Cover page

Title: **Arabidopsis aspartic protease ASPG1 affects seed dormancy, seed longevity and seed germination**

Running title: **role of ASPG1 in seed dormancy and germination**.

Corresponding author: Yan Wu, College of Life Sciences, Wuhan University, Wuhan 430072, China. Tel & Fax: +86 2768753805. E-mail: wuy@whu.edu.cn

Subject area: growth and development.

Number of figures: 8.

Number and type of supplementary material: 14, PDF and EXCEL.

Arabidopsis aspartic protease ASPG1 affects seed dormancy, seed longevity and seed germination

Wenzhong Shen, Xuan Yao, Tiantian Ye, Sheng Ma, Xiong Liu, Xiaoming Yin and Yan Wu*

State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan 430072, China

*Corresponding author: E-mail, wuy@whu.edu.cn.

Abbreviations: ABA, abscisic acid; AP, aspartic protease; CDT, controlled deterioration test; Col, Columbia; Com, complementation; Cvi, Cape Verde Islands; GA, gibberellic acid; GUS, β-glucuronidase; Ler, Landsberg erecta; OE, overexpression; PAC, paclobutrazol; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription PCR; RNAi, RNA interference; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSP, seed storage protein.

ABSTRACT

Seed storage proteins (SSPs) provide free amino acids and energy for the process of seed germination. Although degradation of SSPs by the aspartic proteases (APs) isolated from seeds have been documented *in vitro*, there is still no genetic evidence for involvement of APs in seed germination. Here we report that the aspartic protease ASPG1 (ASPARTIC PROTEASE IN GUARD CELL 1) plays an important role in the process of dormancy, viability and germination of Arabidopsis seeds. We show that *aspg1-1* mutants have enhanced seed dormancy and reduced seed viability. Significant increase in expression of DELLA genes which act as repressors in the GA (gibberellic acid) signal transduction pathway were detected in *aspg1-1* during seed germination. Seed germination of *aspg1-1* mutants was more sensitive to the treatment of PAC (paclobutrazol, a GA biosynthesis inhibitor). In contrast, seed germination of *ASPG1* overexpression (OE) transgenic lines showed resistant to PAC. The degradation of SSPs in germinating seeds was severely impaired in *aspg1-1* mutants. Moreover, the development of *aspg1-1* young seedlings was arrested when grown on the nutrient-free medium. Thus ASPG1 is important for seed dormancy, seed longevity and seed germination. And its function is associated with degradation of SSPs and regulation of GA signaling in Arabidopsis.

Keywords: ABA, aspartic protease, GA, seed dormancy, seed germination, seed longevity, seed storage protein.

INTRODUCTION

Seed dormancy controls the distribution of germination in space and time through preventing germination of mature seeds during unsuitable ecological conditions (Née et al. 2017a). The primary seed dormancy is generally initiated during seed maturation and it reaches a high level in freshly harvested seeds. A dormant seed may gradually lose its dormancy in subsequent period of dry seed storage (so-called after-ripening) and eventually enter into a non-dormant state (Graeber et al. 2012). In winter annual species such as *Arabidopsis thaliana* seed dormancy can be broken by stratification (Baskin and Baskin 2004). With releasing from dormancy seed germination begins with water uptake (imbibition) and ends with radicle protrusion through the testa and endosperm (seed coat) (Han and Yang 2015). In Arabidopsis, the essential role of the endosperm in inhibiting the germination of a dormant seed has been documented in reports (Lee et al. 2010, Lee and Lopez-Molina 2013). To explore the influence of seed coat on germination, a “seed coat bedding assay” has been used for monitoring the growth of embryos cultured on a layer of endosperm tissue with the testa attached. Results from the “seed coat bedding assay” demonstrated that the biosynthesized and then released ABA (abscisic acid) from the endosperm of dormant seeds could block embryonic growth (Lee et al. 2010, Kang et al. 2015). Although the testa and endosperm play a significant role in maintaining seed dormancy, the cause of dormancy is mainly determined by the inherent characteristics of embryos (Finch-Savage and Leubner-Metzger 2006).

GA and ABA are two major hormones antagonistically controlling seed dormancy and germination (Gubler et al. 2005, Graeber et al. 2012, Shu et al. 2016). ABA accumulates abundantly and maintains at a high level in dormant seeds. However, ABA contents in seeds gradually decrease and drop to a low level during germination. Increased endogenous ABA level in the plants overexpressing *NCED6* (9-CIS-EPOXYCAROTENOID DIOXYGENASE 6), a gene encoding a rate-limiting enzyme in ABA biosynthesis, results in enhanced seed dormancy in Arabidopsis (Martínez-Andújar et al. 2011). Seeds of the *nced* mutants germinate faster than those of wild-type (Frey et al. 2012). ABA signaling plays a crucial role in controlling seed dormancy. The PP2C (PROTEIN PHOSPHATASE 2C), ABI1 (ABA INSENSITIVE 1) and ABI2 (ABA INSENSITIVE 2) are major repressors of ABA

signaling. ABI1 and ABI2 interact with and inactivate SnRK2 (SNF1-RELATED KINASE 2), a positive regulator of ABA response. In presence of ABA, the action of ABI1 and ABI2 is inhibited after binding to the ABA receptor PYR/PYL (PYRABACTIN RESISTANCE/PYRABACTIN-LIKE). The seeds of dominant-negative *abi1-1* and *abi2-1* mutants showed reduced dormancy, because their mutated ABI1 and ABI2 proteins failed to interact with PYR/PYL receptors (Park et al. 2009). In contrast to ABA, GA contents in seeds start to build up during imbibition and stratification (Rodríguez-Gacio et al. 2009, Weitbrecht et al. 2011, Arc et al. 2013). With GA level increased, dormancy breaks down and seed germination is initiated (Holdsworth et al. 2008). Two GA-deficient mutants, *ga1* and *ga2*, both have enhanced seed dormancy phenotype. Addition of exogenous GA is able to improve seed germination in *ga1* and *ga2* mutants (Lee et al. 2002). The DELLA proteins such as RGA (REPRESSOR OF *ga1-3*) and GAI (GIBBERELLIC ACID INSENSITIVE) are negative regulators in the GA signal transduction pathway. Mutations in other repressors of GA signaling such as *RGL2 (RGA-LIKE2)* and *SPY (SPINDLY)*, can rescue the non-germination phenotype of *ga1* (Jacobsen and Olszewski 1993, Lee et al. 2002).

A group of dormancy-specific genes have been identified in studies and their mutants have strong dormant phenotype. Amongst these genes the function of DOG1 (DELAY OF GERMINATION 1) is best characterized (Bentsink et al. 2006). DOG1 regulates seed dormancy via ABA-dependent and ABA-independent pathway (Nakabayashi et al. 2012, Graeber et al. 2014, Huo et al. 2016, Née et al. 2017b). The transcription of *DOG1* can be affected by HUB1/RDO4 (HISTONE MONOUBIQUITINATION1/REDUCED DORMANCY4) and RDO2 (REDUCED DORMANCY2), two positive players in the regulation of seed dormancy (Liu et al. 2007, 2011). DOG18/RDO5 (DELAY OF GERMINATION 18/REDUCED DORMANCY 5), a protein phosphatase 2C, positively regulates seed dormancy through suppressing expression of RNA binding proteins such as APUM (ARABIDOPSIS PUMILIO) (Xiang et al. 2014, 2016). In seed maturation ABI3, LEC1 (LEAFY COTYLEDON 1), LEC2 (LEAFY COTYLEDON 2) and FUS3 (FUSCA 3) are four central regulators of seed dormancy establishment (Stone et al. 2001, Chiu et al. 2012, Ding et al. 2014).

During seed development and maturation plants accumulate and store seed storage

proteins (SSPs), which will subsequently be mobilized for providing free amino acids and nutrients to seed germination and seedling growth. In Arabidopsis the major SSPs are comprised of 12S globulins and 2S albumins (Tan-Wilson and Wilson 2012). Most of identified proteolytic enzymes degrading SSPs in seed germination are cysteine proteases, others such as serine, aspartic and metalloproteases are also reported (Tan-Wilson and Wilson 2012). Plants often ensure early initiation of SSPs mobilization by depositing active proteases during seed maturation (Wang et al. 2014). Many studies have implied that aspartic proteases in dry seeds might be responsible for the first step of SSPs mobilization (Dunaevsky et al. 1989, Belozersky et al. 1989, Capocchi et al. 2000, Jones 2005).

Aspartic proteases (APs) are a group of proteolytic enzymes which use an activated water molecule bound to two aspartate residues for catalyzing their peptide substrates. APs contain two highly conserved aspartates in the active sites and are optimally active at acidic environment (Simões and Faro 2004). As widely studied in animals APs are found to control a wide range of biological functions and processes, including cell growth, cell death, protein turnover and immune defense (Brik and Wong 2003). The knowledge on the functions of APs in plants, however, remains limited. In our previous report we have shown that aspartic protease ASPG1 (ASPARTIC PROTEASE IN GUARD CELL 1, At3G18490) confers drought avoidance in Arabidopsis (Yao et al. 2012). Overexpressing *ASPG1* results in enhanced ABA sensitivity in guard cells and reduced water loss in *ASPG1*-overexpression (*ASPG1-OE*) transgenic plants (Yao et al. 2012). In this study, we further explored the function of ASPG1 in Arabidopsis development. Seed dormancy and seed germination were altered in loss-of-function mutant *aspg1-1*. When no exogenous nutrient was supplied, severely impaired seedling growth and delayed mobilization of SSPs was detected in *aspg1-1* mutants. We suggest that ASPG1 affects seed dormancy, seed longevity and seed germination in Arabidopsis.

RESULTS

ASPG1 is involved in seed dormancy of Arabidopsis

To in depth investigate the role of *ASPG1* in Arabidopsis, we generated RNA interference (RNAi) transgenic plants to knock down the expression of *ASPG1* in wild-type Col (Col-0)

background. The reduction of *ASPG1* expression was quantitatively analyzed. As the result showed that most of RNAi transgenic lines were of efficient silencing in *ASPG1* expression. Especially, the expression level of *ASPG1* in RNAi line #3 (*RNAi3*) and line #5 (*RNAi5*) were declined more than 94% with comparing to that in Col (Figure S1).

Because in our previous study no significant phenotypes were observed in analyzing the growing seedlings and adult plants of knockout *aspg1* mutants (Yao et al. 2012), we turned our attention to phenotypic analysis of the mutants during seed development and seed germination. Occasionally we found that *aspg1-1* seeds germinated much slower than Col seeds when their siliques naturally fell onto the surface of the soil. This incident prompted us to investigate the effect of ASPG1 on seed dormancy. We examined the germination phenotype of developing seeds which were still embedded in green siliques of *aspg1-1* and RNAi plants. The detached green siliques were sterilized and placed on the water-agarose plates, and then the germination rates were quantified at the 10th day of growth (Figure 1A). Over 50% germination rate was scored with Col or overexpressing *ASPG1* transgenic lines (*OE1* and *OE2*). However, significant reduction in germination rate was measured with *aspg1-1* mutants and RNAi lines (Figure 1B). To confirm the role of ASPG1 in seed germination we generated and analyzed the complementation lines (*Com*), the lines expressing *ASPG1* coding sequences controlled by its promoter in *aspg1-1* background. The retarded germination phenotype of *aspg1-1* seeds was rescued in the *Com1* and *Com2* lines (Figure 1A, B).

To monitor the state of dormancy in mature seeds of various genetic backgrounds, freshly harvested seeds from opened siliques were grown on the water-agarose plates for 5 days. Respectively, their germination rates were measured. As high as 90% of germination rate was scored with Col, *OE1*, *OE2*, *Com1* and *Com2*; less than 35% germination rate was measured with *aspg1-1*, *RNAi3* and *RNAi5* (Figure 1C, D). Based on these results we speculated that seeds of *aspg1-1* and the RNAi lines (*RNAi3* and *RNAi5)* might have deeper dormancy. Arabidopsis seed dormancy can be released after being stored at room temperature (22°C) for weeks (Graeber et al. 2012). We compared the germination phenotypes of dry stored seeds in different genetic backgrounds. Freshly harvested seeds of *aspg1-1* and RNAi lines could lose their dormancy by being stored in desiccated condition at room temperature (dry storage). Being stored dry for 2 days the germination of *aspg1-1* seeds was markedly improved and the

germination rate reached 52%. In contrast, the germination rate of freshly harvested (0 day of dry storage) seeds of *aspg1-1* was only 29%. When *aspg1-1* seeds were stored dry for 7 days nearly 90% of them were able to germinate (Figure 1D; Figure S2). Similar phenotype was observed with the germinations of RNAi lines. In addition to dry storage, exogenous GA application is also efficient in breaking seed dormancy (Peng et al. 1999, Rodríguez-Gacio et al. 2009). To test the effect of GA on seed germination of *aspg1-1* and RNAi lines we sowed seeds on water-agarose plates containing GA_3 (1.0 μM). At the day-5, over 90% germinations of *aspg1-1* mutants and RNAi lines was observed on the plates containing GA_3 (Figure 1C, E; Figure S2). Thus GA_3 was evidently helpful for overcoming deep dormancy in seeds of *aspg1-1* mutants and RNAi lines. Because the stratification can promote seed germination (Nordborg and Bergelson 1999, Shu et al. 2013), we analyzed the germination phenotype of freshly harvested seeds under the condition of stratification. All tested seeds were stratified in the darkness at 4°C for 2 days prior to incubating on the water-agarose plates. At the day-5 of incubation up to 72% germinations were observed with the freshly harvested and then stratified seeds of *aspg1-1* mutants. By contrast, the germination rate of not-stratified (Mock) *aspg1-1* seeds was as low as 29% (Figure 1C, F; Figure S2). Similar result was obtained in examining seed germination of RNAi lines. Taken together, dry storage, application of exogenous GA_3 and stratification, all are efficient treatments to defeat deep dormancy of freshly harvested seeds of *aspg1-1* mutants and RNAi lines.

Furthermore, we analyzed the germination phenotype of seeds which were dry stored for longer time (such as for 1-week, 2-week, and 6-month) under the condition without stratification (No stratification) or with stratification (Stratification) (Figure S3). Although the delay in seed germination was persisted in *aspg1-1* mutants and RNAi lines, the dry storage was obviously beneficial for recovering their seed germination (Figure S3).

ASPG1 promotes embryonic growth

Arabidopsis embryos are embedded in surrounding seed coat which consists of a single cell layer of endosperm and an outer layer of dead tissue, the testa. Embryos isolated from some dormant seeds of Arabidopsis mutants are actually not dormant (coat-enhanced dormancy), while in other species embryo itself is dormant (Bewley 1997, Piskurewicz et al.

2016). Thus we sought to find out whether the seed coat or an embryo itself is responsible for the altered seed dormancy in *aspg1-1* mutants. Seed coats of freshly harvested seeds from Col, *aspg1-1* and *OE2* were carefully removed and then the embryos were isolated. The dissected embryos were incubated on the water-agarose plates. The radicle length of each growing embryo was measured and analyzed statistically. Initially, there was no difference in the average radicle length of embryos among *aspg1-1*, Col and *OE2* (Figure 2A, B). After growing for 3 days the difference in radicle growth was observed. The average radicle length of Col embryos was 0.76 mm and the average radicle length of *aspg1-1* embryos was only 0.61 mm (Figure 2A, B). A seed coat bedding assay is useful to identify active components controlling germination from endosperm or embryo (Lee et al. 2010). To examine the influence of *aspg1-1* seed coat (testa and endosperm) on dormancy, we dissected embryos from freshly harvested seeds of Col, *OE2*, and *aspg1-1* and then placed them on a layer of *aspg1-1* seed coats. In contrast to in *aspg1-1* embryos, the faster radicle growth in Col and *OE2* embryos was scored (Figure S4). Thus we can concluded that the embryos instead of the seed coats may take main responsibility for the delayed radicle growth of *aspg1-1* seeds.

To verify the impact of aspartic proteases including ASPG1 to the growth of embryos, we carried out a pharmacologic assay using the pepstatin A, a potent inhibitor that specifically blocks aspartic protease activity (Kulkarni and Rao 2009, Yao et al. 2012). To exclude the interference of seed coats on limiting pepstatin A uptake we conducted this assay with the seed coats removed. Embryos isolated from Col seeds were sown on the water-agarose plates containing pepstatin A (0.2 μM) and their growth was completely blocked (Figure 2C). This result indicated that the role of the aspartic protease in facilitating the growth of dissected embryos should not be ignored.

Seed longevity was affected in *aspg1-1* mutants

Seed longevity and seed dormancy are two major characteristics determining seed quality. Seed longevity is defined as seed viability after dry storage. Arabidopsis seeds may lose their germination ability completely after a few years of dry storage (Rajjou and Debeaujon 2008, Nguyen et al. 2012). To explore the impact of ASPG1 on seed longevity, we compared the phenotype of seed ageing and seedling growth in Col, *aspg1-1*, and OE lines. To examine

natural seed ageing, all tested seeds were stored at 22°C in open and dry air for 6 months or 1 year or even longer, and then they were incubated on the water-agarose plates for 7 days. The phenotype of seed germination and abnormal seedlings was quantitatively analyzed. The 6-month stored seeds of Col, *aspg1-1* and OE were well germinated on the water-agarose plates (Figure 3A, B). Being stored for 1 year, nearly 100% of seeds of Col and *OE2* germinated. However, there were about 80% of *aspg1-1* seeds could germinate (Figure 3A, B). As the storage time extended to longer the viability of *aspg1-1* seeds was declined much faster than that of Col and *OE2* seeds (Figure 3A, B). Additionally, we scored higher rate of abnormal seedlings in *aspg1-1* than in Col and OE lines (Table S1). To confirm that *aspg1-1* mutants had a higher proportion of dead seeds, we further tested the viability of non-germinated seeds using the method of 2,3,5-triphenyltetrazolium chloride (TTC) staining. Seeds could not be stained when they were dead. We found that the majority of non-germinated seeds had lost their viability (Figure S5A; Table S1). The controlled deterioration test (CDT) system is able to accelerate seed ageing by increasing the temperature and relative humidity of seed storage environment (Tesnier et al. 2002, Nguyen et al. 2015). We then used the CDT system to analyze the germination phenotype of *aspg1-1*, *RNAi3* and *RNAi5* seeds. First, we treated seeds with 80% relative humidity at 40°C for weeks; next, the germination phenotype was monitored weekly. Being kept in the CDT system for 1 week the germination rates of *aspg1-1*, *RNAi3* and *RNAi5* seeds were dropped to 60% or even less; however, the germination rate of Col seeds was retained at 80% (Figure 3C; Figure S5B). As the time of CDT treatment extended, the germination rates in *aspg1-1* mutants and RNAi lines were distinctly declined. Being kept in the CDT system for 2 weeks seed germination of *aspg1-1* mutants and RNAi lines were nearly undetectable (Figure 3C; Figure S5). These results together demonstrated that seeds of *aspg1-1* mutants and RNAi lines were susceptible to artificial ageing treatment, suggesting the significance of ASPG1 for maintaining seed longevity.

ASPG1 is preferentially expressed in developing organs and germinating seeds

To better understand the function of ASPG1 we examined the expression profiles of *ASPG1* in developing organs and germinating seeds in the transgenic lines harboring the plasmid ASPG1pro-GUS. Previously we have reported the predominant guard cell expression

of ASPG1pro-GUS (Yao et al. 2012). However, we wanted to investigate more details about *ASPG1* expression property, especially in seed development and germination. Results showed that not only in consistent with our previous report (Yao et al. 2012) that GUS signal was observed in seedlings, but abundant GUS signal was also detected in the early-stage of floral buds and flowers (Figure 4A), developing siliques (Figure 4B), as well as germinating seeds (Figure 4C). Although we did not detect the GUS signal in mature embryos of ASPG1pro-GUS seeds, we observed it in the cotyledons of 24-hour imbibed seeds (Figure 4C). In addition, supply of exogenous GA_3 or treatment of stratification prior to imbibition could significantly induce expression of ASPG1pro-GUS in embryos (Figure 4C). Similar expression patterns of *ASPG1* in Col were consistently obtained using the quantitative analysis. Results demonstrated that *ASPG1* expression was detectable mainly in developing organs such as the inflorescences and siliques at the fifth day of pollination (Figure 4D). The expression level of *ASPG1* in germinating seeds was 5.0-fold higher than that in non-imbibed mature seeds (Figure 4D). In addition, the expression level of *ASPG1* in mature seeds which were stratified or treated with GA_3 (1.0 μM) was 3.0-fold higher than that in non-treated seeds (Figure 4D). These results were in fact consistent with the annotated public microarray data (The BAR, http://bar.utoronto.ca/; Winter et al. 2007, Bassel et al. 2008) (Figure S6). With analyzing the microarray data, we noticed that among aspartic proteases family members *ASPG1* is one of the major genes which are highly expressed during seed development and seed germination (Figure S7; Table S4). Thus the expression characteristic of *ASPG1* implies its functional specificity in seed development and germination.

Genes involved in the regulation of dormancy and GA signaling pathway were differentially modulated in *aspg1-1* seeds

To further explore the function of ASPG1 in seed dormancy and germination, we analyzed the impact of the mutation of *ASPG1* on expression of key regulators of dormancy and germination in dry and imbibed seeds. The results demonstrated that expression levels of a set of key positive regulators of seed dormancy, including *DOG1*, *DOG18*, *RDO2*, and *RDO4*, were obviously increased in *aspg1-1* dry seeds. In a similar fashion, expression of four key positive transcriptional regulators such as *LEC1*, *LEC2*, *FUS3*, and *ABI3* were all significantly

enhanced in *aspg1-1* dry seeds (Figure 5). DOG1 is a key regulator for seed dormancy in Arabidopsis and other species. Deep dormancy of Arabidopsis seeds is associated with high level of *DOG1* transcription (Huo et al. 2016). The expression level of *DOG1* in *aspg1-1* dry seeds was already 2.5-fold higher than that in dry seeds of Col and *OE2*. Strikingly, after imbibition the difference in *DOG1* expression level between in *aspg1-1* and Col reduced at various degrees (Figure 5). Similar results were scored in analyzing expression of other positive regulators of seed dormancy, such as *DOG18*, *RDO2*, *RDO4*, *ABI3*, *FUS3*, *LEC1* and *LEC2* (Figure 5). It is widely recognized that ABA and GA are primary hormones that antagonistically regulate seed dormancy and germination (Shu et al. 2016). Thus we compared expression of ABA- and GA-biosynthesis genes as well as their signaling components in *aspg1-1* and in Col seeds during imbibition. Two of ABA biosynthesis genes such as *ABA1* and *ABA2* showed similar expression level in seeds of *aspg1-1* and Col. The expression levels of another two ABA biosynthesis genes, *NCED6* and *NCED9*, were at least 6.0-fold higher in *aspg1-1* dry seeds than in Col dry seeds. However, the expression levels of *NCED6* and *NCED9* in imbibed seeds of *aspg1-1* were not much different from those in Col imbibed seeds (Figure 5). We measured the endogenous ABA contents in dry seeds of *aspg1-1* and Col, and we found that ABA contents in dry seeds of *aspg1-1* and Col had no significant difference (Figure S8). As for ABA signaling components we compared expression levels of *ABI4*, *ABI5*, *ABF3*, and *RD29B* genes in seeds of *aspg1-1* and Col; no obvious differences were detected (Figure 5). Additionally, we analyzed expression levels of GA biosynthesis genes such as *KAO1*, *KAO2*, *GA20ox1* and *GA3ox1* in seeds of Col, *aspg1-1* and *OE2*. No significant difference showed in expression of *KAO1* and *KAO2* genes between in Col and *aspg1-1*. However, clearly decreased expression levels of *GA20ox1* and *GA3ox1* were detected in *aspg1-1*. We also analyzed expression of GA signaling repressor genes including *GAI, RGA*, *RGL2*, and *RGL3* (encoding DELLA transcriptional regulators). Comparing to *OE2*, higher expression level of *GAI, RGA*, *RGL2*, and *RGL3* were detected in *aspg1-1* seeds (Figure 5). Considering DELLA proteins are important for repressing testa rupture during seed germination (Piskurewicz and Lopez-Molina, 2009), our data implicated that ASPG1 function might be executed through altering the transcription of a set of key regulators including GA signaling components in seeds.

***aspg1-1* seeds were sensitive to PAC treatment**

Because the transcription of GA signaling repressors and GA biosynthesis genes were differentially modulated in *aspg1-1* seeds during germination (Figure 5), we thus explored the germination phenotypes of *aspg1-1* mutants and RNAi lines under the conditions of GA_3 and PAC (paclobutrazol, a GA biosynthesis inhibitor) treatment. We observed that the germination of two-week-stored *aspg1-1* seeds was completely inhibited in the presence of PAC (5.0 μM), whereas 86% of seed germination was scored with Col at the day-7 of growth (Figure 6). We noticed that the inhibitory influence of PAC on germinations of *aspg1-1* and RNAi lines was in a dose-dependent manner (Figure S9). Compared with Col the influence of PAC on germinations of OE lines was evidently less severe (Figure 6; Figure S9). Interestingly, the addition of GA_3 (10.0 μM) to the PAC containing plates could successfully rescue the inhibited germination of *aspg1-1* and RNAi lines (Figure 6A, E).

ASPG1 promotes break down of seed storage protein

Degradation of seed storage proteins (SSPs) is a crucial physiological process during seed germination and early seedling establishment. It is believed that approximate 84% of total proteins in Arabidopsis seeds are present in the form of legumin type 12S globulin and napin type 2S albumin storage proteins (Gruis et al. 2002, Higashi et al. 2006). Some aspartic proteases are required for proteolytic processing or degrading SSPs (Otegui et al. 2006, Tamura et al. 2007). We sought to examine the involvement of ASPG1 in the degradation of SSPs during seed germination. Total proteins extracted from germinations of two-week-stored seeds were analyzed using one-dimensional SDS-PAGE. Profiles of seed proteins from Col, *aspg1-1* and *OE2* were presented in Figure 7. The contents of SSPs had no obvious difference between in Col and in *aspg1-1* seeds before imbibition (Figure 7; Figure S10). In general, SSPs start to be broken down to provide resources for synthesis of new proteins after imbibition (Tan-Wilson and Wilson 2012). In our analysis, we found that the amount of legumin-type SSPs in germinating seeds of *aspg1-1* was retained, comparing to those in germinating seeds of Col and *OE2*. The most obvious change, in terms of seed storage protein type in germinating *aspg1-1* seeds, were polypeptides with molecular masses at 25, 32, and

40 kD (Figure 7). Thus the degradation of legumin type 12S globulin protein in *aspg1-1* seeds was probably altered. We also found that the amount of new peptides with molecular masses at 78 kD in germinating seeds of *aspg1-1* was reduced. It suggested that the biosynthesis of new peptides in germinating seeds of *aspg1-1* might be affected.

ASPG1 is important for early seedling growth

As that mobilization and degradation of SSPs is vital for young seedling establishment and growth, we examined the growth phenotype of *aspg1-1* young seedlings when their own storage reserve were unique nutrient sources. Two-week-stored seeds were sown on water-agarose plates (without any supply of exogenous nutrients) and MS plates, respectively. Results showed that seedlings of *aspg1-1* and RNAi lines growing on MS plates were all normal (Figure 8). Seedlings of *aspg1-1* and RNAi lines growing on water-agarose plates for 1 week appeared normal (Figure 8A). However, when young seedlings of *aspg1-1*, *RNAi3*, and *RNAi5* were continuously growing on water-agarose plates for 3 weeks their leaves became to pale even to transparent; and their survival rates were significantly declined (Figure 8B). Therefore, we measured the total chlorophyll content in the seedlings and found that the chlorophyll content in seedlings of *aspg1-1*, *RNAi3*, and *RNAi5* was drastically decreased (Figure 8C). Overall, our results demonstrated that with consuming their own reserve, survival ability of *aspg1-1* mutants and RNAi lines were obviously reduced.

DISCUSSION

Seed development and seed germination are two crucial stages in the life cycle of plants. Seed dormancy is induced during seed development, and then is completely broken during seed germination. Lots of studies have sought to decipher the complex regulatory network that controls seed dormancy and germination. ABA and GA are two major plant hormones in establishing and releasing seed dormancy. During seed development and maturation ABA accumulates and induces seed dormancy. At the same time, seeds start to build up and store seed storage proteins (SSPs) and proteases which are required for seed germination and seedling growth (Tan-Wilson and Wilson 2012, Shu et al. 2016). However, our knowledge about the role of SSPs and proteases in mature seeds during the process of dormancy and

germination is limited.

ASPG1 plays negative role in seed dormancy but promotes seed germination

The ASPG1 expression pattern that was annotated from the public microarray data (Figure S6; Figure S7) and the ASPG1pro-GUS distribution characteristics (Figure 4) suggested the impact of ASPG1 on seed development, maturation and germination. To explore the function of ASPG1, we first analyzed the germination phenotype of the mutants and *OE* lines. We found that loss of ASPG1 function could increase the level of seed dormancy (Figure 1; Figure S2), and the deep dormancy phenotype of freshly harvested seeds from *aspg1-1* and RNAi plants could be rescued by the treatments such as dry storage, application of exogenous GA_3, and stratification (Figure 1; Figure S2). Although dry storage and stratification process could improve the germination of *aspg1-1* seeds on water-agarose plates, their germination always progressed slower than that of Col (Figure 1; Figure S3), thus the role of ASPG1 in seed germination was implicated. Seed dormancy can be imposed by the embryo, or the seed coat (endosperm and testa), or a combination of both factors to an extent (Debeaujon et al. 2000). Previous researches showed that ABA can be released from endosperm and in turn to inhibit seed germination (Lee et al. 2012, Lee and Lopez-Molina 2013). In our case, both endosperm and testa, isolated from freshly harvested *aspg1-1* seeds, had limited influence on embryonic growth (Figure 2; Figure S4). In fact, the delayed embryonic growth of *aspg1-1* mutants might be caused mainly by embryos themselves, not by the endosperm. It has been reported that the aspartic protease inhibitor VrAPI, purified from the seeds of *Vigna radiate*, can inhibit seed germination by controlling the activity of endogenous aspartic proteases (Kulkarni and Rao 2009). In consistent with their finding, we observed that the growth of Col embryos was completely inhibited when placed on the inhibitor pepstatin A containing plates (Figure 2). These results suggested the importance of activities of aspartic proteases including ASPG1 in seed germination. In addition, we found that both radicle extension and cotyledon greening were facilitated in the growth of dissected embryos of *OE* lines (Figure 2). Hence, the role of ASPG1 in promoting seed germination shouldn't be ignored.

Maintaining dormancy and longevity are two crucial traits for a seed. Seed dormancy and longevity have been thought to be positively correlated in plants (Nguyen et al. 2012, Sano et

al. 2016). This theory is mainly based on the performance of those mutants possessing low level of dormancy, such as *lec1*, *abi3*, *green seed* mutant (enhancer of *abi3-1*), *tt* (*TRANSPARENT TESTA*), *ats* (*ABERRANT TESTA*) and *dog1* (Debeaujon et al. 2000, Clerkx et al. 2003, Bentsink et al. 2006, Sugliani et al. 2009). All these mutants have reduced dormancy level that is associated with reduced seed longevity. However, a study discussing natural variations for seed longevity reveals a negative correlation between dormancy and longevity (Nguyen et al. 2012). The transgenic plants in Ler (Landsberg erecta) background carrying the *DOG1* allele of Cvi (Cape Verde Islands) exhibit increased seed dormancy and reduced seed longevity (Nguyen et al. 2012). Therefore, DOG1 may not only control seed dormancy but also influence seed longevity (Nguyen et al. 2012, Nguyen et al. 2015). In this study, we showed that loss of ASPG1 function enhanced seed dormancy and decreased seed viability (Figure 1; Figure 3). The reduction of seed longevity and increase in seed dormancy in *aspg1-1* mutants may be attributed to higher expression level of *DOG1* in *aspg1-1* dry seeds. Taken together, ASPG1 may play a negative role in the control of seed dormancy and act as a positive factor in seed longevity and germination.

ASPG1-modulated seed dormancy and germination is associated with GA signaling

Seed dormancy is induced during seed maturation. Mutants of seed maturation regulators such as ABI3, FUS3, LEC1 and LEC2 are of poor seed quality with declined dormancy level (Stone et al. 2001, Holdsworth et al. 2008, Chiu et al. 2012, Ding et al. 2014). In our analysis, we found that genes such as *ABI3*, *FUS3*, *LEC1* and *LEC2* were all highly expressed in *aspg1-1* dry seeds (Figure 5). Their higher expression levels were correlated to the delayed germination phenotype of *aspg1-1* mutants (Figure S3). In addition, the expression of *DOG1*, as well as other positive regulators of dormancy including *DOG18*, *RDO2* and *RDO4*, was significantly higher in *aspg1-1* dry seeds (Figure 5). DOG1 and DOG18 protein levels in freshly harvested seeds are associated with seed dormancy level (Nakabayashi et al. 2012, Xiang et al. 2014). Hence, higher level of *DOG18*, *RDO2* and *RDO4* expression in *aspg1-1* seeds may explain their enhanced dormancy phenotype. Numerous studies suggest that accumulation of aspartic proteases during seed development is significant for initiating seed germination (Elpidina et al. 1990, Marttila et al. 1995, Müntz et al. 2001). We speculate that disruption of

ASPG1 function alters expression of seed dormancy genes such as *DOG1*, *RDO2*, and *RDO4*; thus to produce deeper dormancy in *aspg1-1* seeds and in turn, to affect seed germination of *aspg1-1* mutants.

ABA is required to induce and maintain seed dormancy during seed maturation (Shu et al. 2016). Although the expression levels of ABA biosynthesis genes *NCED6* and *NECD9* were increased in *aspg1-1* dry seeds, no difference in expression of ABA responsive genes between in 2-week dry stored seeds of *aspg1-1* and Col were observed (Figure 5). We did not detect any obvious difference in endogenous ABA concentration between *aspg1-1* and Col dry seeds (Figure S8). RDO5 regulates seed dormancy through ABA-independent pathway (Xiang et al. 2014). The *reduced dormancy 5* (*rdo5*) has strong reduced seed dormancy phenotype. Mutation in RDO5 neither affects ABA level in dry and imbibed seeds nor ABA sensitivity during seed germination. It will be interesting to further investigate whether ASPG1 regulates seed dormancy through ABA-dependent pathway or not. In contrast to ABA, the GA signaling was severely impaired in *aspg1-1* during germination. DELLA proteins are negative regulators of GA responses (Yoshida et al. 2014). During seed germination expression levels of DELLA components were significantly increased in *aspg1-1* but slightly reduced in *OE2* (Figure 5). Among these DELLA components, the most significant change was observed in the expression of *RGL2*, a main negative regulator for seed germination (Cao et al. 2005, Yoshida et al. 2014). Hence, loss of ASPG1 function could produce repression in GA signaling during seed germination. Mutants containing higher level of active GA or robust GA signaling may have stronger resistance to PAC (Zhang et al. 2011, Shu et al. 2013). Indeed, we found that overexpression of *ASPG1* led to higher level of resistance to PAC, while *aspg1-1* and RNAi lines showed more sensitive to PAC (Figure 6; Figure S9). Furthermore, exogenous supply of GA_3 could recover the impaired germination phenotype of *aspg1-1* under PAC treatment (Figure 6). These results are supportive to the suggestion that ASPG1 plays an important role in seed dormancy and seed germination; the perturbed seed dormancy and seed germination in *aspg1-1* mutants is attributed to the repression of GA signaling.

The ubiquitin-proteasome system (UPS) plays a regulatory role in seed development and seed germination. Degradation of phytohormone specific transcription factors such as RGL1 and RGL2 by UPS is one of the mechanisms in facilitating seed germination (Wang and Deng

2011). Recently, the UPS-independent degradation system in the regulation of hormonal signaling has been reported. DAG9 (DEGRADATION OF PERIPLASMIC PROTEINS 9), a serine protease, modulates the interaction of cytokinin and light signal via mediating the degradation of ARR4 (ARABIDOPSIS RESPONSE REGULATOR 4) (Chi et al. 2016). Acting as an aspartic protease, ASPG1 might play its role in modulation of GA signaling, in which ASPG1 might execute its function through degradation of hormonal transcriptional regulators. Future study to confirm this hypothesis would enrich our understanding of the way how ASPG1 regulates hormonal signaling.

ASPG1 is involved in degradation of seed storage proteins during seed germination

During seed germination seed storage proteins (SSPs) are degraded by proteases, which convert the insoluble storage proteins into soluble peptides and free amino acids. The free amino acids are mobilized to the embryonic axis for supporting the growth of embryos and the initiation of seed germination (Shutov and Vaintraub 1987, Müntz et al. 2001). Numerous studies have proposed possible involvement of aspartic proteases in processing SSPs (Hondt et al. 1993, Runeberg-Roos et al. 1994, Gruis et al. 2002, Otegui et al. 2006). We have reported the aspartic protease activity of ASPG1 in our previous study (Yao et al. 2012). Here, we suggest that ASPG1 is involved in degradation of seed storage proteins (SSPs) during seed germination. The insignificant difference in the content of SSPs in *aspg1-1*, *OE2* and Col (Figure 7; Figure S10) indicates that loss of ASPG1 function may not be sufficient to affect accumulation of SSPs, and the functional redundancy among aspartic proteases in Arabidopsis may explain this phenomenon. Because *ASPG1* highly expressed in the earlier stage of seed development (Figure 4), it may play its role in the process of SSPs polypeptides precursors. However, little is known about the function of aspartic proteases on mobilization of SSPs during seed germination. Some aspartic proteases isolated and purified from dormant or germinating seeds of wheat (Belozersky et al. 1989, Capocchi et al. 2000, Tamura et al. 2007), rye (Brijs et al. 1999) can digest their respective SSPs *in vitro*. However, there is no genetic evidence in that aspartic proteases are directly involved in degradation of SSPs. In this study, we found that loss-of-function in ASPG1 could result in delayed degradation of SSPs during seed germination (Figure 7). And the impaired breakdown of SSPs in *aspg1-1* germinations

contributed to the reduced survival rate of its young seedlings grown in the condition without additional nutrient supplied (Figure 8). According to our data, we propose that ASPG1 plays a positive role in degradation of SSPs during seed germination. Searching for the putative targets of thioredoxin (TRX) in Arabidopsis the interaction between ASPG1 and AtTRX3 (THIOREDOXIN 3) has been detected in the proteomic analysis (Marchand et al. 2004, Darabi and Seddigh 2015). Studies have attested that TRX protein can act as a signaling component in the early stage of seed germination. TRX facilitates mobilization of reserves in seeds by reducing SSPs which enhances their solubility and susceptibility to proteolysis (Yano et al. 2001, Wong et al. 2004, Guo and Yin 2007, Shahpiri et al. 2008). In cells of germinating seeds, proteins such as TRX3 and some proteases are sorted and transported to the protein storage vacuoles (PSVs) after being synthesized in the endoplasmic reticulum (ER) (Onda et al. 2011, Gao et al. 2015). The ER localization of ASPG1 has been detected in mesophyll cells in a transient assay (Yao et al. 2012). To promote degradation of SSPs, ASPG1 may execute its function via association with TRX3 in PSVs of seed cells. Aspartic proteases are optimally active at acidic environment (Simões and Faro 2004). During seed germination the acidic condition in PSVs (He et al. 2007) may facilitate ASPG1 activity, promote degradation of SSPs and/or support other proteases such as aleurain (Gao et al. 2015) for breaking down SSPs.

Overall, our findings in this study indicate that ASPG1 is involved in the control of seed quality and probably facilitates seed germination through promoting the degradation of SSPs. Future studies to attest the interaction of ASPG1 and TRX3 and to address the involvement of ASPG1 in the degradation of SSPs will enrich our knowledge on the roles of aspartic proteases in seed germination.

MATERIAL AND METHOD

Plant materials and plasmid construction

All Arabidopsis plants used in this study were in the Columbia-0 (Col-0) background. The generation of transgenic plants overexpressing *ASPG1* (At3G18490) and the source of the mutant *aspg1-1* had been described previously (Yao et al. 2012). For generating the complementation lines, the plasmid pBI101-ASPG1pro-ASPG1 was constructed by cloning full length *ASPG1* genomic DNA (3444 bp) with its upstream promoter (1942 bp) into the pBI101

vector at SbfI/XmaI cloning sites. And then, the plasmid pBI101-ASPG1pro-ASPG1 was transformed into *aspg1-1* plants using the floral dipping method (Clough and Bent 1998). The RNAi lines were generated by following the method described by Wesley et al. (2001). Two inverted 550 bp fragments of *ASPG1* and an intron from pKANNIBAL vector (CSIRO Plant industry, Canberra, ACT, Australia) were cloned into the pBA002 binary vector (Kost et al. 1998) at the XhoI/SpeI cloning sites, therefore the plasmid pBA002-ASPG1-RNAi was constructed. Subsequently, this plasmid was transformed into Col-0 plants using the floral dipping method. All plants were grown under same conditions (16 h light/8 h dark at 22°C). Primers used for plasmid construction are listed in Supplementary Table S2.

Dormancy and germination assay

For seed dormancy analysis, developing siliques at the long-green stage (15 days after pollination) were collected from the consistent position of the inflorescence of each plant. Once the siliques turned brown and opened, mature seeds were harvested and used for analyses. Harvested seeds were stored in the open air with approximately 45% relative humidity at 22°C. Surface-sterilized seeds were sown on water-agarose plates which are made from ddH_2O plus 0.6% agarose at pH 5.7, and the seeds were treated with or without stratification at 4°C for 2 days (Barrero et al. 2010). Seeds were grown in a growth chamber under 16 h light/8 h dark at 22°C. Seeds with emerging radicles were defined as germination. GA_3 (G7645, Sigma-Aldrich, USA), PAC (paclobutrazol, P687, Phytotech, USA), or pepstatin A (P5318, Sigma-Aldrich, USA) were added to the water-agarose medium as that was needed for doing the treatment. To statistically analyze the phenotype of seed dormancy and seed germination, more than 100 seeds, which were harvested at the same time from 3 individual plants of each genotype, were used for each experiment. And all assays were performed more than three times.

RNA extraction and gene expression analysis

To analyze the expression level of genes, the method of quantitative reverse transcription PCR (qRT-PCR) was used. Total RNA was extracted from dry and imbibed seeds using RNAprep Pure Plant Kit (DP441, TIANGEN, China). The first-strand cDNA was synthesized by the Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen, USA). Then,

the cDNAs were amplified using Advanced SYBR Green supermix (Bio-Rad, USA) with CFX connect real-time PCR detection system 185-5201 (Bio-Rad, USA). The Arabidopsis *SECRETION-ASSOCIATED RAS SUPER FAMILY 1* (*SAR1*, At4g02080) was used as the internal control (Dekkers et al. 2012). The relative gene expression level was analyzed using the CFX manager software (Bio-Rad, USA). Three biological repeats were carried out for each experiment. Each experiment was performed at least three times with similar results and one representative result is shown. Primers used for qRT-PCR are listed in Supplementary Table S3.

Embryo growth and seed coat bedding assays

The method for the seed coat bedding assay was modified from the protocol described by Lee et al. (2013). Seed coats and embryos were isolated from freshly harvested seeds which had been imbibed for 2 hours. A fine syringe needles (0.45x28.5 mm, 5 mL, Hongda, China) were used for the dissection. In the process of the dissection seeds were all placed on a wet Whatman 3MM paper. Isolated coatless embryos were transferred to water-agarose plates or laid on a layer of seed coats on the surface of water-agarose plates. The embryos were incubated in a growth chamber under 16h light/8 h dark at 22°C. The images of embryo growth were acquired using the Nikon SMZ1500 stereomicroscope (Nikon, Japan). The length of emerged radicles was measured using the Image-Pro Plus software (http://www.mediacy.com/imageproplus, Media Cybernetics, USA). Seeds from three individual plants of each genotype were harvested at the same time. At least 60 embryos which were isolated from seeds of each genotype were used for each experiment. And this experiment was repeated for three times, in order to obtain statistical analytic data.

Histochemical GUS assay

The transgenic plants carrying plasmid ASPG1pro-GUS were generated as described previously (Yao et al. 2012). To determine the expression pattern of ASPG1pro-GUS, the embryos were dissected from two-week-stored seeds which were treated with or without stratification; afterwards they were transferred to water-agarose plates for 0 h or for 24 h. As for the GA treatment the GA_3 (1.0 μM) was added to the plates. The ethanol was added to the

plates as for the controls (Mock). The GUS assay was performed following the method described by Jefferson et al. (1987). The GUS assay solution was made with 50 mM sodium phosphate buffer (pH 7.2), 0.2% Triton X-100, 5 mM Ferrocyanide, 5 mM Ferricyanide, and 2 mM 5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronic acid. The images were acquired using the Nikon SMZ1500 stereomicroscope (Nikon, Japan).

Analysis for seed storage proteins

Total soluble proteins in dry and imbibed seeds were extracted following the protocol described by Chibani et al. (2006) with minor modification. Seeds (100 mg) were homogenized into power in liquid nitrogen. The powder was suspended in 1.2 mL of a thiourea/urea lysis buffer which composed 7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-propane- sulfonate), 18 mM Tris-HCl (Trizma HCl), 14 mM Trizma Base, 215 µL of protease inhibitor cocktail complete Mini (Roche Diagnostics) from one tablet dissolved in 1.5 mL of sterilized water, 53 units mL^{-1} DNase I, 4.9 K units mL^{-1} Rnase A, and 0.2% (v/v) Triton X-100. After incubating for 10 min at 4°C, protein extracts were centrifuged (15,000g for 10 min) at 4°C. The supernatants were subjected to a second centrifugation (15,000g for 10 min) and stored at -20°C. The concentration of purified proteins was measured using Bradford Protein Assay Kit (P0006C, Beyotime, China), and BSA (bovine serum albumin) was used as the standard. 30 µg protein of each sample was loaded for electrophoresis (140 V, 45 min) in the SDS-PAGE gel (4-20%, ExpressPlus) (Genscript, China). The gel was stained using Coomassie Brilliant Blue R250 (ST031, Beyotime, China) for visualization. The image was taken using Syngene Bio imaging system (InGenius, Syngene, UK).

Natural and artificial ageing assay

To evaluate seed longevity, we performed natural and artificial ageing assays. As for natural ageing assay, seeds harvested from 3 individual plants of each genotype were stored at room temperature for 0.5-, or 1.0-, or 2.0-, or 3.0-, or 4.0-year before they were tested for the viability in germination. As for artificial ageing assay, the controlled deterioration test (CDT) was

performed according to the protocol by Tesnier et al. (2002). Briefly, freshly harvested seeds were stored in the open air at room temperature for 2 weeks, and then they were transferred to an opened 1.5 mL Eppendorf tube and stored above on a saturated NaCl solution in a closed desiccator (80% relative humidity and temperature at 40°C). After treated for 0-, or 1-, or 2-, or 3-week the germination assay was performed. Seed longevity was determined on the basis of seed germination and seedling abnormality. Seedlings were judged as "abnormal" if they were showing malformation. Normally growing seedlings from germinations of the 0.5-year-old Col seeds were used as the control. The malformation phenotypes were categorized as such: no cotyledons, asymmetrical cotyledons, narrow vitrified cotyledons, chlorotic or albino cotyledons, no cauline apex, no root, and short or elongated hypocotyls. To examine the viability of non-germinated seeds in the ageing assay, the embryos of non-germinated seeds were isolated and incubated in 1% (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, T8877) at 30°C in the darkness for 2 days, according to the method described by Wharton (1955). Viable seeds can be stained red whereas dead seeds cannot be stained.

Measurements of ABA contents

Two-week-stored seeds (100 mg) of Col and *aspg1-1* were used for the experiment of measuring ABA contents. This experiment was carried out according to the method described in our previous report (Yao et al. 2012). Three biological replicates were conducted.

Seedling survival and growth under no nutrient supply

Two-week-stored seeds were grown on the water-agarose plates or on the Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 1% sucrose and 0.6% agarose. Survival rates were calculated after 3 weeks of incubation. We determined dying seedlings as their leaves were turned pale or transparent. To evaluate the rate of survival seedlings the chlorophyll contents were measured. All seedlings which were under survival rate analysis were collected and used for determination of chlorophyll contents. Total chlorophyll was extracted in 85% acetone as described by Porra et al. (1989). The chlorophyll content was determined at settings of 639 nm and 645 nm of the spectrophotometer (SpectraMax M2,

Molecular Devices, USA). All experiments were performed three times independently.

SUPPLEMENTARY DATA

The following supplementary materials are available online.

Supplementary Figure S1. The expression level of *ASPG1* in Col, *aspg1-1* mutants, complementation, overexpression and RNAi lines was quantitatively analyzed.

Supplementary Figure S2. Dry storage, GA_3 or stratification rescued the deep dormancy phenotype shown in *aspg1-1* and RNAi lines.

Supplementary Figure S3. Seed germination of *aspg1-1* and RNAi lines was delayed.

Supplementary Figure S4. Limited influence of *aspg1-1* seed coat on embryonic growth was observed.

Supplementary Figure S5. Seeds of *aspg1-1* lost their viability faster after natural ageing and were more sensitive to artificial ageing treatment.

Supplementary Figure S6. The expression pattern of *ASPG1* in various tissues was analyzed based on the data from the public microarray database.

Supplementary Figure S7. The relative expression levels of Arabidopsis aspartic protease genes in developing, dry and germinating seeds were annotated based on the data from the public microarray databases.

Supplementary Figure S8. The endogenous ABA contents in dry seeds were quantified statistically.

Supplementary Figure S9. The PAC treatment was effective on germinations of Col, *aspg1-1*, RNAi lines and OE lines.

Supplementary Figure S10. The storage protein contents in germinating seeds were evaluated.

Supplementary Table S1. The average percentages of germinated seeds, non-germinated seeds, dead seeds and abnormal seedlings in ageing assays.

Supplementary Table S2. Primer sequences used for plasmid constructions in this study.

Supplementary Table S3. Primer sequences used for qRT-PCR analysis in this study.

Supplementary Table S4. Relative expression levels of aspartic protease family members in developing, dry and germinating seeds of Arabidopsis.

ACKNOWLEDEMENTS

We thank the members of the Wu Lab for their technical help and comments on this manuscript. We thank the 'Large-scale Instrument and Equipment Sharing Foundation of Wuhan University' for supporting the use of the instruments in the College of Life Sciences in Wuhan University. This work was supported by grants to Y.W. from Nation Natural Science Foundation of China (31270333 and 90817013), the Major State Basic Research Program from the Ministry of Science and Technology of China (2013CB126900).

FIGURE LEGENDS

Figure 1. The dormancy in seeds of *aspg1-1* mutants and RNAi lines was enhanced.

(A-B) Seeds in the developing siliques of *aspg1-1* and RNAi lines had lower germination rates than those in Col. (A) Immature long-green siliques (15 days after pollination) of Col, *OE1*, *OE2*, *Com1*, *Com2*, *aspg1-1*, *RNAi3*, and *RNAi5* were grown on water-agarose plates for 10 days. Scale bar: 3 mm. (B) Quantitative analysis of germination rates of seeds in siliques. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01). (C-F) Mature seeds of *aspg1-1* showed deep dormancy, which could be rescued by GA_3 (1.0 μM) or stratification. (C) Freshly harvested seeds (0 day of dry storage) were germinating for 5 days on water-agarose plates containing 1.0 μM GA_3 or ethanol (Mock). Before incubation, seeds were treated with or without stratification. Scale bar: 2 mm. (D) Germination rates of after-ripening seeds were analyzed. Seeds which were stored at 22°C for indicated time (day) were grown on water-agarose plates for 5 days. (E and F) GA_3 or stratification treatment could rescue deep dormancy phenotype of *aspg1-1* mutants and RNAi lines. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment).

Figure 2. Retarded embryonic growth showed in *aspg1-1*.

(A-B) Embryonic growth of *aspg1-1* was delayed. Embryos were dissected from freshly harvested seeds of Col, *aspg1-1*, and *OE2* and grown on water-agarose plates. HAI: hour after incubation. Scale bar: 0.3 mm. (B) The length of growing radicles was measured. Data represent the mean ± SD of three biological replicates (n > 60 for each experiment). (C) The embryonic growth was inhibited by pepstatin A (0.2 μM). DMSO was added to the control

plates (Mock). DAI: day after incubation. Scale bar: 0.5 mm.

Figure 3. Seed longevity was affected in *aspg1-1*.

(A-B) Seed viability of *aspg1-1* lost much quicker than that of Col. Seeds stored at 22°C for indicated time (year) were germinated on water-agarose plates. (A) Photos were taken at the day-7 of growth. Scale bar: 0.5 cm. (B) Seed germination rate was quantified at the day-7 of growth. Data represent the mean ± SD of biological replicates (n > 100 for each experiment). (C) Seeds of *aspg1-1*, *RNAi3*, and *RNAi5* were more sensitive to artificial ageing treatment (controlled deterioration test, CDT). Seeds were stored in 80% relative humidity at 40°C for indicated time (week) and then, they were sown on water-agarose plates. The germination rates were measured at the day-7 of growth. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01).

Figure 4. The tissue-specific expression profile of ASPG1pro-GUS was analyzed.

(A) The expression of ASPG1pro-GUS was detectable in inflorescences (A1, scale bar: 1 mm); younger floral buds (A2, scale bar: 0.5 mm); flowers at stage 10, 12, and 14 (A3-A5, scale bar: 0.5 mm); anthers at stage 8 to stage 14 (A6-A12, scale bar: 50 μm). (B) Stronger ASPG1pro-GUS signal showed in younger siliques. DAP: day after pollination. (B1-B2, scale bars: 0.5mm; B3, 20μm). (C) Application of GA_3 or treatment of stratification could increase the expression level of ASPG1pro-GUS in 24-hour-imbibed embryos. C1: embryo before imbibition; C2: embryo imbibed for 24 hours on water-agarose plate; C3: embryo imbibed for 24 hours on water-agarose plate containing 1.0 μM GA_3; C4: embryo stratified for 2 days before imbibition. Scale bar: 20 μm. (D) The expression level of *ASPG1* in correspondent Arabidopsis tissues was quantitatively measured.

Figure 5. Expression levels of a set of genes in dry and imbibed seeds were analyzed.

The expression levels of dormancy-related key genes, GA biosynthesis genes and signaling components were altered in *aspg1-1* seeds. Two-week stored seeds which were imbibed for indicated hours were used in this experiment. *SAR1* expression level was used as the internal control. Data represent the mean ± SD of three biological replicates.

Figure 6. Phenotypes of seed germination were analyzed in tested lines under the treatments of PAC and GA_3.

(A) Two-week-stored seeds were sown on water-agarose plates containing PAC (5.0 μM) or

GA_3 (10.0 μM) or PAC (5.0 μM) + GA_3 (10.0 μM). The same volume of solvent (ethanol) was added to the control plates. And then the plates were placed in the growth chamber. The pictures were taken at the day-5 of germination. Scale bars: 0.5 mm. (B-E) The emerged radicles were quantified at the indicated time points (DAI: day after incubation). Data are means ± SDs of three biological replicates (n > 100 for each experiment).

Figure 7. The seed storage proteins were analyzed.

Total proteins were extracted from germinating seeds of Col, *aspg1-1* and *OE2*. Equal number of seeds were imbibed on water-agarose plates for 0 h, 24 h, and 48 h (HAI: hour after imbibition). Arrowheads indicate the differences shown in the SDS-PAGE gel.

Figure 8. Falling off growth ability showed in *aspg1-1* mutants and RNAi lines.

(A) The seedling survival ability was obviously declined in *aspg1-1* mutants and RNAi lines when they were growing on the water-agarose plates for 3 weeks. However, all tested seeds were growing similarly on MS plates. Photos were taken at the 1st week and the 3rd week of growth. Scale bars: 0.5 cm. (B) The survival rate was measured at the 3rd week of growth. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01). (C) The chlorophyll content was measured at the 3rd week of growth. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01).

Supplementary data figure legend

Figure S1. The expression level of *ASPG1* in Col, *aspg1-1* mutants, complementation, overexpression and RNAi lines was quantitatively analyzed.

The expression level of *ASPG1* in 7-day-old seedlings of Col, *aspg1-1*, RNAi (*RNAi1* to *RNAi8*), overexpression (*OE1*, *OE2*) and complementation lines (*Com1*, *Com3*) was quantified using the method of qRT-PCR. The expression level of *ACTIN2* (*At3G18780*) was used as the internal control. Data represent the mean ± SD of three biological replicates. This experiment was repeated three times.

Figure S2. Dry storage, GA_3 or stratification rescued the deep dormancy phenotype shown in *aspg1-1* and RNAi lines.

Freshly harvested seeds (0 DAH, day after harvest) and seeds were stored at 22°C for

indicated time (DAH) were grown for 5 days on the water-agarose plates containing GA_3 (1.0 μM) or on the water-agarose plates without GA_3 (Mock, Stratification). The stratification was performed in the darkness at 4°C for 2 days. 1-8: Col, *aspg1-1*, *RNAi3*, *RNAi5*, *OE2*, *OE1*, *Com2*, *Com1*. Scale bar: 1 cm.

Figure S3. Seed germination of *aspg1-1* and RNAi lines was delayed.

(A-F) Seed germination of Col, *aspg1-1*, *RNAi3*, *RNAi5*, *Com1*, *Com2*, *OE1* and *OE2* lines was assessed on the water-agarose plates without (A-C) or with (D-F) stratification treatment. Seeds which were dry stored for 1 week, 2 weeks, or 6 months were subjected to analysis. Germination rates were measured at indicated time (hour) after growing in a growth chamber. The stratification was performed in the darkness at 4°C for 2 days. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment). (G) The germination of *aspg1-1* and RNAi seeds which were not treated with stratification was delayed. Photos were taken at the 48-hour after incubation. Scale bar: 0.3 mm. (H) The germination of *aspg1-1* and RNAi seeds which were treated with stratification was delayed. Photos were taken at the 32-hour after incubation. Scale bar: 0.3 mm.

Figure S4. Limited influence of *aspg1-1* seed coat on embryonic growth was observed.

(A) Embryos dissected from Col, *aspg1-1*, *OE2* seeds were placed on a layer of *aspg1-1* seed coat bedding. HAI: hour after incubation. Scale bar: 0.3 mm. (B) The radicle length of embryos were measured. Data represent the mean ± SD of three biological replicates (n > 60 for each experiment).

Figure S5. Seeds of *aspg1-1* lost their viability faster after natural ageing and were more sensitive to artificial ageing treatment.

(A) Viability test for seeds after natural ageing with 2,3,5-triphenyltetrazolium chloride (TTC). After 7 days of germination, the embryos of non-germinated seeds were isolated and stained with TTC. 1: viable seeds (0.5-year-storage of Col embryos); 2: dead seeds (Col embryos which were killed by subjecting to an autoclave at 121°C for 20min; 3-11: embryos of Col, *aspg1-1*, *OE2* non-germinated seeds in natural ageing assay. Scale bar: 0.5 mm. (B) Before being sown on the water-agarose plates, seeds were stored in 80% relative humidity at 40°C for indicated time (week). Photos were taken at the day-7 after incubation. 1-8: Col, *aspg1-1*, *RNAi3*, *RNAi5*, *OE2*, *OE1*, *Com3*, *Com1*. Scale bar: 1 cm.

Figure S6. The expression pattern of *ASPG1* in various tissues was analyzed based on the data from the public microarray database.

Relative expression level of *ASPG1* in various tissues was annotated based on the data downloaded from the public microarray database (The BAR, http://bar.utoronto.ca/). h, hour; w/, with; w/o, without.

Figure S7. The relative expression levels of Arabidopsis aspartic protease genes in developing, dry and germinating seeds were annotated based on the data from the public microarray databases.

Expressions of 59 aspartic protease family members in Arabidopsis dry and germinating seeds have been predicted by MEROPS (https://www.ebi.ac.uk/merops/). Based on the public microarray data from The BAR (http://bar.utoronto.ca/), we annotated the relative expression levels of Arabidopsis aspartic protease genes in developing, dry and germinating seeds. More detailed information is listed in Supplementary Table S4. Developing seeds: seeds stage 10; Imbibed seeds: 12 hours imbibed seeds; Dry seeds: dry seeds before imbibition.

Figure S8. The endogenous ABA contents in dry seeds were quantified statistically.

Dry seeds (two-week-stored) of Col and *aspg1-1* were used for this experiment. Three biological replicates were performed (The P-value was calculated based on *t*-test, P>0.05). DW: dry weight.

Figure S9. The PAC treatment was effective on germinations of Col, *aspg1-1*, RNAi lines and OE lines.

(A) Two-week-stored seeds were germinated on the water-agarose plates containing different concentration of paclobutrazol (PAC). Photos were taken at the day-5 of germination. Scale bar: 0.5 mm. (B) The germination phenotype shown in the panel A was statistically quantified. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01).

Figure S10. The storage protein contents in germinating seeds were evaluated.

Total soluble proteins were extracted from imbibed seeds of Col, *aspg1-1*, and *OE2*. The storage protein contents were measured. Seeds of equal dry weight (DW) were used for this experiment. Data represent the mean ± SD of three biological replicates. HAI: hour after

imbibition.

LITERATURE CITED

Arc, E., Sechet, J., Corbineau, F., Rajjou, L. and Marion-Poll, A. (2013) ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination. *Front. Plant Sci*. 4: 63.

Barrero, J.M., Millar, A.A., Griffiths, J., Czechowski, T., Scheible, W.R., Udvardi, M. et al. (2010) Gene expression profiling identifies two regulatory genes controlling dormancy and ABA sensitivity in Arabidopsis seeds. *Plant J*. 61: 611-622.

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

Bassel, G.W., Fung, P., Chow, T.F., Foong, J.A., Provart, N.J. and Cutler, S.R. (2008) Elucidating the germination transcriptional program using small molecules. *Plant Physiol*. 147: 143.

Belozersky, M.A., Sarbakanova, S.T. and Dunaevsky, Y.E. (1989) Aspartic proteinase from wheat seeds: isolation, properties and action on gliadin. *Planta* 177: 321-326.

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. USA* 103: 17042-17047.

Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell* 9: 1055-1066.

Brijs, K., Bleukx, W. and Delcour, J.A. (1999) Proteolytic activities in dormant rye (*Secale cereale L*.) grain. *J. Agric. Food Chem*. 47: 3572-3578.

Brik, A. and Wong, C.H. (2003) HIV-1 protease: mechanism and drug discovery. *Org. Biomol. Chem.* 1: 5-14.

Cao, D., Hussain, A., Cheng, H. and Peng, J. (2005) Loss of function of four DELLA genes leads to light- and gibberellin-independent seed germination in Arabidopsis. *Planta* 223: 105-113.

Capocchi, A., Cinollo, M., Galleschi, L., Saviozzi, F., Calucci, L., Pinzino, C. et al. (2000) Degradation of gluten by proteases from dry and germinating wheat (*Triticum durum*) seeds: an *in vitro* approach to storage protein mobilization. *J. Agric. Food Chem*. 48: 6271-6279.

Chi, W., Li, J., He, B., Chai, X., Xu, X., Sun, X. et al. (2016) DEG9, a serine protease, modulates cytokinin and light signaling by regulating the level of ARABIDOPSIS RESPONSE REGULATOR 4. *Proc. Natl. Acad. Sci. USA* 113: E3568-E3576.

Chibani, K., Ali-Rachedi, S., Job, C., Job, D., Jullien, M. and Grappin, P. (2006) Proteomic analysis of seed dormancy in Arabidopsis. *Plant Physiol*. 142: 1493-1510.

Chiu, R.S., Nahal, H., Provart, N.J. and Gazzarrini S. (2012) The role of the Arabidopsis *FUSCA3* transcription factor during inhibition of seed germination at high temperature. *BMC Plant Biol*. 12: 15.

Clerkx, E.J., Vries, H.B., Ruys, G.J., Groot, S.P. and Koornneef, M. (2003) Characterization of green seed, an enhancer of *abi3-1* in Arabidopsis that affects seed longevity. *Plant Physiol.* 132: 1077-1084.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*. 16: 735-743.

Darabi, M. and Seddigh, S. (2015) Bioinformatic characterization of aspartic protease (AP)

enzyme in seed plants. *Plant Syst. Evol*. 301: 2399-2417.

Debeaujon, I., Léon-Kloosterziel, K.M. and Koornneef, M. (2000) Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. *Plant Physiol.* 122: 403-414.

Dekkers, B.J.W., Willems, L., Bassel, G.W., Bolderen-Veldkamp, R.P., Ligterink, W., Hilhorst, H.W.M. et al. (2012) Identification of reference genes for RT-qPCR expression analysis in Arabidopsis and Tomato seeds. *Plant Cell Physiol.* 53: 28-37.

Ding, Z.J., Yan, J.Y., Li, G.X., Wu, Z.C., Zhang, S.Q. and Zheng, S.J. (2014) WRKY41 controls Arabidopsis seed dormancy via direct regulation of *ABI3* transcript levels not downstream of ABA. *Plant J*. 79: 810-823.

Dunaevsky, Y.E., Sarbakanova, S.T. and Belozersky, M.A. (1989) Wheat seed carboxypeptidase and joint action on gliadin of proteases from dry and germinating seeds. *J. Exp. Bot*. 40: 1323-1329.

Elpidina, E.N., Dunaevsky, Y.E. and Belozersky M.A. (1990) Protein bodies from buckwheat seed cotyledons: isolation and characteristics. *J. Exp. Bot.* 41: 969-977.

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

Frey, A., Effroy, D., Lefebvre, V., Seo, M., Perreau, F., Berger, A. et al. (2012) Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. *Plant J*. 70: 501-512.

Gao, C., Zhuang, X., Cui, Y., Fu, X., He, Y., Zhao, Q. et al. (2015) Dual roles of an Arabidopsis ESCRT component FREE1 in regulating vacuolar protein transport and autophagic degradation. *Proc. Natl. Acad. Sci. USA* 112: 1886-1891.

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

Graeber, K., Linkies, A., Steinbrecher, T., Mummenhoff, K., Tarkowská D., Turečková, V. et al. (2014) *DELAY OF GERMINATION1* mediates a conserved coat-dormancy mechanism for the temperature-and gibberellin-dependent control of seed germination. *Proc. Natl. Acad. Sci. USA* 111: 3571-3580.

Gruis, D.F., Selinger, D.A., Curran, J.M. and Jung, R. (2002) Redundant proteolytic mechanisms process seed storage proteins in the absence of seed-type members of the vacuolar processing enzyme family of cysteine proteases. *Plant Cell* 14: 2863-2882.

Gubler, F., Millar, A.A. and Jacobsen, J.V. (2005) Dormancy release, ABA and pre-harvest sprouting. *Curr. Opin. Plant Biol*. 8: 183-187.

Guo, H.X. and Yin, J. (2007) Effects of antisense TRX s gene on hydrolysis of storage proteins in germination of transgenic wheat (*Tritirum aestivum L*.). *Acta. Agron. Sin.* 33: 885-890.

He, F., Huang, F. Wilson, K.A. and Tan-Wilson, A. (2007) Protein storage vacuole acidification as a control of storage protein mobilization in soybeans. *J. Exp. Bot.* 58: 1059-1070.

Han, C. and Yang, P. (2015) Studies on the molecular mechanisms of seed germination. *Proteomics* 15: 1671-1679.

Higashi, Y., Hirai, M.Y., Fujiwara, T., Naito, S., Noji, M. and Saito, K. (2006) Proteomic and transcriptomics analysis of Arabidopsis seeds: molecular evidence for successive processing of seed proteins and its implication in the stress response to sulfur nutrition.

Plant J. 48: 557-571.

Holdsworth, M.J., Bentsink, L. and Soppe, W.J. (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytol.* 179: 33-54.

Hondt, K.D., Bosch, D., van Damme, J., Goethals, M., Vandekerckhove, J. and Krebbers, E. (1993) An aspartic proteinase present in seeds cleaves Arabidopsis 2S albumin precursors *in vitro*. *J. Bio. Chem.* 268: 20884-20891.

Huo, H., Wei, S. and Bradford, K.J. (2016) *DELAY OF GERMINATION1* (*DOG1*) regulates both seed dormancy and flowering time through microRNA pathways. *Proc. Natl. Acad. Sci. USA* 113: 2199- 2206.

Jacobsen, S.E. and Olszewski, N.E. (1993) Mutations at the *SPINDLY* locus of Arabidopsis alter gibberellin signal transduction. *Plant Cell* 5: 887-896.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. 6: 3901-3907.

Jones, B.L. (2005) Endoproteases of barley and malt. *J. Cereal. Sci*. 42: 139-156.

Kang, J., Yim, S., Choi, H., Kim, A., Lee, K.P., Lopez-Molina, L. et al. (2015) Abscisic acid transporters cooperate to control seed germination. *Nat. Commun*. 6: 8113.

Kost, B., Spielhofer, P. and Chua, N.H. (1998) A GFP-mouse talin fusion protein labels plant actin filaments *in vivo* and visualizes the actin cytoskeleton in growing pollen tubes. *Plant J*. 16, 393-401.

Kulkarni, A. and Rao, M. (2009) Differential elicitation of an aspartic protease inhibitor: regulation of endogenous protease and initial events in germination in seeds of *Vigna*

radiata. *Peptides* 30: 2118-2126.

Lee, K.P., Piskurewicz, U., Turecková, V., Strnad, M. and Lopez-Molina, L. (2010) A seed coat bedding assay shows that RGL2-dependent release of abscisic acid by the endosperm controls embryo growth in Arabidopsis dormant seeds. *Proc. Natl. Acad. Sci. USA* 107: 19108-19113.

Lee, K.P., Piskurewicz, U., Turečková, V., Carat, S., Chappuis, R., Strnad, M. et al. (2012) Spatially and genetically distinct control of seed germination by phytochromes A and B. *Genes Dev*. 26: 1984-1996.

Lee, K.P. and Lopez-Molina, L. (2013) A seed coat bedding assay to genetically explore *in vitro* how the endosperm controls seed germination in *Arabidopsis thaliana*. *J. Vis. Exp*. 81: 50732.

Lee, S., Cheng, H., King, K.E., Wang, W., He, Y., Hussain, A. et al. (2002) Gibberellin regulates Arabidopsis seed germination via *RGL2*, a *GAI*/*RGA*-like gene whose expression is up-regulated following imbibition. *Genes Dev*. 16: 646-658.

Liu, Y., Koornneef, M. and Soppe, W.J. (2007) The absence of histone H2B monoubiquitination in the Arabidopsis *hub1* (*rdo4*) mutant reveals a role for chromatin remodeling in seed dormancy. *Plant Cell* 19: 433-444.

Liu, Y., Geyer, R., van Zanten, M., Carles, A., Li, Y., Hörold, A. et al. (2011) Identification of the Arabidopsis *REDUCED DORMANCY 2* gene uncovers a role for the polymerase associated factor 1 complex in seed dormancy. *PLoS One* 6: e22241.

Marchand, C., Le Maréchal, P., Meyer, Y., Miginiac-Maslow, M., Issakidis-Bourguet, E. and Decottignies, P. (2004) New targets of Arabidopsis thioredoxins revealed by proteomic

analysis. *Proteomics* 4: 2696-2706.

Martínez-Andújar, C., Ordiz, M.I., Huang, Z., Nonogaki, M., Beachy, R.N. and Nonogaki, H. (2011) Induction of 9-cis-epoxycarotenoid dioxygenase in *Arabidopsis thaliana* seeds enhances seed dormancy. *Proc. Natl. Acad. Sci. USA* 108: 17225-17229.

Marttila, S., Jones, B.L. and Mikkonen, A. (1995) Differential localization of two acid proteinases in germinating barley (*Hordeum vulgare*) seed. *Physiol. Plantarum* 93: 317-327.

Müntz, K., Belozersky, M.A., Dunaevsky, Y.E., Schlereth, A. and Tiedemann, J. (2001) Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. *J. Exp. Bot.* 52: 1741-1752.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with Tobacco tissue cultures. *Physiol. Plantarum* 15: 473-497.

Nakabayashi, K., Bartsch, M., Xiang, Y., Miatton, E., Pellengahr, S., Yano, R. et al. (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.

Née, G., Xiang, Y. and Soppe, W.J. (2017a) The release of dormancy, a wake-up call for seeds to germinate. *Curr. Opin. Plant Biol*. 35: 8-14.

Née, G., Kramer, K., Nakabayashi, K., Yuan, B., Xiang, Y., Miatton, E. et al. (2017b) DELAY OF GERMINATION1 requires PP2C phosphatases of the ABA signaling pathway to control seed dormancy. *Nat. Commun.* 8: 72.

Nguyen, T.P., Keizer, P., van Eeuwijk, F., Smeekens, S. and Bentsink, L. (2012) Natural variation for seed longevity and seed dormancy are negatively correlated in Arabidopsis.

Plant Physiol. 160: 2083-2092.

Nguyen, T.P., Cueff, G., Hegedus, D.D., Rajjou, L. and Bentsink, L. (2015) A role for seed storage proteins in Arabidopsis seed longevity. *J. Exp. Bot*. 66: 6399-6413.

Nordborg, M. and Bergelson, J. (1999) The effect of seed and rosette cold treatment on germination and flowering time in some *Arabidopsis thaliana* (Brassicaceae) ecotypes. *Am. J. Bot.* 86: 470-475.

Onda, Y. Nagamine, A., Sakurai, M., Kumamaru, T., Ogawa, M. and Kawagoe, Y. (2011) Distinct roles of protein disulfide isomerase and P5 sulfhydryl oxidoreductases in multiple pathways for oxidation of structurally diverse storage proteins in Rice. *Plant Cell* 23: 210-223.

Otegui, M.S., Herder, R., Schulze, J., Jung, R., and Staehelin, L.A. (2006) The proteolytic processing of seed storage proteins in Arabidopsis embryo cells starts in the multivesicular bodies. *Plant Cell* 18: 2567-2581.

Park, S.Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y. et al. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYP/PYL family of START proteins. *Science* 324: 1068-1071.

Peng, J., Richards, D.E., Moritz, T., Caño-Delgado, A. and Harberd, N.P. (1999) Extragenic suppressors of the Arabidopsis *gai* mutation alter the dose-response relationship of diverse gibberellin responses. *Plant Physiol.* 119: 1199-1207.

Piskurewicz, U. and Lopez-Molina, L. (2009) The GA-signaling repressor RGL3 represses testa rupture in response to changes in GA and ABA levels. *Plant Signal. Behav*. 4: 63-65.

Piskurewicz, U., Iwasaki, M., Susaki, D., Megies, C., Kinoshita, T. and Lopez-Molina, L.

(2016) Dormancy-specific imprinting underlies maternal inheritance of seed dormancy in *Arabidopsis thaliana*. *eLife* 5: e19573.

Porra, R.J., Thompson, W.A. and Kriedemann, P.E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta.* 975: 384-394.

Rajjou, L. and Debeaujon, I. (2008) Seed longevity: survival and maintenance of high germination of high germination ability of dry seeds. *C. R. Biol*. 331: 796-805.

Rodríguez-Gacio, M.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.

Runeberg-Roos, P., Kervinen, J., Kovaleva, V., Raikhel, N.V. and Gal, S. (1994) The aspartic proteinase of barley is a vacuolar enzyme that processes probarley lectin *in vitro*. *Plant Physiol.* 105: 321-329.

Sano, N., Rajjou, L., North, H.M., Debeaujon, I., Marion-Poll, A. and Seo, M. (2016) Staying alive: molecular aspects of seed longevity. *Plant Cell Physiol*. 57: 660-674.

Shahpiri, A., Sevensson, B. and Finnie, C. (2008) The NADPH-dependent thioredoxin reductase/thioredoxin system in germinating barley seeds: gene expression, protein profiles, and interactions between isoforms of thioredoxin *h* and thioredoxin reductase. *Plant Physiol.* 146: 789-799.

Shu, K., Zhang, H., Wang, S., Chen, M., Wu, Y., Tang, S. et al. (2013) ABI4 regulates primary seed dormancy by regulating the biogenesis of abscisic acid and gibberellins in Arabidopsis. *PLoS Genet*. 9: e1003577.

Shu, K., Liu, X.D., Xie, Q. and He, Z.H. (2016) Two faces of one seed: hormonal regulation of dormancy and germination. *Mol. Plant* 9: 34-45.

Shutov, A.D. and Vaintraub, I.A. (1987) Degradation of storage proteins in germinating seeds. *Phytochem*. 26: 1557-1566.

Simões, I. and Faro, C. (2004) Structure and function of plant aspartic proteinases. *Eur. J. Biochem.* 271: 2067-2075.

Stone, S.L., Kwong, L.W., Yee, K.M., Pelletier, J., Lepiniec, L., Fischer, R.L. et al. (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. *Proc. Natl. Acad. Sci. USA* 98: 11806-11811.

Sugliani, M., Rajjou, L., Clerkx, E.J.M., Koornneef, M. and Soppe, W.J.J. (2009) Natural modifiers of seed longevity in the Arabidopsis mutants *abscisic acid insensitive3-5 (abi3-5)* and *leafy cotyledon1-3 (lec1-3)*. *New Phytol*. 184: 898-908.

Tamura, T., Terauchi, K., Kiyosaki, T., Asakura, T. Funaki, J., Matsumoto, I. et al. (2007) Differential expression of wheat aspartic proteinases, WAP1 and WAP2, in germinating and maturing seeds. *J. Plant Physiol*. 164: 470-477.

Tan-Wilson, A.L. and Wilson, K.A. (2012) Mobilization of seed protein reserves. *Physiol. Plant* 145: 140-153.

Tesnier, K., Strookman-Donkers, H.M., van Pijlen, J.G., van der Geest, A.H.M., Bino, R.J. and Groot, S.P.C. (2002) A controlled deterioration test for *Arabidopsis thaliana* reveals genetic variation in seed quality. *Seed Sci. Technol.* 30: 149-165.

Wang, F. and Deng, X.W. (2011) Plant ubiquitin-proteasome pathway and its role in gibberellin signaling. *Cell Res.* 21: 1286-1294.

Wang, W.Q., Ye, J.Q., Rogowska-Wrzesinska, A., Wojdyla, K.I., Jensen, O.N., Møller, I.M. et al. (2014) Proteomic comparison between maturation drying and prematurely imposed drying of *Zea mays* seeds reveals a potential role of maturation drying in preparing proteins for seed germination, seedling vigor, and pathogen resistance. *J. Proteome Res*. 13: 606-626.

Weitbrecht, K., Müller, K. and Leubner-Metzger, G. (2011) First off the mark: early seed germination. *J. Exp. Bot*. 62: 3289-3309.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M. Rouse, D.T., Liu, Q. et al. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* 27: 581-590.

Wharton M.J. (1955) The use of tetrazolium test for determining the viability of seeds of the genus *Brassica*. *Proc. Int. Seed Test Assoc*. 20: 82-88.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V. and Provart, N.J. (2007) An “electronic fluorescent pictograph” browser for exploring and analyzing large-scale biological data sets. *PLoS ONE* 2: e718.

Wong, J.H., Cai, N., Balmer, Y., Tanaka, C.K., Vensel, W.H., Hurkman, W.J. et al. (2004) Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. *Phytochem*. 65: 1629-1640.

Xiang, Y., Nakabayashi, K., Ding, J., He, F., Bentsink, L. and Soppe, W.J.J. (2014) *REDUCED DORMANCY5* encodes a protein phosphatase 2C that is required for seed dormancy in Arabidopsis. *Plant Cell* 26: 4362-4375.

Xiang, Y., Song, B., Née, G., Kramer, K., Finkemeier, I. and Soppe, W.J.J. (2016)

Sequence polymorphisms at the *REDUCED DORMANCY5* pseudophosphatase underlie natural variation in Arabidopsis dormancy. *Plant Physiol*. 171: 2659-270.

Yano, H., Wong, J.H., Cho, M.J. and Buchanan, B.B. (2001) Redox changes accompanying the degradation of seed storage proteins in germinating rice. *Plant Cell Physiol.* 42: 879-883.

Yao, X., Xiong, W., Ye, T. and Wu, Y. (2012) Overexpression of the aspartic protease *ASPG1* gene confers drought avoidance in Arabidopsis. *J. Exp. Bot*. 63: 2579-2593.

Yoshida, H., Hirano, K., Sato, T., Mitsuda, N., Nomoto, M., Maeo, K. et al. (2014) DELLA protein functions as a transcriptional activator through the DNA binding of the INDETERMINATE DOMAIN family proteins. *Proc. Natl. Acad. Sci. USA* 111: 7861-7866.

Zhang, Z.L., Ogawa, M., Fleet, C.M., Zentella, R., Hu, J.H., Heo, J.O. et al. (2011) SCARECROW-LIKE 3 promotes gibberellin signaling by antagonizing master growth repressor DELLA in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 108: 2160-2165.

Figure 1

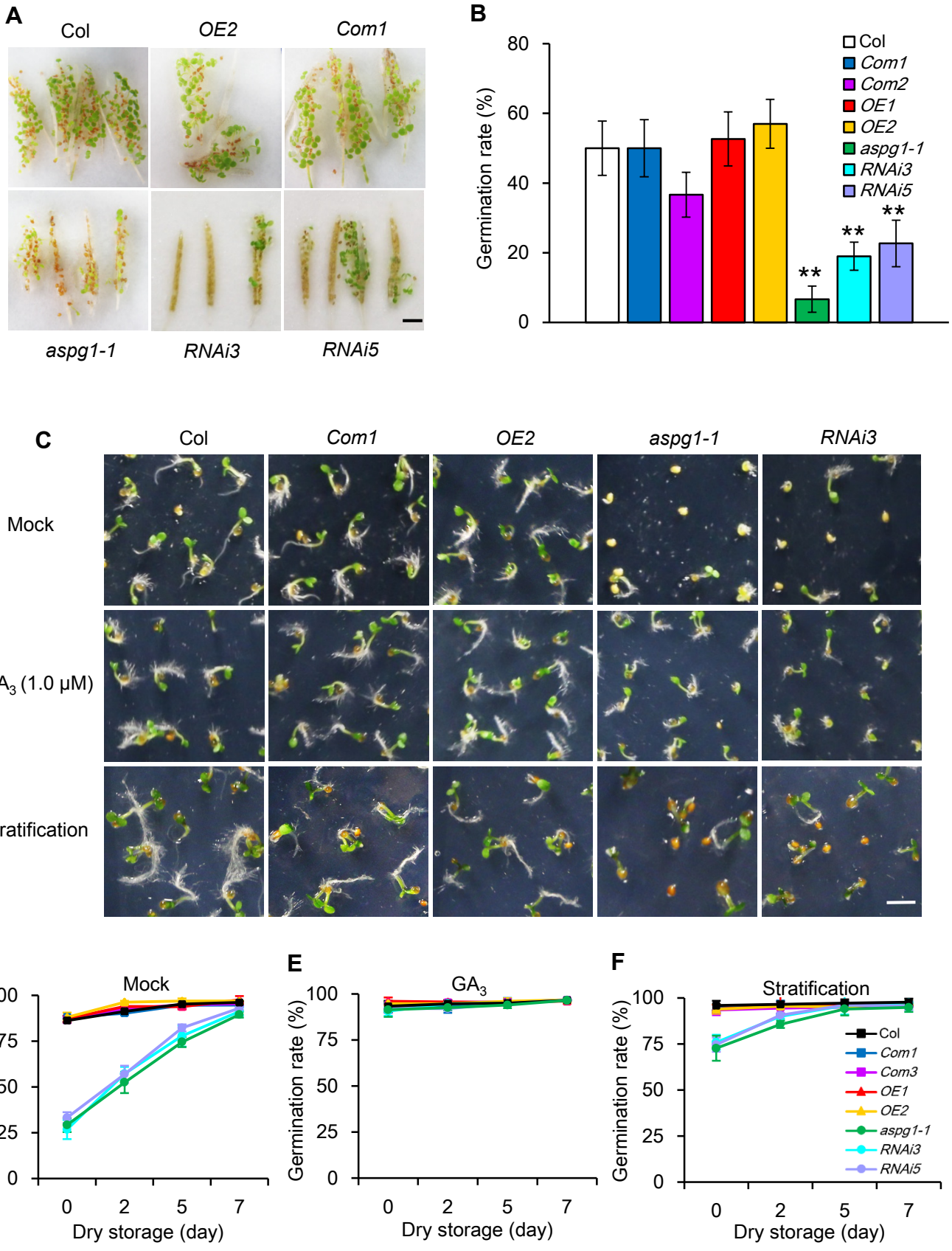


Figure 1. The dormancy in seeds of *aspg1-1* mutants and RNAi lines was enhanced.
(A-B) Seeds in the developing siliques of *aspg1-1* and RNAi lines had lower germination rates than those in Col. (A) Immature long-green siliques (15 days after pollination) of Col, *OE1*, *OE2*, *Com1*, *Com2*, *aspg1-1*, *RNAi3*, and *RNAi5* were grown on water-agarose plates for 10 days. Scale bar: 3 mm. (B) Quantitative analysis of germination rates of seeds in siliques. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01). (C-F) Mature seeds of *aspg1-1* showed deep dormancy, which could be rescued by GA_3 (1.0 μM) or stratification. (C) Freshly harvested seeds (0 day of dry storage) were germinating for 5 days on water-agarose plates containing 1.0 μM GA_3 or ethanol (Mock). Before incubation, seeds were treated with or without stratification. Scale bar: 2 mm. (D) Germination rates of after-ripening seeds were analyzed. Seeds which were stored at 22°C for indicated time (day) were grown on water-agarose plates for 5 days. (E and F) GA_3 or stratification treatment could rescue deep dormancy phenotype of *aspg1-1* mutants and RNAi lines. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment).

Figure 2

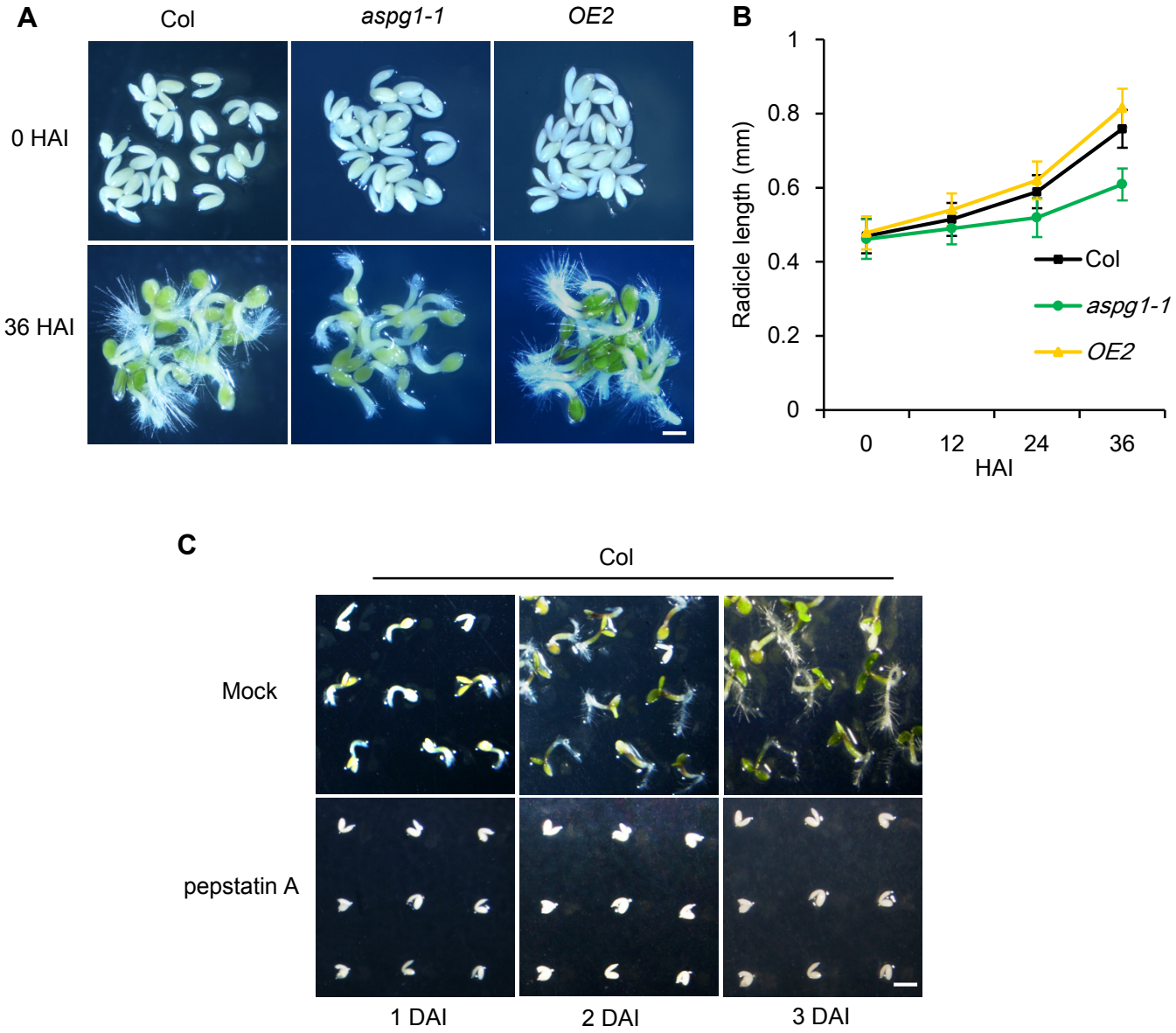


Figure 2**.** Retarded embryonic growth showed in *aspg1-1***.**

(A-B) Embryonic growth of *aspg1-1* was delayed. Embryos were dissected from freshly harvested seeds of Col, *aspg1-1*, and *OE2* and grown on water-agarose plates. HAI: hour after incubation. Scale bar: 0.3 mm. (B) The length of growing radicles was measured. Data represent the mean ± SD of three biological replicates (n > 60 for each experiment). (C) The embryonic growth was inhibited by pepstatin A (0.2 μM). DMSO was added to the control plates (Mock). DAI: day after incubation. Scale bar: 0.5 mm.

Figure 3

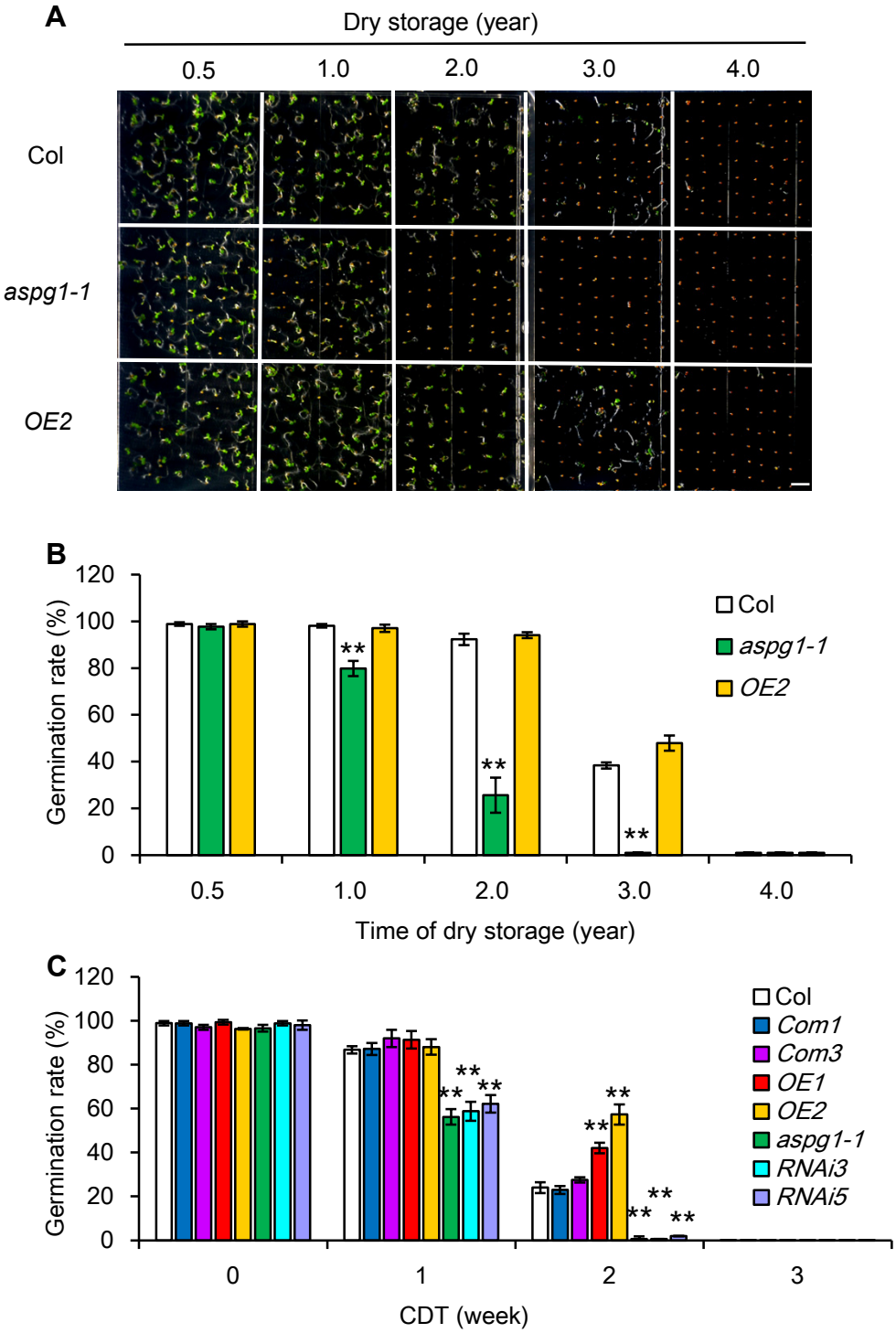
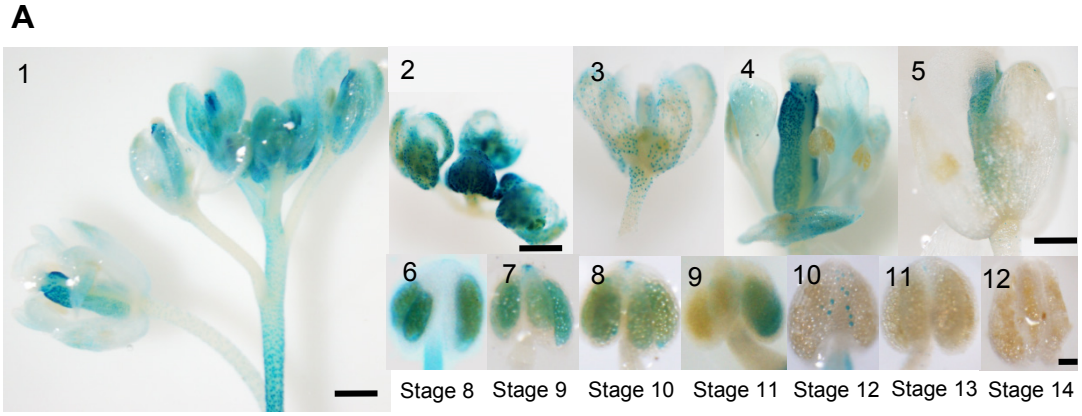
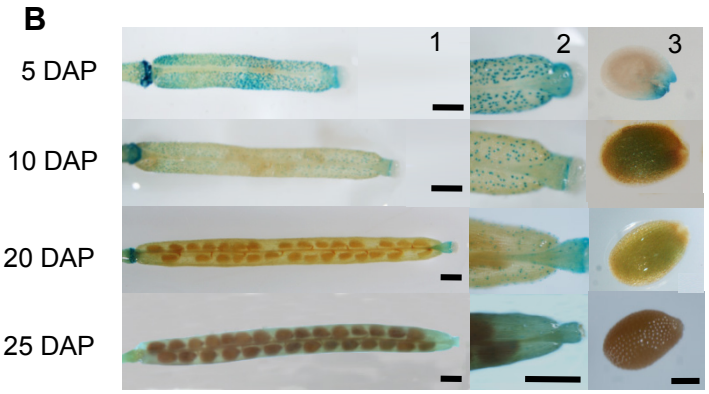


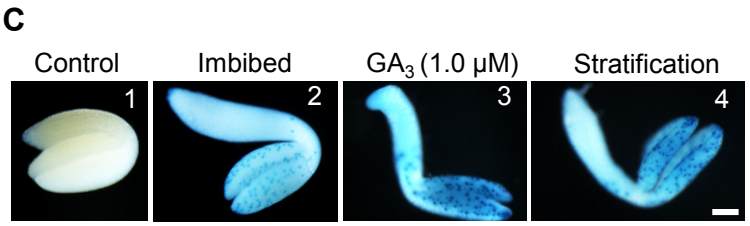
Figure 3. Seed longevity was affected in *aspg1-1*.

(A-B) Seed viability of *aspg1-1* lost much quicker than that of Col. Seeds stored at 22°C for indicated time (year) were germinated on water-agarose plates. (A) Photos were taken at the day-7 of growth. Scale bar: 0.5 cm. (B) Seed germination rate was quantified at the day-7 of growth. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment). (C) Seeds of *aspg1-1*, *RNAi3*, and *RNAi5* were more sensitive to artificial ageing treatment (controlled deterioration test, CDT). Seeds were stored in 80% relative humidity at 40°C for indicated time (week) and then, they were sown on water-agarose plates. The germination rates were measured at the day-7 of growth. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, ** P < 0.01).

Figure 4







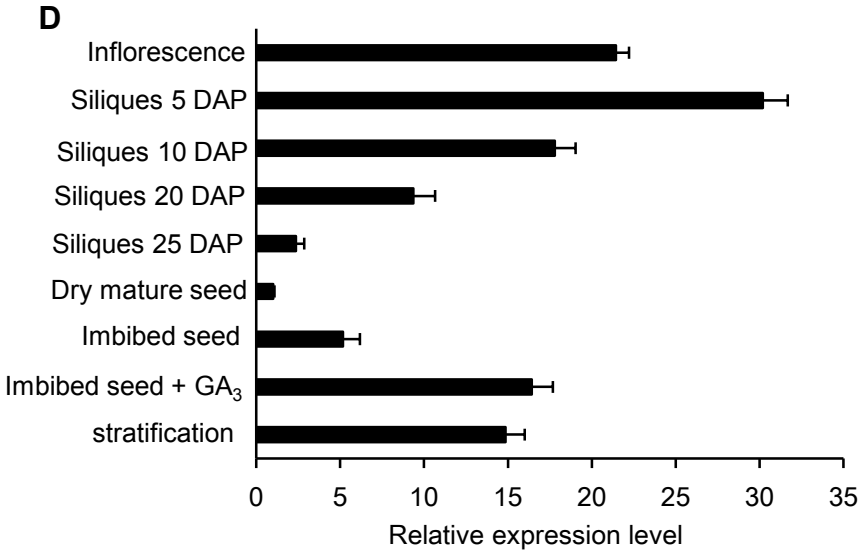


Figure 4. The tissue-specific expression profile of ASPG1pro-GUS was analyzed.
(A) The expression of ASPG1pro-GUS was detectable in inflorescences (A1, scale bar: 1 mm); younger floral buds (A2, scale bar: 0.5 mm); flowers at stage 10, 12, and 14 (A3-A5, scale bar: 0.5 mm); anthers at stage 8 to stage 14 (A6-A12, scale bar: 50 μm). (B) Stronger ASPG1pro-GUS signal showed in younger siliques. DAP: day after pollination. (B1-B2, scale bars: 0.5 mm; B3: 20 μm). (C) Application of GA_3 or treatment of stratification could increase the expression level of ASPG1pro-GUS in 24-hour-imbibed embryos. C1: embryo before imbibition; C2: embryo imbibed for 24 hours on water-agarose plate; C3: embryo imbibed for 24 hours on water-agarose plate containing 1.0 μM GA_3; C4: embryo stratified for 2 days before imbibition. Scale bar: 20 μm. (D) The expression level of ASPG1 in correspondent Arabidopsis tissues was quantitatively measured.

Figure 5

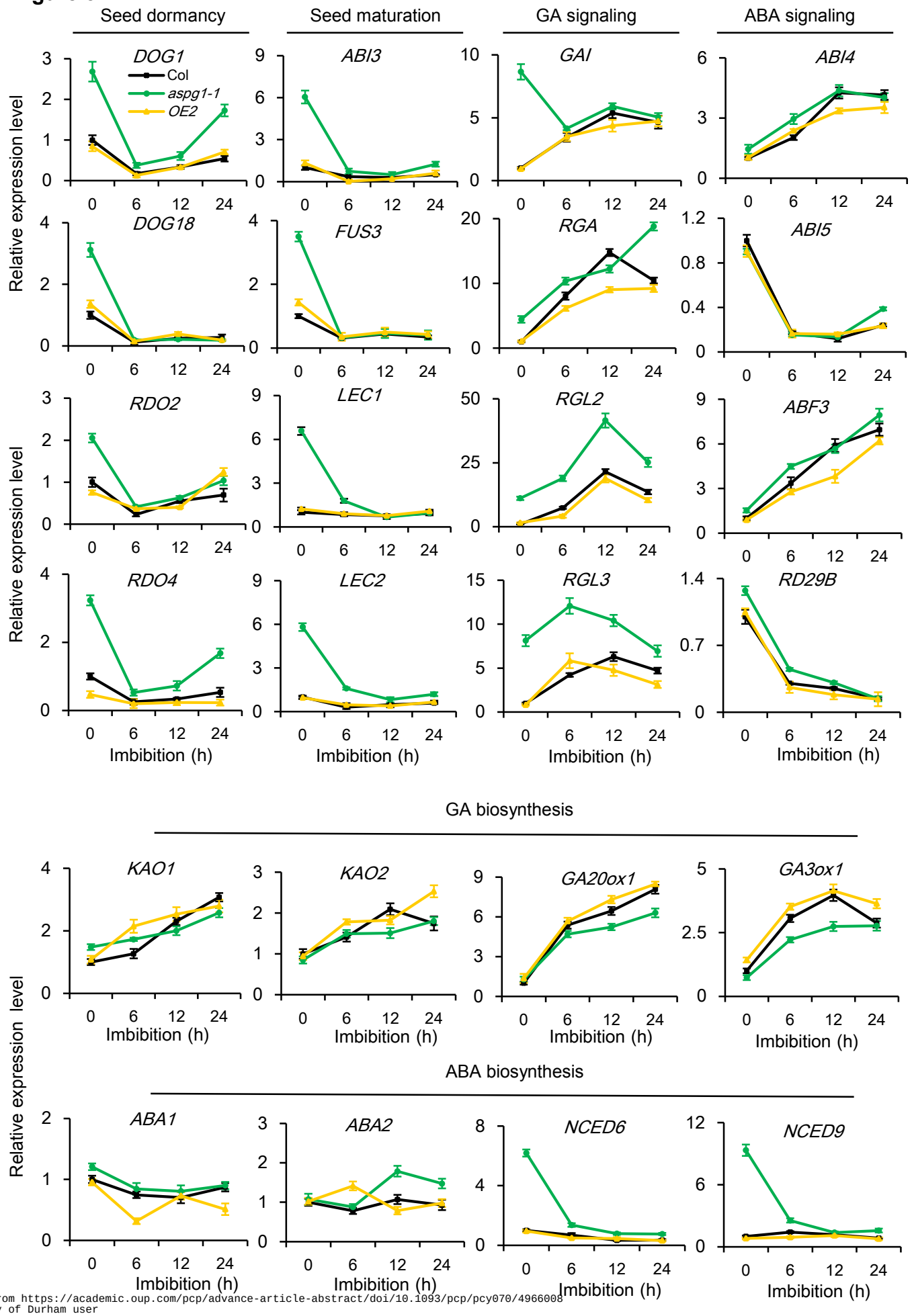


Figure 5. Expression levels of a set of genes in dry and imbibed seeds were analyzed.

The expression levels of dormancy-related key genes, GA biosynthesis genes and signaling components were altered in *aspg1-1* seeds. Two-week stored seeds which were imbibed for indicated hours were used in this experiment. *SAR1* expression level was used as the internal control. Data represent the mean ± SD of three biological replicates.

.

Figure 6

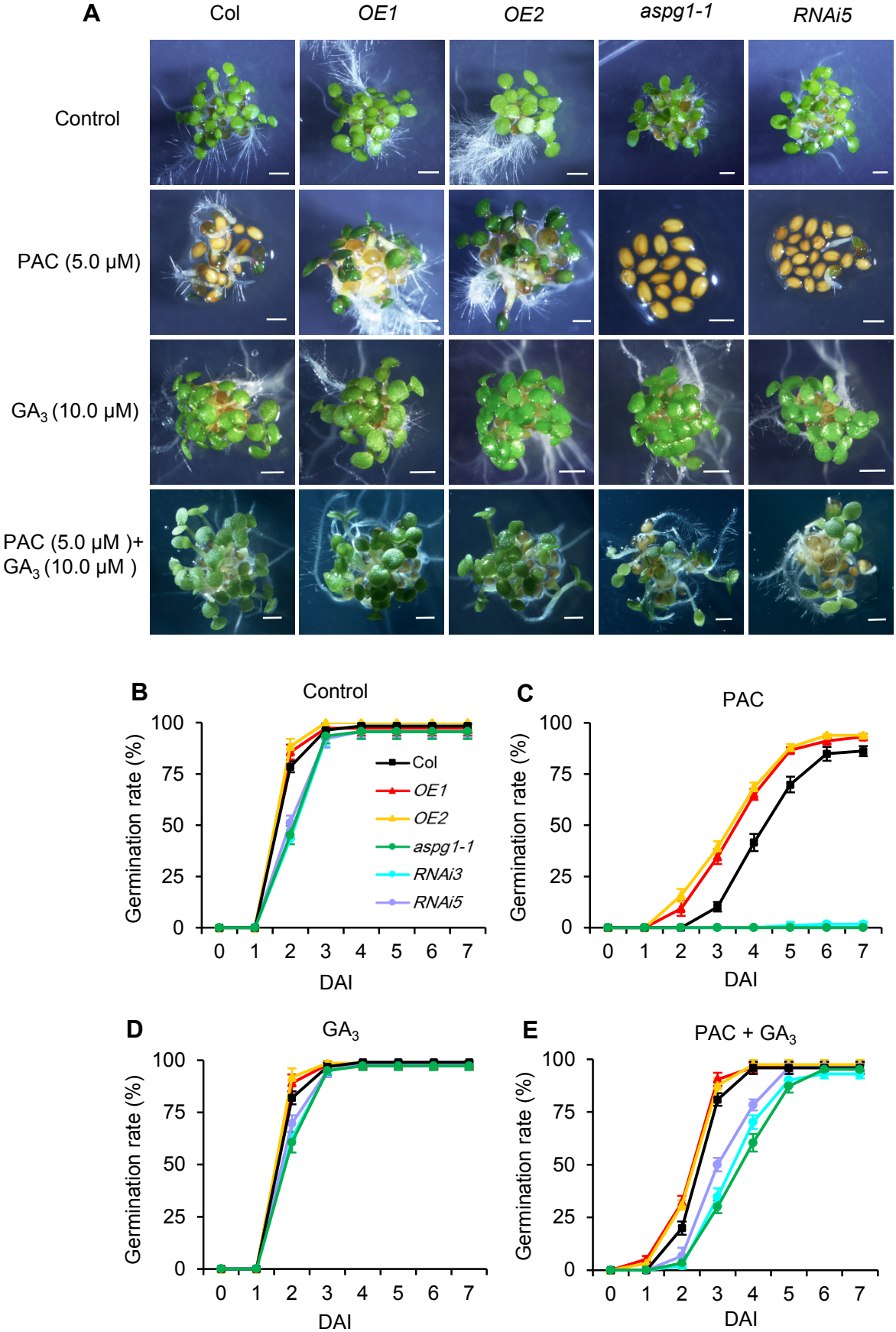


Figure 6. Phenotypes of seed germination were analyzed in tested lines under the treatments of PAC and GA_3.

(A) Two-week-stored seeds were sown on water-agarose plates containing PAC (5.0 μM) or GA_3 (10.0 μM) or PAC (5.0 μM) + GA_3 (10.0 μM). The same volume of solvent (ethanol) was added to the control plates. And then the plates were placed in the growth chamber. The pictures were taken at the day-5 of germination. Scale bars: 0.5 mm. (B-E) The emerged radicles were quantified at the indicated time points (DAI: day after incubation). Data are means ± SDs of three biological replicates (n > 100 for each experiment).

Figure 7

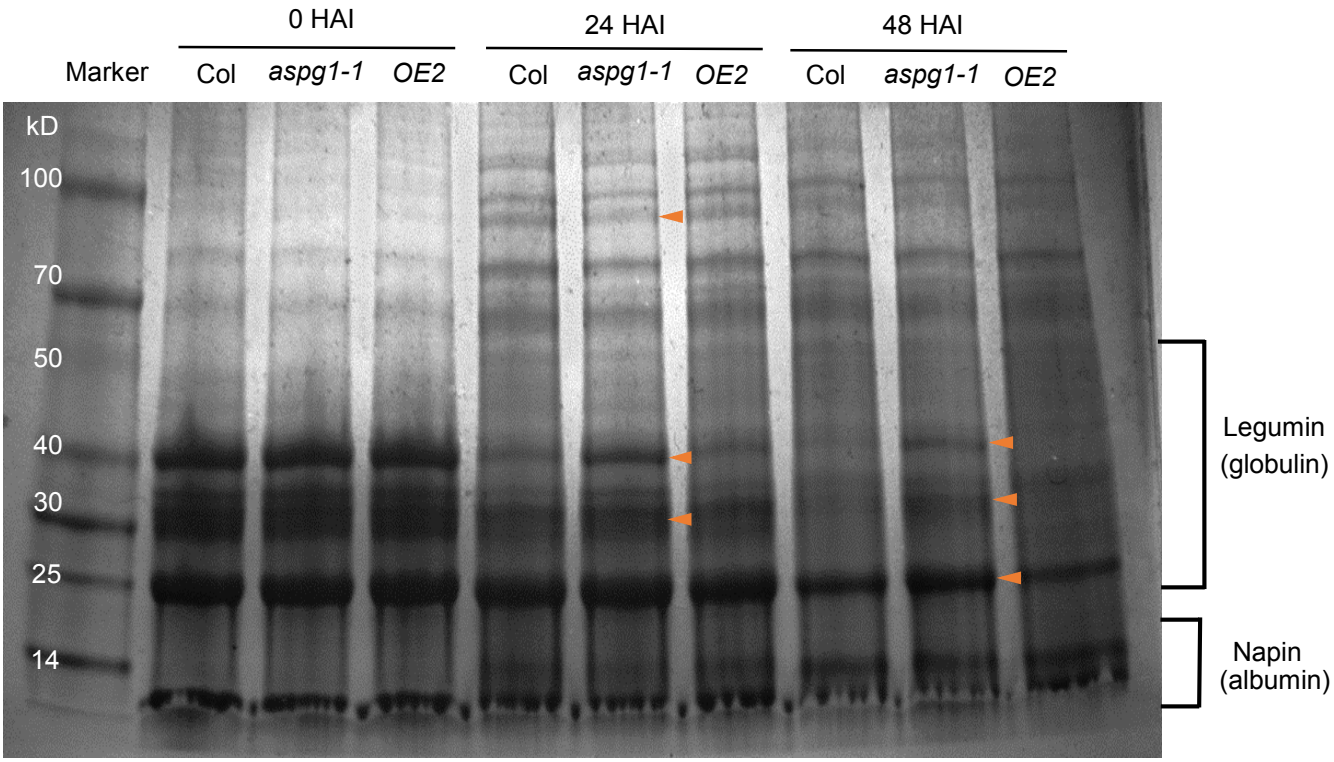


Figure 7. The seed storage proteins were analyzed.

Total proteins were extracted from germinating seeds of Col, *aspg1-1* and *OE2*. Equal number of seeds were imbibed on water-agarose plates for 0 h, 24 h, and 48 h (HAI: hours after imbibition). Arrowheads indicate the differences shown in the SDS-PAGE gel.

Figure 8

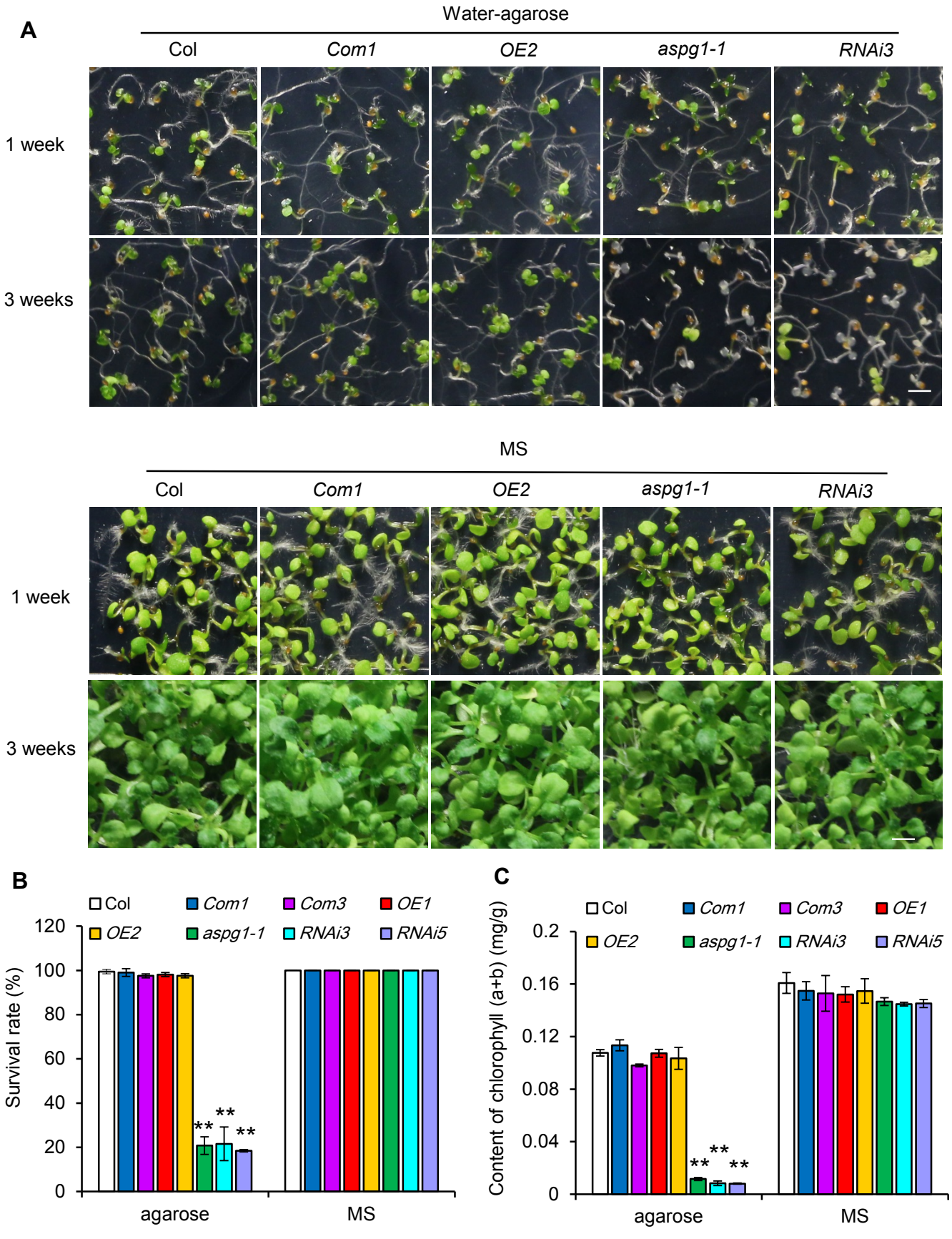


Figure 8. Falling off growth ability showed in *aspg1-1* mutants and RNAi lines.

(A) The seedling survival ability was obviously declined in *aspg1-1* mutants and RNAi lines when they were growing on the water-agarose plates for 3 weeks. However, all tested seeds were growing similarly on MS plates. Photos were taken at the 1st week and the 3rd week of growth. Scale bars: 0.5 cm. (B) The survival rate was measured at the 3rd week of growth. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01). (C) The chlorophyll content was measured at the 3rd week of growth. Data represent the mean ± SD of three biological replicates (n > 100 for each experiment, **P< 0.01).