr4h1-1* and *atsdr4h2-1*. Seed were after-ripened for about 2 months and sown on 1/2 MS plate after surface-sterilizing. (B) Testa-ruptured seeds (black arrow) and radicle-extruded seeds (white arrow) of *atsdr4-1*. (C, D) Red light-dependent germinations test were done as a skim of (A). (C) Seeds with ruptured testa or (D) Radicle-extruded seed were counted as germinating seeds. (E) Germination rate was counted 4 days after treatment of far-red light only or far-red light following red light. (F) The plates were placed under constant white light for 2, 3, 4, and 6 days and germination rate was counted. Mean values and standard errors of three biological repeats are shown. Each biological repeat contains 60 to 200 seed for each genotype.

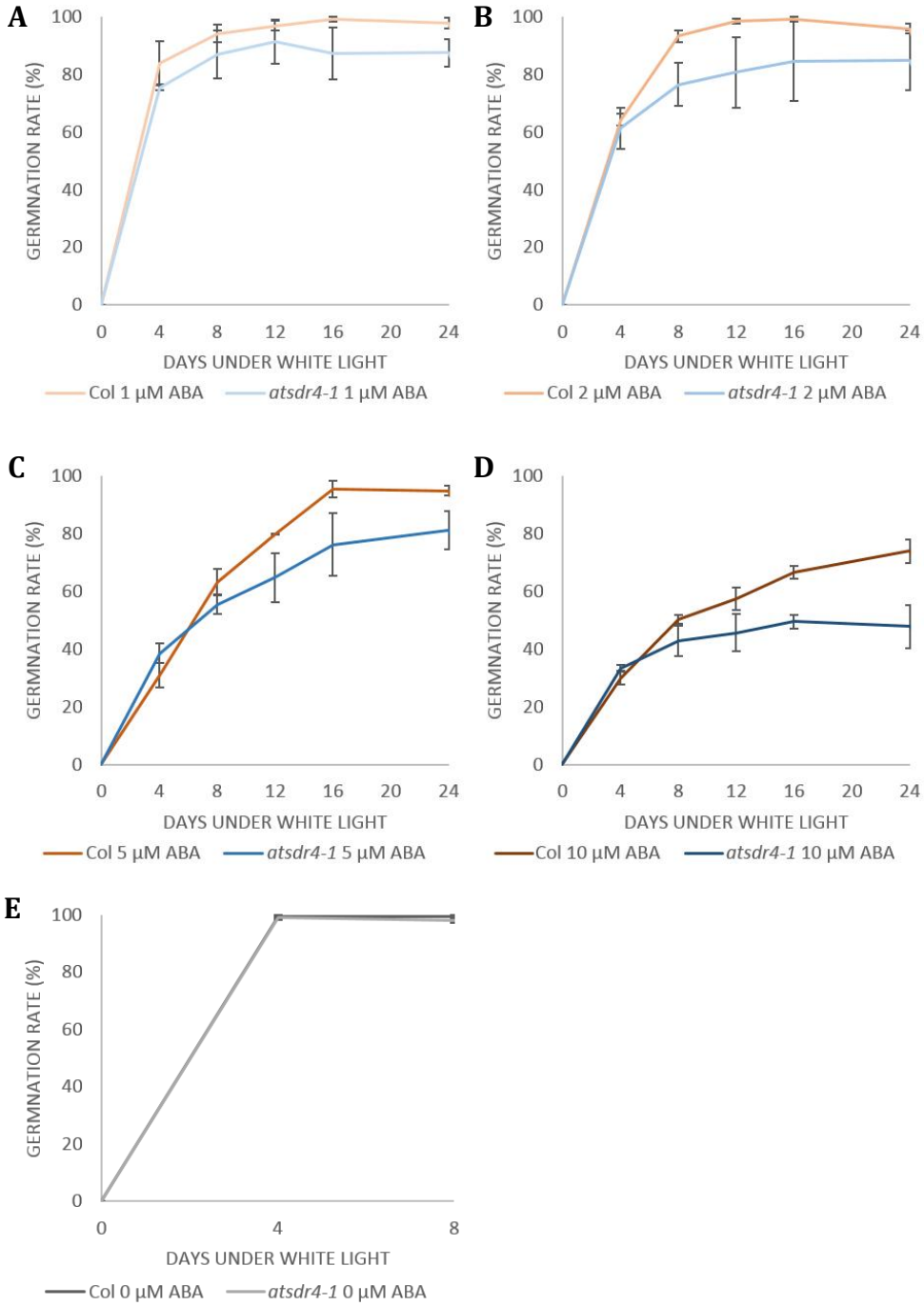
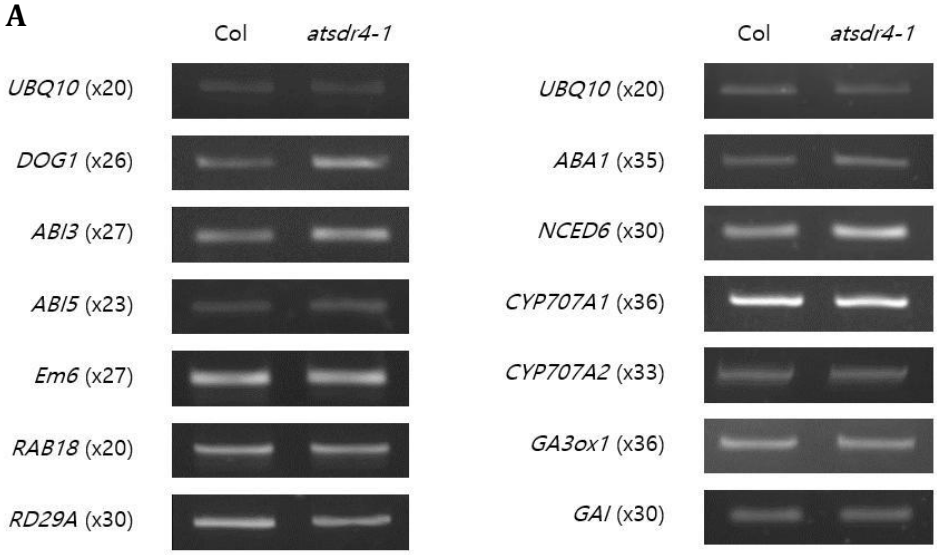

A
GERMNATION RATE (%)
DAYS UNDER WHITE LIGHT
Col 1 µM ABA
atsdr4-1 1 µM ABA
B
GERMNATION RATE (%)
DAYS UNDER WHITE LIGHT
Col 2 µM ABA
atsdr4-1 2 µM ABA
C
GERMNATION RATE (%)
DAYS UNDER WHITE LIGHT
Col 5 µM ABA
atsdr4-1 5 µM ABA
D
GERMNATION RATE (%)
DAYS UNDER WHITE LIGHT
Col 10 µM ABA
atsdr4-1 10 µM ABA
E
GERMNATION RATE (%)
DAYS UNDER WHITE LIGHT
Col 0 µM ABA
atsdr4-1 0 µM ABA

Figure 8. ABA-sensitive germination phenotype of WT and *atsdr4-1*

Germination test was performed using after-ripened (AR) seed of WT and *atsdr4-1* on ABA media. AR seeds were sown on the 1/2 MS plate containing (A) 1 μM, (B) 2 μM, (C) 5 μM, (D) 10 μM and (E) 0 μM of ABA. Each plates underwent 3 days of stratification and were placed under constant white light.

A


Col
atsdr4-1
UBQ10 (x20)
DOG1 (x26)
ABI3 (x27)
ABI5 (x23)
Em6 (x27)
RAB18 (x20)
RD29A (x30)
Col
atsdr4-1
UBQ10 (x20)
ABA1 (x35)
NCED6 (x30)
CYP707A1 (x36)
CYP707A2 (x33)
GA3ox1 (x36)
GAI (x30)

B

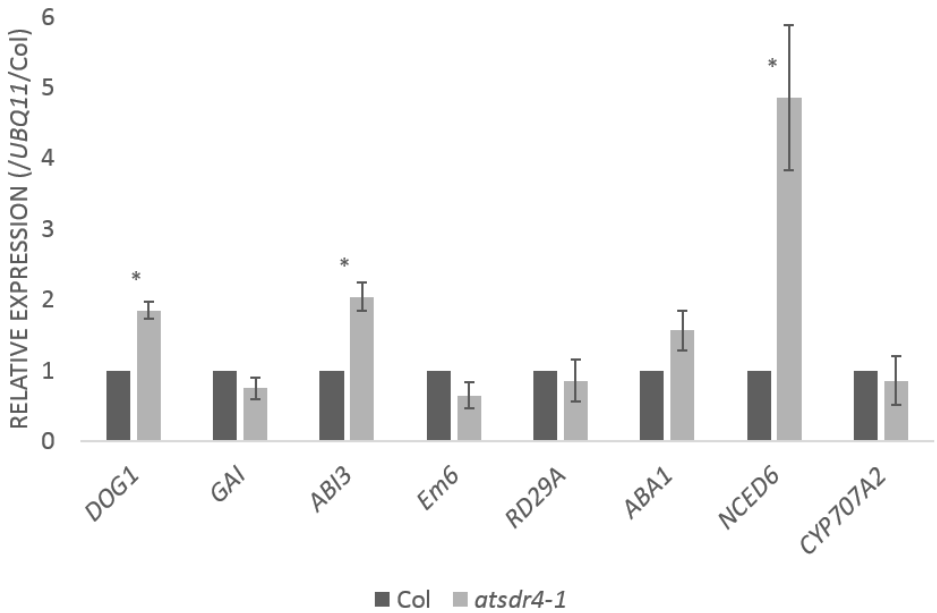

RELATIVE EXPRESSION (/UBQ11/Col)
0
1
2
3
4
5
6
*
*
*
DOG1
GAI
ABI3
Em6
RD29A
ABA1
NCED6
CYP707A2
Col
atsdr4-1

Figure 9. mRNA expression of *DOG1* and ABA or GA-related genes in WT and *atsdr4-1* FH seed

(A) RT-PCR was performed using cDNA of WT and *atsdr4-1* FH seed. The numbers next to the gene mean PCR cycle numbers. (B) qRT-PCR was performed and the gene expression level was normalized with the expression level of *UBQ11*. All expression levels of Col were set as 1. Mean values and standard errors of three biological repeats are shown. Student's t-test were done and the genes with $p<0.05$ were marked with asterisks.

4. DISCUSSION

AtJmj4 could be a minor regulator of seed dormancy

The expression of *AtJmj4*, one of JmjC domain-containing protein-coding gene, is higher in FH seed than other tissues and the expression of *pAtJmj4::GUS* was observed from various parts in the embryo of FH seed. *atjmj4-1* and *atjmj4/5/6/7/8/9* did not show any dormancy phenotype in their normal growth condition, long-day and 22 ℃. Two alternative systems were used to expand the window of seed dormancy; the low temperature (12 ℃) and the high dormancy genetic background (Col^{DOG1_Cvi}). Interestingly, *atjmj4-1* and *atjmj4/5/6/7/8/9* showed reduced seed dormancy only at 12 ℃, but not in Col^{DOG1_Cvi} background that offer much wider window of seed dormancy. This suggests that no remarkable dormancy difference between Col and *atjmj4-1* at 22 ℃ was the temperature matter, not the narrow window matter. In addition, the dormancy test at 12 ℃ reveals that only *AtJmj4* contributes to seed dormancy among Clade II JmjC domain-containing protein-coding genes.

The result indicates that the contribution of *AtJmj4* to seed dormancy is quite weak and *AtJmj4* would work at low temperature to regulate seed dormancy in fine manner. Considering the dormancy

phenotype of *atjmj4-1* is only visible at 12 ℃ and the altered expression of *DOG1* and GA- or ABA-dependent genes was not observed, it could be suggested that *AtJmj4* controls cold-related genes to regulate seed dormancy. Though *DOG1* is regarded as the key gene of seed dormancy in Arabidopsis, there is a possibility that other genes could regulate seed dormancy. The study on cold-related genes in *atjmj4-1* mutant would be able to uncover the mechanism of seed dormancy regulation by *AtJmj4*.

AtSDR4 is putative negative regulator of seed dormancy

OsSDR4 regulates seed dormancy and prevents pre-harvest sprouting in rice. The study on the homologs of *OsSDR4* in various species has begun lately (Subburaj et al., 2016). In Arabidopsis, three *OsSDR4* homologs were identified (Choi, 2012): *AtSDR4, AtSDR4H1*, and *AtSDR4H2*. Dormancy test using FH seed of *atsdr4-1, atsdr4h1-1*, *atsdr4h2-1* and their multiple mutants revealed that *AtSDR4* regulates seed dormancy negatively while *atsdr4h1-1* and *atsdr4h2-1* do not contribute to the dormancy phenotype.

The result of qRT-PCR suggested that the strong seed dormancy of *atsdr4-1* may be the consequence of the elevated

expression level of ABA-synthesis or signaling genes. High *DOG1* mRNA level supports the high seed dormancy phenotype of *atsdr4-1*. *NCED6* mRNA level was upregulated indicating increase of ABA production. Interestingly, the level of *ABI3* mRNA was also elevated. It seems that *AtSDR4* negatively controls *ABI3* whether the opposite phenomenon is observed: *OsVp1*, the homolog of *AtABI3*, regulates *OsSDR4* in rice. It suggests that the regulation of seed dormancy by *AtSDR4, DOG1* and *ABI3* in Arabidopsis is different from by *OsSDR4, OsDOG1-like* genes and *OsVp1* in rice. Furthermore, a dormancy test performed in our condition revealed that *dog1 atsdr4-1* double mutant FH seed possesses very strong seed dormancy as much as, or more than *atsdr4-1,* even the double mutant lacks *DOG1*. It has known that the absence of *DOG1* is not overcame by high ABA level (Nakabayashi et al., 2012). If the strong seed dormancy of *atsdr4-1* is only caused by the upregulation of ABA pathway, the phenotype of *dog1 atsdr4-1* cannot be explained. Thus, the further study is needed to uncover the relationship between *DOG1*, *AtSDR4* and *OsSDR4*.

Germination tests were performed using *atsdr4-1, atsdr4h1-1* and *atsdr4h2-1* AR seed. Their perception of far-red and red light was normal, and no significant germination phenotypes of *atsdr4-1* and *atsdr4h1-1* were observed. Testa rupture of *atsdr4h2-1* was a little bit

faster than other genotypes; *atsdr4h2-1* has a possibility to regulate seed germination in fine manner. *atsdr4-1* showed marginally delayed radicle extrusion and revealed delayed germination on the ABA media. This could be explained by its hypersensitivity to ABA rather than its large amount of endogenous ABA. In every germination tests, enough after-ripening over 1 month and 3 days of stratification was done to remove endogenous ABA at most. Thus, it is more convincing to say that the retarded radicle-extrusion phenotype of *atsdr4-1* on ABA media is due to its minor ABA hypersensitivity. To verify this hypothesis, additional experiments such as measuring of endogenous ABA would be needed. Since the expression of *pAtSDR4::GUS* was observed in the endosperm, the adjacent layer of radicle, there is a possibility that *AtSDR4* may function in the endosperm layer, regulating ABA signaling.

Taken together, *AtSDR4* might play an important role in seed dormancy in negative manner by repressing ABA pathway in FH seed, and possess a probability to regulate ABA signaling in AR seed. Further studies would be reveal the relationship of *AtSDR4*, *DOG1* and *ABI3*, as well as *OsSDR4*. It is expected that *AtSDR4* will be another key to uncover the mystery of seed dormancy in Arabidopsis. On the other side, following gene expression study on ABA signaling genes of

atsdr4-1 AR seed would reveal the role of *AtSDR4* in regulating ABA signaling in AR seed.

V. REFERENCES

Alonso-Blanco, C., Bentsink, L., Hanhart, C.J., Blankestijn-de Vries, H., and Koornneef, M. (2003). Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. Genetics 164, 711 – 729.

Baskin, J.M., and Baskin, C.C. (2004). A classification system for seed dormancy. Seed Sci. Res. *14*, 1 – 16.

Bentsink, L., Jowett, J., Hanhart, C.J., and Koornneef, M. (2006). Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. *103*, 17042 – 17047.

Bentsinka, L., and Koornneef, M. (2008). Seed Dormancy and Germination. Arab. B. 6 e0119. 2008 *6*.

Bewley, J. (1997). Seed Germination and Dormancy. Plant Cell *9*, 1055 – 1066.

Chen, C., Wu, C., Miao, J., Lei, Y., Zhao, D., Sun, D., Yang, G., Huang, J., and Zheng, C. (2014). Arabidopsis SAG protein containing the MDN1 domain participates in seed germination and seedling development by negatively regulating ABI3 and ABI5. J. Exp. Bot. *65*, 35 – 45.

Chiang, G.C.K., Bartsch, M., Barua, D., Nakabayashi, K., Debieu, M., Kronholm, I., Koornneef, M., Soppe, W.J.J., Donohue, K., and De Meaux, J. (2011). DOG1 expression is predicted by the seed-maturation environment and contributes to geographical variation in germination in Arabidopsis thaliana. Mol. Ecol. *20*, 3336 – 3349.

Choi, H.H. (2012). A Study on the Physiological and Molecular Seed Dormancy Phenotypes of Several Arabidopsis Chromatin Modifier Mutants. 석사학위논문, 서울대학교, 서울.

Finch-Savage, W.E., and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination - Finch-Savage - 2006 - New Phytologist - Wiley Online Library. New Phytol. *171*, 501 – 523.

Graeber, K., Nakabayashi, K., Miatton, E., Leubner-Metzger, G., and Soppe, W.J.J. (2012). Molecular mechanisms of seed dormancy. Plant, Cell Environ. *35*, 1769 – 1786.

Hong, E.H., Jeong, Y.M., Ryu, J.Y., Amasino, R.M., Noh, B., and Noh, Y.S. (2009). Temporal and spatial expression patterns of nine Arabidopsis genes encoding Jumonji C-domain proteins. Mol. Cells *27*, 481 – 490.

Huijser, P., and Schmid, M. (2011). The control of developmental phase transitions in plants. Development *138*, 4117 – 4129.

Jeong, J.H., Song, H.R., Ko, J.H., Jeong, Y.M., Kwon, Y.E., Seol, J.H., Amasino, R.M., Noh, B., and Noh, Y.S. (2009). Repression of FLOWERING LOCUS T chromatin by functionally redundant histone H3 lysine 4 demethylases in Arabidopsis. PLoS One *4*.

Kucera, B., Cohn, M.A., and Leubner-Metzger, G. (2005). Plant hormone interactions during seed dormancy release and germination. Seed Sci. Res. *15*, 281 – 307.

Lin, S.Y.S., Sasaki, T., and Yano, M. (1998). Mapping quantitative trait loci controlling seed dormancy and heading date in rice, Oryza sativa L., using backcross inbred lines. Theor. Appl. Genet. *96*, 997 – 1003.

Meng, L., and Feldman, L. (2010). A rapid TRIzol-based two-step method for DNA-free RNA extraction from Arabidopsis siliques and dry seeds. Biotechnol. J. *5*, 183 – 186.

Nakabayashi, K., Bartsch, M., Xiang, Y., Miatton, E., Pellengahr, S., Yano, R., Seo, M., and Soppe, W.J.J. (2012). The Time Required for Dormancy Release in Arabidopsis Is Determined by DELAY OF GERMINATION1 Protein Levels in Freshly Harvested Seeds. Plant Cell *24*, 2826 – 2838.

Piskurewicz, U., Turečková, V., Lacombe, E., Lopez-Molina, L., Turecková, V., Lacombe, E., and Lopez-Molina, L. (2009). Far-red light inhibits germination through DELLA-dependent stimulation of ABA synthesis and ABI3 activity. EMBO J. *28*, 2259 – 2271.

Qian, S., Wang, Y., Ma, H., and Zhang, L. (2015). Expansion and functional divergence of the JmjC gene family: significance of duplications in ancestral angiosperms and vertebrates. Plant Physiol. *168*, 1321 – 1337.

Raz, V., Bergervoet, J.H.W., and Koornneef, M. (2001). Sequential steps for developmental arrest in Arabidopsis seeds. Development *128*, 243 – 252.

Rodríguez-Gacio, M.D.C., Matilla-Vázquez, M. a, and Matilla, A.J. (2009). Seed dormancy and ABA signaling: the breakthrough goes on. Plant Signal. Behav. *4*, 1035 – 1049.

Shi, Y., and Whetstine, J.R. (2007). Dynamic Regulation of Histone Lysine Methylation by Demethylases. Mol. Cell *25*, 1 – 14.

Shinomura, T., Nagatani, a., Chory, J., and Furuya, M. (1994). The Induction of Seed Germination in Arabidopsis thaliana Is Regulated Principally by Phytochrome B and Secondarily by Phytochrome A. Plant Physiol. *104*, 363–371.

Subburaj, S., Cao, S., Xia, X., and He, Z. (2016). Phylogenetic Analysis , Lineage-Specific Expansion and Functional Divergence of seed dormancy 4 -Like Genes in Plants. PLoS One *31690*, 1–24.

Sugimoto, K., Takeuchi, Y., Ebana, K., Miyao, A., Hirochika, H., Hara, N., Ishiyama, K., Kobayashi, M., Ban, Y., Hattori, T., et al. (2010). Molecular cloning of Sdr4, a regulator involved in seed dormancy and domestication of rice. Proc. Natl. Acad. Sci. U. S. A. *107*, 5792–5797.

Yamaguchi-Shinozaki, K. (1994). A Novel cis-Acting Element in an Arabidopsis Gene Is Involved in Responsiveness to Drought, Low-Temperature, or High-Salt Stress. Plant Cell Online *6*, 251–264.

VI. ABSTRACT IN KOREAN

애기장대 종자 휴면 관련 유전자들에 관한 연구

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종자 휴면(seed dormancy)는 종자가 생존하기 적합한 환경을 만날 때까지 발아를 미루는 현상을 이른다. 종자 휴면에 영향을 주는 환경적, 유전적 요소들에 대한 연구가 일부 이루어졌지만. 그 기저 작용 기전은 알려진 바가 많지 않다. 최근 몇몇 후성유전학적 조절자들이 종자 휴면의 조절에 관여한다고 보고되었다. *AtJmj4* 는 애기장대의 히스톤 탈메틸화효소(H3K4 demethylase)를 암호화하는 유전자로, 가장 높은 종자 휴면을 지니는 FH 상태의 종자에서 다량 발현한다. 이에 따라 *AtJmj4* 가 종자 휴면의 조절자일 가능성이 엿보인다. 한편, 벼에서는 종자 휴면 핵심 조절자로 *OsSDR4* 가 알려져 있다. 애기장대에서 아미노산 서열 기준의 *OsSDR4* 의 가장 가까운 상동유전자는 *AtSDR4* 이다. *OsSDR4* 결여

돌연변이는 매우 낮은 종자 휴면을 가지는데, 이에 반해 애기장대의 *OsSDR4* 상동유전자인 *AtSDR4* 결여 돌연변이는 반대로 매우 높은 종자 휴면을 지님이 보고되었다. 추가로 밝혀진 두 개의 상동유전자인 *AtSDR4H1* 과 *AtSDR4H2* 의 종자 휴면과의 관련성은 아직 알려지지 않았다. 본 연구에서는 *AtJmj4* 와 *AtSDR4* 의 종자 휴면에서의 역할에 대하여 연구하였다. 일반적인 애기장대 생육 환경인 22 ℃ 장일 조건에서 *AtJmj4* 의 결여는 Columbia 와 Col^{DOG1_Cvi} 에서 종자 휴면을 변화시키지 않았다. 한편 12 ℃의 조건 하에서 *atjmj4-1* 는 야생형에 비해 낮아진 종자 휴면을 보였다. *atsdr4-1, atsdr4h1-1, atsdr4h2-1*와 이들의 다중 돌연변이를 이용한 종자 휴면 실험에서는 *atsdr4-1* 돌연변이가 종자 휴면을 증가시키며, *atsdr4h1-1* 혹은 *atsdr4h2-1* 돌연변이는 그렇지 않음을 확인할 수 있었다. 유전자 발현 분석을 통해 *atsdr4-1* FH 종자에서 대표적인 종자 휴면 조절자인 *DOG1* 뿐만 아니라 앱시스산 합성과 신호전달에 관여하는 유전자들의 발현이 함께 증가함을 발견했다. 종자 발아와 관련된 표현형이 이들 돌연변이체들에 존재하는 지를 알아보기 위한 적색광 아래의 발아 실험에서, *atjmj4-1, atsdr4-1, atsdr4h1-1, atsdr4h2-1* 모두 발아표현형을 가지지는 않는 것으로 드러났다. 다만 *atsdr4-1* 의 경우 어린 뿌리가 다소 느리게 나오는 것으로 밝혀졌다. ABA 배지에서의 발아 실험에 의하면 *atsdr4-1* 의 어린 뿌리가 늦게 나오는 현상은 ABA 에 대한 과민감성에 의한 것으로 보인다. 이 결과들로 미루어 볼 때, *AtJmj4* 는 저온 환경에서만 종자 휴면을 부여하는 약한 조절자이며, *AtSDR4* 는 종자 휴면을 억제하는 중요 조절자로 판단된다.

주요어: 종자 휴면, freshly-harvested seed, *AtJmj4*, *AtSDR4*, *DOG1*, 종자 발아, 앱시스산

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