Gibberellic Acid-Stimulated Arabidopsis6 Serves as an Integrator of Gibberellin, Abscisic Acid, and Glucose Signaling during Seed Germination in Arabidopsis[1][OPEN]

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The DELLA protein REPRESSOR OF ga1-3-LIKE2 (RGL2) plays an important role in seed germination under different conditions through a number of transcription factors. However, the functions of the structural genes associated with RGL2-regulated germination are less defined. Here, we report the role of an Arabidopsis (*Arabidopsis thaliana*) cell wall-localized protein, Gibberellic Acid-Stimulated Arabidopsis6 (AtGASA6), in functionally linking RGL2 and a cell wall loosening expansin protein (Arabidopsis expansin A1 [AtEXPA1]), resulting in the control of embryonic axis elongation and seed germination. *AtGASA6*-overexpressing seeds showed precocious germination, whereas transfer DNA and RNA interference mutant seeds displayed delayed seed germination under abscisic acid, paclobutrazol, and glucose (Glc) stress conditions. The differences in germination rates resulted from corresponding variation in cell elongation in the hypocotyl-radicle transition region of the embryonic axis. *AtGASA6* was down-regulated by *RGL2*, *GLUCOSE INSENSITIVE2*, and *ABSCISIC ACID-INSENSITIVE5* genes, and loss of *AtGASA6* expression in the *gasa6* mutant reversed the insensitivity shown by the *rgl2* mutant to paclobutrazol and the *gin2* mutant to Glc-induced stress, suggesting that it is involved in regulating both the gibberellin and Glc signaling pathways. Furthermore, it was found that the promotion of seed germination and length of embryonic axis by *AtGASA6* resulted from a promotion of cell elongation at the embryonic axis mediated by *AtEXPA1*. Taken together, the data indicate that *AtGASA6* links *RGL2* and *AtEXPA1* functions and plays a role as an integrator of gibberellin, abscisic acid, and Glc signaling, resulting in the regulation of seed germination through a promotion of cell elongation.

The transition from the dormant embryonic stage to seed germination is pivotal in the plant lifecycle (Bewley, 1997) and influenced by both environmental and intrinsic signals (Koornneef et al., 2002), including the two phytohormones, abscisic acid (ABA) and GA (Gubler et al., 2005). The roles of ABA in modulating seed development, dormancy, germination, and plant adaptation to abiotic environmental stresses, have been well studied (Gubler et al., 2005; Finkelstein et al., 2008), and it has been found that ABA specifically inhibits endosperm rupture rather than testa rupture in the seed germination process (Müller et al., 2006; Lee et al., 2010). ABA signaling components include *ABSCISIC ACID-INSENSITIVE3* (*ABI3*), which encodes a B3 domain transcription factor, and *ABI5*, encoding a basic Leu zipper transcription factor, both of which are necessary for growth arrest when germinating seeds encounter unfavorable conditions (Giraudat et al., 1992; Finkelstein and Lynch, 2000). *ABI5* functions downstream of *ABI3* and is essential for executing ABA-dependent growth arrest, which occurs after the breakage of seed dormancy but before autotrophic growth (Lopez-Molina et al., 2002). Such growth arrest occurs through the recruitment of de novo late embryogenesis programs and confers osmotic tolerance to harsh environmental conditions (Lopez-Molina et al., 2002).

GA promotes seed germination and growth, and its biosynthesis and responses are highly coordinated during seed germination (Ogawa et al., 2003). Complex regulatory events in the GA signaling pathway include cross talk with other hormones and the regulation of genes involved in promoting cell elongation and division (Ogawa et al., 2003). Accumulation of GA during seed germination is accompanied by a reduction in ABA levels, suggesting that GA and ABA play antagonistic roles in this process (Olszewski et al., 2002). GA signaling is known to be regulated by a group of

repressors collectively called DELLA proteins, including REPRESSOR OF ga1-3 (RGA), GA-INSENSITIVE (GAI), and REPRESSOR OF ga1-3-LIKE1/2/3 (RGL1/2/3; Tyler et al., 2004), of which RGL2 seems to be the major DELLA factor involved in repressing seed germination (Lee et al., 2002; Tyler et al., 2004; Cao et al., 2005). Recent studies have shown that RGL2 stimulates ABA biosynthesis by inducing the expression of *XERICO* and *ABI5*, whereas ABA enhances RGL2 expression (Ko et al., 2006; Zentella et al., 2007; Piskurewicz et al., 2008), indicating that RGL2 mediates the interaction of GA and ABA during seed germination.

During seed germination, sugar signaling also plays an important role by interacting with phytohormones, especially ABA (León and Sheen, 2003). Arabidopsis (*Arabidopsis thaliana*) *HEXOKINASE1* (*HXK1*) has been characterized as a Glc sensor (Jang et al., 1997; Moore et al., 2003). *HXK1*-dependent and -independent Glc signaling leads to increased ABA levels by inducing the expression of ABA synthesis genes (*ABA1/2/3*) and enhancing signaling through *ABI3* and *ABI5* to control seed germination (Ramon et al., 2008). Sugar signaling has also been reported to modulate the effects of other phytohormones, including cytokinin, ethylene, and auxin (Ramon et al., 2008). However, the interaction between GA and sugar signaling in the control of seed germination is not clear, and factors mediating the GA and sugar signaling interaction remain to be identified.

To date, in a wide range of plant species, the GA-Stimulated Arabidopsis (GASA) peptide family, which comprises Cys-rich peptides, has been identified as GA responsive (Roxrud et al., 2007), and tomato (*Lycopersicon esculentum*) *GA-stimulated transcript1* (*GAST1*) has been identified as the first *GASA* gene member (Shi et al., 1992). GASA proteins contain a putative signal peptide at the N terminus for targeting to the secretory pathway and a conserved C-terminal region of approximately 60 amino acids that contains 12 Cys residues at conserved sites. Fourteen *GASA* genes have been identified in Arabidopsis (Roxrud et al., 2007). Most of them are regulated by phytohormones, such as GA and ABA, and participate in hormone signaling pathways by mediating hormonal levels and responses (Zhang and Wang, 2008; Zhang et al., 2009; Rubinovich and Weiss, 2010; Sun et al., 2013). Thus far, GASA proteins have been shown to play important roles in various developmental programs, including seed germination (Rubinovich and Weiss, 2010), root formation (Taylor and Scheuring, 1994; Zimmermann et al., 2010), establishment of seed size (Roxrud et al., 2007), stem growth and flowering time (Ben-Nissan et al., 2004; Zhang et al., 2009), fruit development and ripening (de la Fuente et al., 2006; Moyano-Cañete et al., 2013), fiber development (Liu et al., 2013), and leaf expansion (Sun et al., 2013). Some GASAs have been reported to be involved in cellular processes, such as the promotion or inhibition of cell elongation and cell division; however, the detailed cellular regulation mechanisms through which *GASA*s operate have not been established.

In this study, we report the characterization of the Arabidopsis GASA protein AtGASA6 and describe both its involvement in hormonal cross talk during seed germination and the mechanism by which it affects cell elongation in germinating seeds.

RESULTS

AtGASA6 and Arabidopsis *Expansin A1* Are Up-Regulated in Imbibed *della* Mutant Seeds

Although GA and DELLA proteins are considered to play central regulatory roles in seed germination, the downstream structural components of this pathway, especially the putative genes affecting cell wall properties during cell elongation, have not been well clarified. Previous transcriptome data suggest that several *GASA* and *EXPANSIN* family genes, which encode putative cell wall localized proteins, likely function in GA-mediated seed germination (Gonzali et al., 2006; Stamm et al., 2012). To verify this, we evaluated their expressions in imbibed *della* mutant seeds and found that only *AtGASA6* (At1g74670) and Arabidopsis *expansin A1* (*AtEXPA1*; At1g69530) are up-regulated in *rga24-*, *gai-t6-*, *rgl2-1-*, and *gai-t6/rga-24*-imbibed seeds (Fig. 1). In addition, the GeneMANIA program predicted that the expression patterns of *AtGASA6* and *AtEXPA1* are similar to those of *RGL2* and that these three genes are coexpressed (Supplemental Fig. S1; Zuberi et al., 2013), implying that both *AtGASA6* and *AtEXPA1* may function with *RGL2*.

Reverse transcription (RT)-PCR was performed to determine the temporal and spatial expression patterns of *AtGASA6*, which is expressed in various organs, including roots, inflorescence stems, rosette and cauline leaves, and flowers as well as mature siliques (Supplemental Fig. S2A). To confirm these results, *pGASA6::GUS* plants (Columbia-0 [Col-0]) were generated and examined. During the seedling stage, GUS activity was observed in petioles (Supplemental Fig. S2Ba), the vascular tissue of rosette leaves (Supplemental Fig. S2Bb) and cotyledons (Supplemental Fig. S2Bc), the root meristem (Supplemental Fig. S2Bd), and emerging lateral roots (Supplemental Fig. S2Be). During the reproductive stage, strong GUS activity was observed in ovules and anthers (Supplemental Fig. S2Bf). Interestingly, GUS activity was observed in developing and immature seeds at stages 12 (Supplemental Fig. S2Bg), 13 (Supplemental Fig. S2Bh), 15 (Supplemental Fig. S2Bi), and 16 (Supplemental Fig. S2Bj) but not in mature seeds at stage 17 (Supplemental Fig. S2Bk). GUS staining was apparently specific in the hypocotyl-radicle transition zone of the embryonic axis in germinated seeds (Supplemental Fig. S2Bl), although no staining was observed in the endosperm layer and testa (Supplemental Fig. S2Bm). Thus, the GUS expression patterns were in close agreement with the RT-PCR analyses (Supplemental Fig. S2A). Importantly, *AtGASA6* was found to be highly expressed in developing seeds
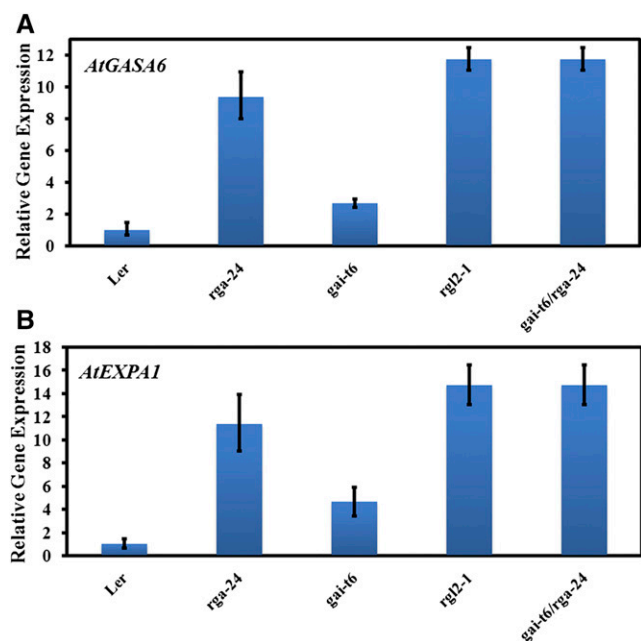
Figure 1. DELLA proteins inhibit *AtGASA6* and *AtEXPA1* expression in germinating seeds. Relative transcript levels of *AtGASA6* (A) and *AtEXPA1* (B) in germinating wild-type (Col-0) and *della* mutant seeds (*rga-24*, *gai-t6*, *rgl2-1*, and *gai-t6/rga-24*). All seeds were collected at 10 h after seeds were transferred into light at 22°C from dark at 4°C. The transcript levels were normalized to *UBQ1* gene expression. Results are averages ± SE (*n* = 3). All experiments were repeated at least three times with similar results.

and the embryonic axis of imbibed seeds, suggesting that it may play roles in seed germination.

AtGASA6 Is a Positive Regulator of Seed Germination under Stress Conditions

To elucidate the function of the *AtGASA6* gene, a transfer DNA (T-DNA) insertion line in the third exon (Fig. 2A), *gasa6-1*, was identified from the Arabidopsis Biological Resource Center. RT-PCR showed that *gasa6-1* is an *AtGASA6* knockdown mutant (Fig. 2B), and phenotypic analysis revealed that germination was slightly delayed in the *gasa6-1* mutant under normal conditions but significantly delayed as a consequence of exposure to high concentration of Glc, ABA, or the GA antagonist paclobutrazol (PAC; Fig. 2D). The difference in germination status between *gasa6-1* mutant and wild-type seeds under various conditions was also documented (Fig. 2E).

To determine the function of the *AtGASA6* gene during seed germination, *AtGASA6-overexpressing* (OE) and RNA interference (RNAi) suppressed transgenic Arabidopsis lines were generated for further analysis. RT-PCR analysis confirmed that *AtGASA6* expression levels were increased or decreased according to the particular transgenic line (Fig. 2C). Additionally, the expression levels of the closest homologous genes of

AtGASA6, which are classified to the same subfamily (Zhang and Wang, 2008), in *AtGASA6-RNAi* lines were not affected significantly by RNAi (Supplemental Fig. S3). Finally, the *AtGASA6-OE* lines *OE#2-1*, *OE#5-5*, and *OE#6-1* and *AtGASA6-RNAi* lines *RNAi (R)#3-5*, *R#4-3*, and *R#6-7* were chosen for subsequent experiments. Under normal conditions, although the seed germination rate of the *AtGASA6-OE* lines seemed somewhat faster than that of the wild type and the germination rate of the *AtGASA6-RNAi* seeds was slightly slower, the differences were not statistically significant (Fig. 2E). However, the seed germination rates were clearly different when the various *AtGASA6* genotypes were challenged using specific stress conditions. Compared with wild-type seeds, *AtGASA6-OE* seeds germinated faster, and the RNAi seeds germinated significantly more slowly under 6% (w/v) Glc, 0.5 μM PAC, or 1 μM ABA conditions (Fig. 2E). To assess whether the germination difference was caused by osmotic stress, seed germination rates were tested using 6% (w/v) mannitol, and it was found that the OE and RNAi lines did not show significant difference compared with the wild type (Fig. 2E). The *AtGASA6-RNAi* seeds began to germinate after 46 h on 6% (w/v) Glc and 36 h on 0.5 μM PAC or 1 μM ABA plates, whereas the percentage of germinated *AtGASA6-OE* seeds had reached 20% at these time points (Fig. 2E). As a result of these treatments, seed germination rate differences between the three *AtGASA6* genotypes remained significant until the end of the germination process, indicating that *AtGASA6* is associated with Glc, GA, and ABA signaling pathway-mediated seed germination.

AtGASA6 Expression Is Regulated by Glc, ABA, and GA

It is well documented that GA and ABA act antagonistically in the regulation of seed germination and that ABA and GA levels change substantially during seed imbibition (Karssen et al., 1983; Yamauchi et al., 2004; Okamoto et al., 2006). Because the seed germination rates of *AtGASA6-OE* and RNAi lines were affected by Glc, ABA, or PAC, we hypothesized that *AtGASA6* might be up-regulated by GA and down-regulated by ABA and Glc during seed germination. To test this, wild-type (Col-0) seeds were planted on minimal medium (Murashige and Skoog medium [MS] without Suc) with 10 μM ABA, 10 μM PAC, 6% (w/v) Glc, or 10 μM GA$_3$ after 10 h of exposure to light. Gene expression analyses showed that *AtGASA6* transcripts were up-regulated almost 5-fold after 6 h of GA treatment, whereas a very low level of transcript was detected after ABA, PAC, or Glc treatment for 6 h compared with the mock treatment (Fig. 3A), indicating that *AtGASA6* is indeed down-regulated by ABA and Glc and up-regulated by GA in germinating seeds. To determine if the effects of Glc, ABA, and GA on *AtGASA6* expression were developmentally regulated, expression was measured in 14-d-old wild-type seedlings treated with 6% (w/v) Glc, 10 μM ABA, or 10 μM GA$_3$
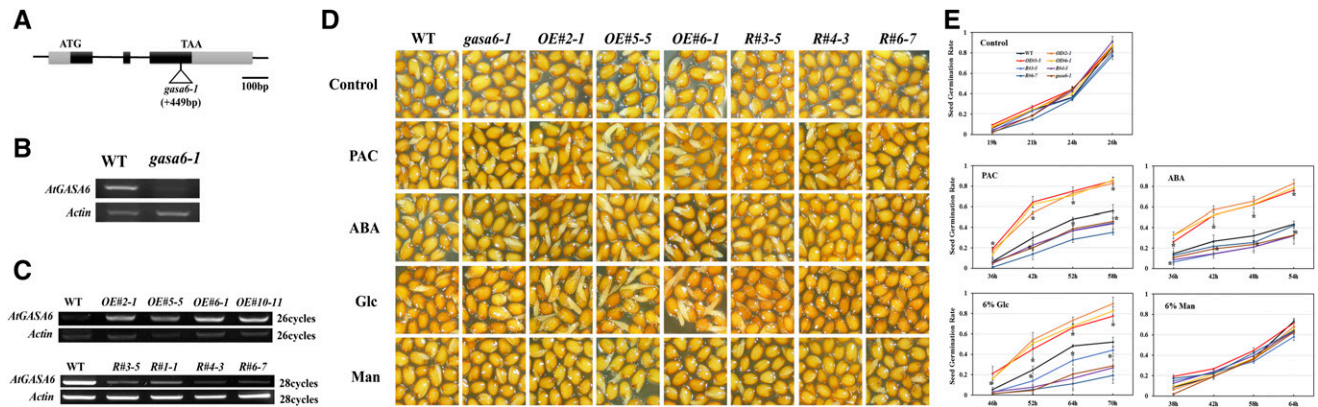
Figure 2. *AtGASA6-OE* seeds are hyposensitive, but RNAi and *gasa6-1* mutant seeds are hypersensitive to Glc, ABA, and PAC. A, The diagram of the T-DNA insertion position in *gasa6-1* mutant. B, Relative transcript levels of *AtGASA6* in 2-week-old *gasa6-1* mutant seedlings detected by RT-PCR; *Actin1* was used to standardize gene expression. C, Relative transcript levels of *AtGASA6* in germinating seeds of *AtGASA6-OE* and RNAi seeds. The transcript levels all were normalized to *Actin1* gene expression. D, Photographs of germination phenotypes of wild-type (WT), *gasa6-1*, *AtGASA6-OE* (*OE#2-1*, *OE#5-5*, and *OE#6-1*), and RNAi (*R#3-5*, *R#4-3*, and *R#6-7*) seeds treated with 0.5 μM PAC, 1 μM ABA, 6% (w/v) Glc, or 6% (w/v) mannitol; minimal medium was used as control. Seeds were photographed at 24, 64, 52, 64, or 48 h, respectively. At least 40 seeds were used. E, Seed germination rates of wild-type (Col-0), *gasa6-1*, *AtGASA6-OE* (*OE#2-1*, *OE#5-5*, and *OE#6-1*), and RNAi (*R#3-5*, *R#4-3*, and *R#6-7*) seeds untreated or treated with 0.5 μM PAC, 1 μM ABA, 6% (w/v) Glc, or 6% (w/v) mannitol; minimal medium was used as control. Three technical replicates were performed for each of three biological replicates. *, Significant differences compared with control seeds (one-way ANOVA was used to analyze the significant difference: $P < 0.01$).

for 3 h, and the effects of all treatments were observed to be the same as during seed germination (Supplemental Fig. S4).

To elucidate the effects of GA and ABA concentrations on *AtGASA6* expression, germinating seeds were treated with 1 or 10 μM PAC and 10 or 100 μM ABA. As expected, the effect of PAC on the expression of *AtGASA6* was the opposite of that of GA, and a significant inhibition effect was positively correlated with concentration. After 1 or 10 μM PAC treatment for 6 h, *AtGASA6* transcript levels declined by approximately 70% or 90%, respectively (Fig. 3B). Similarly, the down-regulation effect of high ABA concentrations was more severe than that of the lower concentration, and *AtGASA6* transcripts were barely detectable after treatment with 100 μM ABA (Fig. 3B). Although GA induced *AtGASA6* expression, there was no significant difference between the 1 and 10 μM GA$_3$ treatments (Fig. 3C).

Glc has been reported to inhibit seed germination in a similar manner to ABA, and this was also apparent in our studies (Fig. 2E). Because GA antagonizes ABA function in seed germination, we speculated that the down-regulating effect of Glc on *AtGASA6* expression might be counteracted by the inducing effect of GA. As expected, we found that a 6% (w/v) Glc treatment inhibited *AtGASA6* transcript levels but that this effect was abolished by 1 or 10 μM GA$_3$ applications (Fig. 3C). Based on the above data that *AtGASA6* is down-regulated by Glc and ABA and up-regulated by GA, we speculated that the lower seed germination rate of *AtGASA6* RNAi and *gasa6-1* mutants under ABA or 6% (w/v) Glc treatment also can be rescued by exogenous GA treatment. As expected, the significant germination

rate differences caused by ABA and Glc among the *AtGASA6* mutants could all be countered by the addition of exogenous GA (Fig. 3, D and E). However, exogenous GA treatment could not eliminate the germination rate differences among the different *AtGASA6* genotype seeds absolutely under normal condition (Supplemental Fig. S5). Taken together, these data confirm the regulation of *AtGASA6* by Glc, ABA, and GA.

AtGASA6 Is an Integrator of Glc, GA, and ABA Signaling Cross Talk

Because *AtGASA6* is regulated by Glc, ABA, and GA, its role in each of the three signaling pathways was further investigated by evaluating *AtGASA6* expression in the seeds of mutant genotypes corresponding to important steps in the different pathways. *ABI3*, *ABI4*, and *ABI5* are critical regulators of seed development and seed germination in the ABA signaling pathway (Lopez-Molina et al., 2001, 2002; Piskurewicz et al., 2008), the DELLA protein RGL2 is an important regulator of seed germination in the GA signaling pathway (Lee et al., 2002; Tyler et al., 2004), and *GLUCOSE INSENSITIVE1* (*GIN1*) and *GIN2* both play important roles in Glc signaling and seed germination (Moore et al., 2003; Lin et al., 2007). After exposure to light for 10 h, germinating seeds were harvested for analysis. In the *della* mutants *rag-24*, *rgl2-1*, and *gai-t6/rga-24*, the *AtGASA6* transcript levels were significantly higher (Fig. 1A), and the same was true for the ABA signaling mutants *abi3*, *abi4*, and *abi5* and Glc signaling mutants *gin1* and *gin2* (Fig. 4A), indicating that *AtGASA6* mRNA levels are regulated by
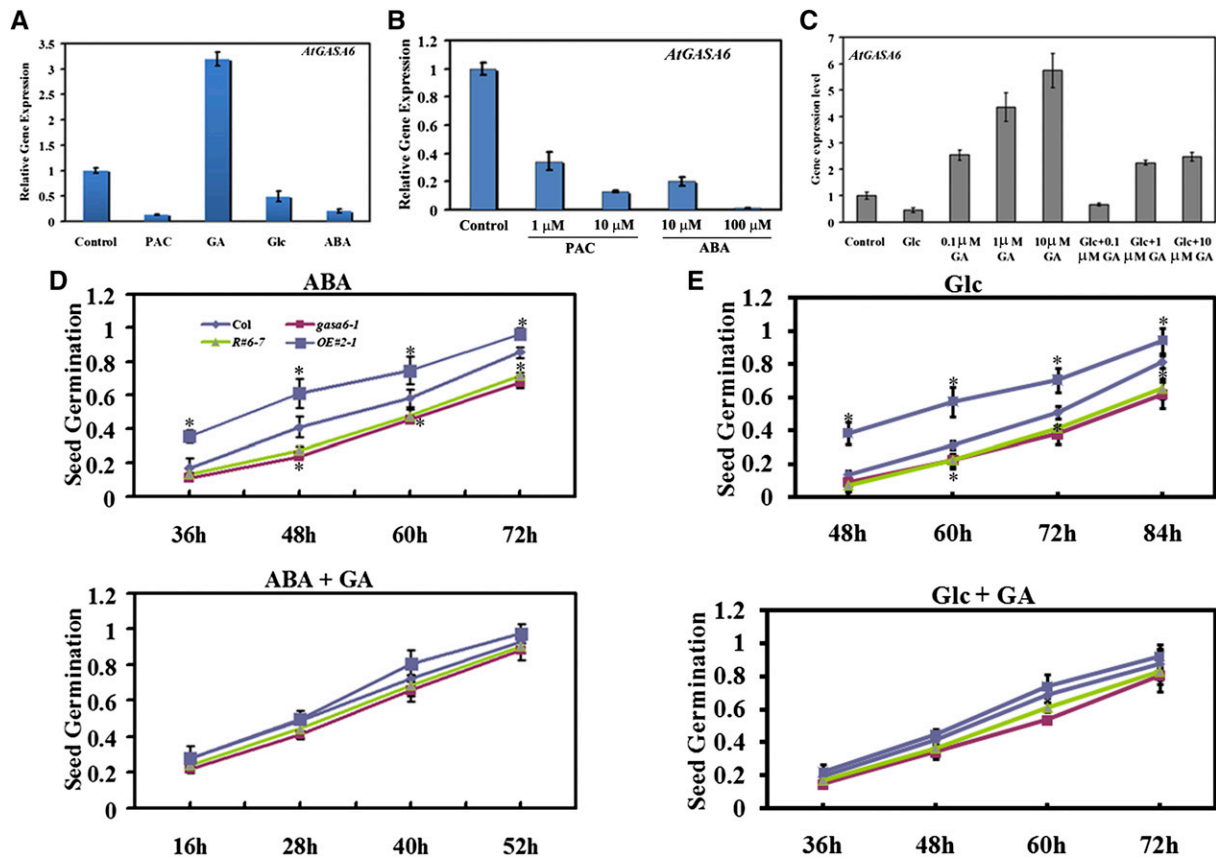
Figure 3. GA treatment abolishes the Glc- and ABA-induced differences in germination of different *AtGASA6* genotypes. A, Relative transcript levels of *AtGASA6* in germinating seeds in response to PAC, GA, Glc, and ABA. Seeds were grown on minimal medium for 4 h at 22°C after seeds were transferred from dark at 4°C and then treated with 10 μM PAC, 10 μM GA, 10 μM ABA, or 6% (w/v) Glc for 6 h; minimal medium was used as control. B, Relative transcript levels of *AtGASA6* in response to different concentrations of PAC or ABA in germinating seeds. Seeds germinated for 4 h at 22°C after seeds were transferred from dark at 4°C and then treated with 1 or 10 μM PAC or 10 or 100 μM ABA for 6 h. C, Relative transcript levels of *AtGASA6* in response to GA plus Glc in germinating seeds. Seeds germinated for 4 h were treated with 6% (w/v) Glc; 0.1, 1, or 10 μM GA alone; or 6% (w/v) Glc for 6 h; minimal medium was used as control. D, Seed germination rate of the wild type (Col-0), *AtGASA6-OE* (*OE#2-1*), RNAi (*R#6-7*), and the *gasa6-1* mutant treated with 1 μM ABA alone or plus 1 μM GA. Error bars denote sd. Similar results were obtained in at least three independent experiments, and at least 80 seeds were used. E, Seed germination rate of the wild type (Col-0), *AtGASA6-OE* (*OE#2-1*), RNAi (*R#6-7*), and the *gasa6-1* mutant treated with 6% (w/v) Glc alone or plus 1 μM GA. All transcript levels of gene expression were normalized to *UBQ1* gene expression. Results are averages ± se ($n = 3$). All experiments were repeated at least three times with similar results. *, One-way ANOVA was used to analyze the significant difference: $P < 0.01$.

these components in the GA, ABA, and Glc signaling pathways.

To determine whether *AtGASA6* transcript levels in GA, ABA, or Glc signaling mutants are affected by other signaling factors, a series of treatments was performed. In the wild type, *AtGASA6* expressions were significantly inhibited by more than 50% by PAC, ABA, or Glc, whereas in *abi5* mutant seeds, the inhibitions of *AtGASA6* expression by Glc, PAC, or ABA were alleviated, indicating that the Glc-, GA-, and ABA-mediated *AtGASA6* expressions are partially dependent on ABI5 (Fig. 4B). *AtGASA6* expression was not inhibited by Glc treatment but showed a moderate decrease after ABA or PAC treatment in the *gin2* mutant, indicating that Glc-induced *AtGASA6* inhibition is mediated by *GIN2* (Fig. 4B). Interestingly, under Glc,

ABA, or PAC treatments, the significant down-regulation of *AtGASA6* expression levels in the wild-type plant was alleviated greatly in the *rgl2* mutant, suggesting that *RGL2*, at least partially involved in the Glc, ABA, and GA signaling pathways, regulated *AtGASA6* expression (Fig. 4B). These results indicated that the *AtGASA6* expression is regulated by the cross talk of Glc, ABA, and GA signaling.

Considering the data above, we speculated that *AtGASA6* might function downstream of *RGL2*. To test this, the *gasa6-1* knockdown mutant was crossed with the *rgl2-13* (Col-0) mutant. Under normal and 0.5 μM PAC treatment conditions, the *rgl2-13* mutant seeds germinated faster, and the *rgl2-13/gasa6-1* double-mutant seeds germinated slightly slower than wild-type seeds and similar to that of *gasa6-1* seeds (Fig. 4, C and D),
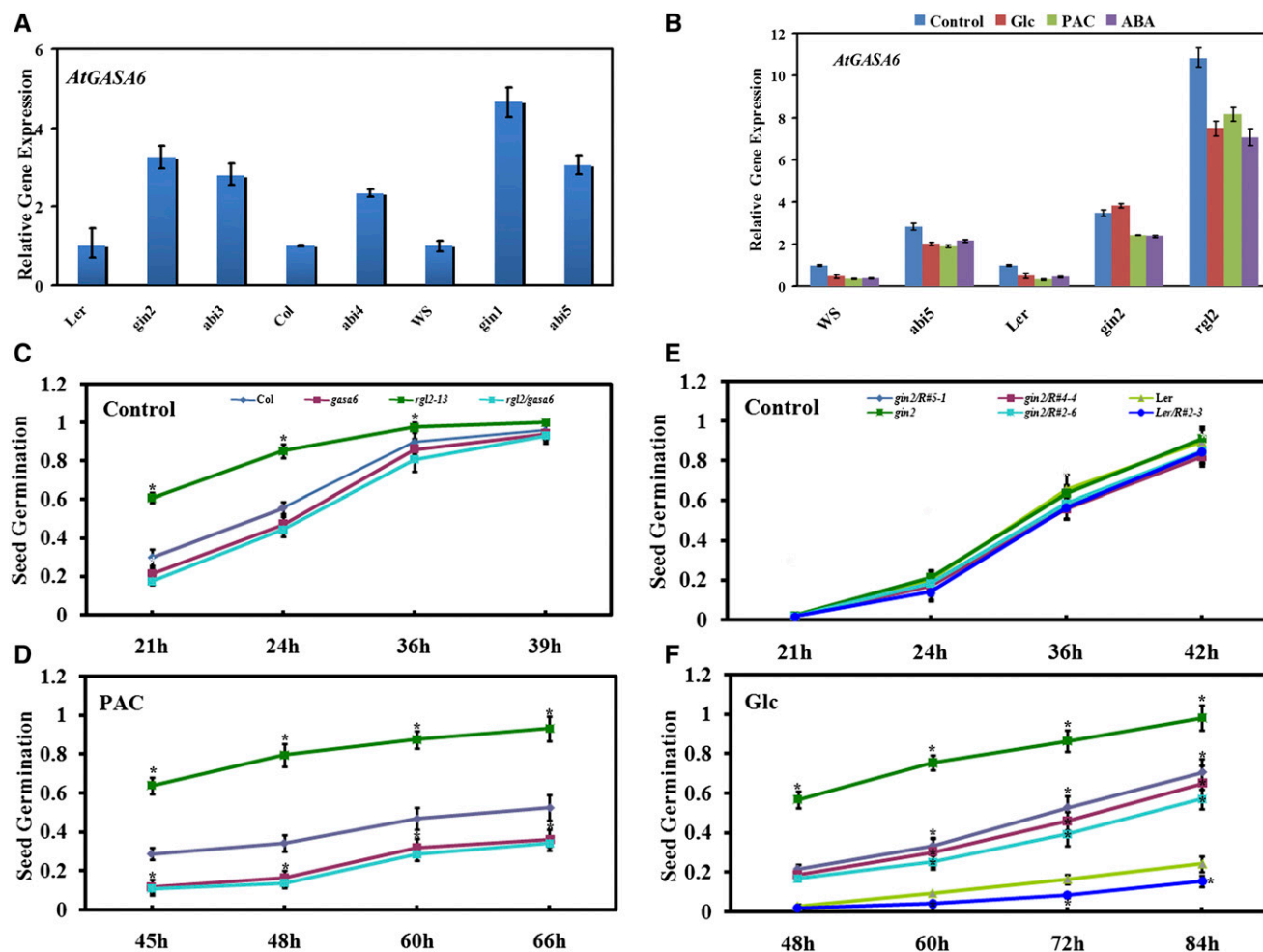
Figure 4. *AtGASA6* functions downstream of *RGL2*, *ABI5*, and *GIN2* in the GA, ABA, and Glc signaling pathways. A, Relative transcript levels of *AtGASA6* in germinating the wild type (L*er*, Col-0, and Ws) and a set of *abi* and *gin* mutant seeds (*abi3-1*, *abi4-102*, *abi5-1*, *gin1-1*, and *gin2-1*). All seeds were collected 10 h after seeds were transferred into light at 22°C from dark at 4°C. B, Relative transcript levels of *AtGASA6* in germinating wild-type (L*er* and Ws) and *rgl2-1*, *abi5-1*, and *gin2-1* mutant seeds treated with 0.5 μM PAC, 10 μM ABA, and 6% (w/v) Glc for 6 h; minimal medium was used as control. C and D, Seed germination assays of Col-0, *gasa6-1*, *rgl2-13*, and *rgl2-13/gasa6-1* treated with 0.5 μM PAC. E and F, Seed germination assays of L*er*, *gin2*, *AtGASA6-RNAi* (L*er/R#2-3*), and *gin2/AtGASA6-RNAi* (*gin2/R#2-1*, *gin2/R#3-2*, and *gin2/R#4-4*) treated with 6% (w/v) Glc. All transcript levels of gene expression were normalized to *UBQ1* gene expression. Results are averages ± SE ($n = 3$). All experiments were repeated at least three times with similar results. *, One-way ANOVA was used to analyze the significant difference: $P < 0.01$.

indicating *AtGASA6* functions downstream of *RGL2* in the GA pathway. To further show that *AtGASA6* operates downstream of *GIN2* in the Glc signaling pathway, *AtGASA6-RNAi* plants in the *gin2* and wild-type (Landsberg *erecta* [L*er*]) backgrounds were generated, and expression levels were detected by quantitative real-time PCR (Supplemental Fig. S6). The responses of *gin2/AtGASA6-RNAi*, *gin2*, and *AtGASA6-RNAi* (L*er*) seeds to 6% (w/v) Glc were tested. The *gin2/AtGASA6-RNAi* seeds showed a significantly lower seed germination response to Glc treatment compared with those of *gin2*, indicating that down-regulation of *AtGASA6* increased the sensitivity of *gin2* to Glc (Fig. 4, E and F). These results suggested that *AtGASA6* indeed functions downstream of *GIN2* and *RGL2* and

plays an integral role in Glc, ABA, and GA signaling pathways.

AtGASA6 Is Located Downstream of RGL2 to Integrate Glc and GA Signaling

Because *RGL2* plays an essential role in integrating GA and ABA signaling during seed germination (Piskurewicz et al., 2008; Lee et al., 2010) and because our results suggest that *AtGASA6* plays an integral role in Glc, ABA, and GA signaling pathways and functions downstream of *RGL2* in GA pathway, we hypothesized that the response of *AtGASA6* to Glc and GA is also regulated by *RGL2*. To verify this, mutant seeds of *rgl2-13*, *rgl2-13/gasa6-1*, and *gasa6-1* were
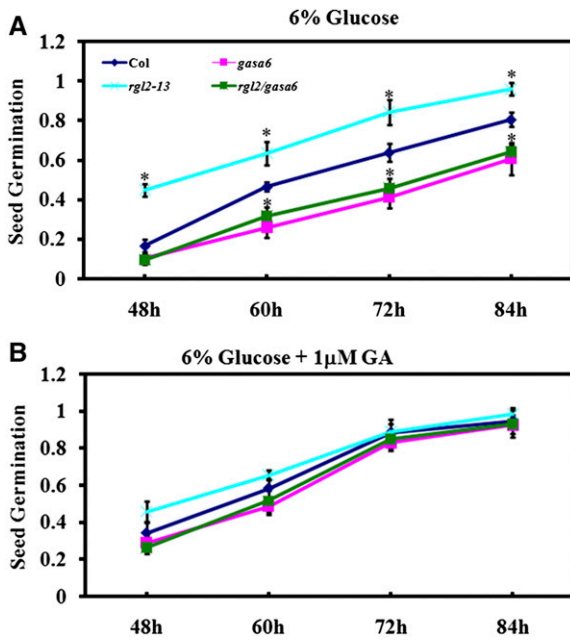
Figure 5. An *AtGASA6* mutation decreases *rgl2-13* insensitivity to Glc, and GA abolishes the Glc-induced germination differences between *rgl2-13* and *rgl2-13/gasa6-1* mutants. Seed germination assays of Col-0, *gasa6-1*, *rgl2-13*, and *rgl2-13/gasa6-1* treated with 6% (w/v) Glc alone (A) or plus 1 μM GA$_3$ (B). Three technical replicates were performed for each of three biological replicates. *, Significant differences compared with control plants (one-way ANOVA was used to analyze the significant difference: $P < 0.01$).

challenged with 6% (w/v) Glc. As expected, *rgl2-13* seeds were insensitive to the treatment; however, importantly, *rgl2-13/gasa6-1* seeds showed the same hypersensitivity to 6% (w/v) Glc as the *gasa6-1* seeds (Fig. 5A). Because the inhibition of *AtGASA6* expression by Glc could be reversed by addition of exogenous GA, the germination difference to 6% (w/v) Glc between *rgl2-13* and *gasa6-1* was predicted to be reduced but not dispelled by addition of exogenous GA. Although the germination rate of *rgl2-13* was not changed, all other mutant germination rates were significantly increased by treating with 6% (w/v) Glc plus 1 μM GA$_3$ compared with 6% (w/v) Glc only. The germination rates of *gasa6-1* and *rgl2-13/gasa6-1* were significantly lower than those of *rgl2-13*, although they were more similar at late germination stages (Fig. 5B). Taken together, these data suggest that *AtGASA6* integrates Glc and GA signaling and functions downstream of the *RGL2*-mediated seed germination pathway.

AtGASA6 Regulates Seed Germination by Promoting Embryonic Axis Elongation

The GUS staining of *AtGASA6* specifically expressed in the hypocotyl-radicle transition zone of the embryonic axis in germinated seeds (Supplemental Fig. S2Bl) indicates that *AtGASA6* might function in embryonic

axis development during seed germination. To confirm this hypothesis, *pAtGASA6::GUS* seeds were germinated under different conditions that are known to change the germination process. GUS activity was observed at different time intervals around the threshold before or after endosperm rupture. Under normal germination conditions, *AtGASA6* was first expressed at the embryonic axis before endosperm rupture. The expression level at the embryonic axis was 45% after exposure to light for 18 h, when the embryonic axis began to rupture the endosperm. This rate rose to 100% after exposure to light for 25 h and after all of the seeds were germinated (Fig. 6), indicating that expression of *AtGASA6* in hypocotyls is a marker for the completion of seed germination. Importantly, seed germination and GUS activity were delayed significantly and synchronously by 6% (w/v) Glc, ABA, or PAC treatments but enhanced by GA treatment. Under the GA condition, *AtGASA6* expression time was advanced to 14 h, and the expression rate reached 100% by 18 h after stratification, whereas Glc, ABA, and PAC treatments delayed the time point significantly, when both *AtGASA6*
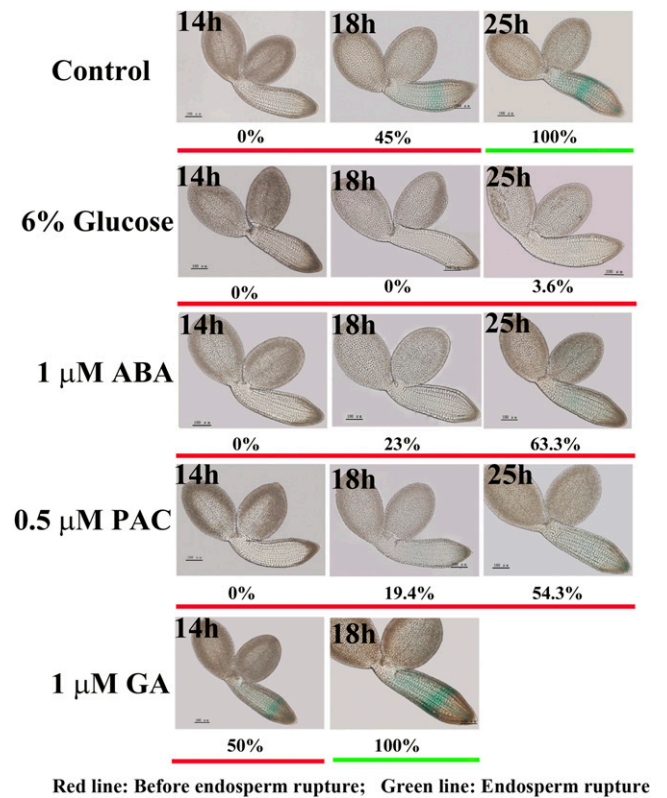

Figure 6. *AtGASA6* is expressed at the embryonic axis to promote endosperm rupture during seed germination. *pGASA6::GUS* seeds were untreated or treated with Glc, ABA, PAC, or GA; minimal medium was used as control. Numbers in the top left indicate time points after stratification, and values (%) at the bottom indicate the percentages of the embryos containing GUS staining. Three technical replicates were performed for each of three biological replicates.

expression and endosperm rupture rates reached 100% (Fig. 6). These results suggest that *AtGASA6* is expressed in the embryo hypocotyl to promote endosperm rupture and together with data showing that

AtGASA6 overexpression promotes seed germination in the presence of ABA, 6% (w/v) Glc, or PAC (Fig. 2, D and E), indicate that *AtGASA6* may promote seed germination by influencing embryo hypocotyl
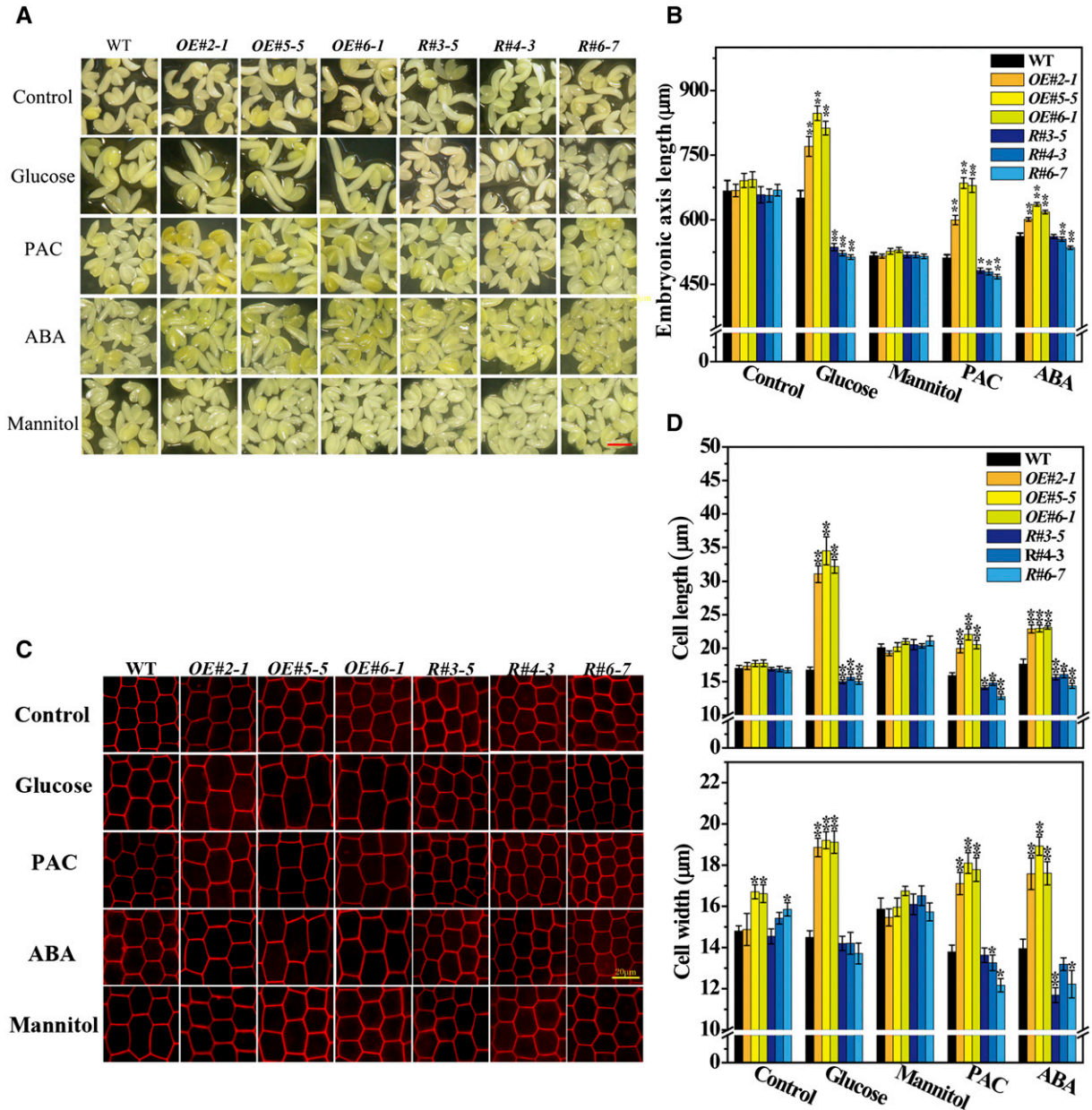

Figure 7. *AtGASA6* accelerates embryonic axis elongation by promoting cell elongation and expansion. A, Photographs of embryonic axis elongation of *AtGASA6-OE* and RNAi seeds treated with 6% (w/v) Glc, 0.5 μM PAC, 1 μM ABA, or 6% (w/v) mannitol using minimal medium as the control and 6% (w/v) mannitol as the osmotic control. Seeds under control, Glc, mannitol, PAC, and ABA at 24, 64, 64, 52, and 48 h, respectively, were photographed. Bar = 2 mm. B, Embryonic axis length of *AtGASA6-OE* and RNAi seeds untreated or treated with 6% (w/v) Glc, 6% (w/v) mannitol, 0.5 μM PAC, or 1 μM ABA. Seeds were harvested at the same time points as in A ($n > 50$). C, Embryonic axis cells of *AtGASA6-OE* and RNAi seeds untreated or treated with 6% (w/v) Glc, 6% (w/v) mannitol, 0.5 μM PAC, or 1 μM ABA. Seeds were harvested at the same time points as in A. Bar = 20 μm. D, Embryonic axis cell length and width in *AtGASA6-OE* and RNAi seeds untreated or treated with 6% (w/v) Glc, 6% (w/v) mannitol, 0.5 μM PAC, or 1 μM ABA. Seeds were harvested at the same time points as in A. The asterisks indicate significant differences compared with control seeds (one-way ANOVA was used to analyze the significant difference). Three technical replicates were performed for each of three biological replicates. WT, Wild type; *, $P < 0.05$; **, $P < 0.01$.

growth. To test this hypothesis, the hypocotyl growth of wild-type, *AtGASA6-OE*, and RNAi seeds treated with Glc, ABA, PAC, or mannitol was observed, and their lengths were measured at the time of endosperm rupture. The hypocotyls of *AtGASA6-OE* lines were longer, but *AtGASA6* RNAi lines were shorter than those of the wild type. Under normal conditions and mannitol treatment, the hypocotyl lengths of all genotypes were similar (Fig. 7, A and B).

It is well documented that completion of germination is the result of cell expansion and not cell division within the hypocotyl-radicle transition region (Sliwinska et al., 2009). Thus, cell length and width of the hypocotyl-radicle transition region of embryo axis were measured to determine whether the effects of *AtGASA6* on embryo axis growth were caused by cell expansion. Cell length was observed to be greater in the *AtGASA6-OE* seeds and smaller in the RNAi seeds under Glc, ABA, and PAC treatments. Moreover, the cell width was also greater in *AtGASA6-OE* seeds (Fig. 7, C and D), indicating that *AtGASA6* accelerates seed germination by promoting cell expansion within the hypocotyl-radicle transition region of the embryonic axis under Glc, ABA, and PAC treatments.

AtGASA6 Regulates Seed Germination and Cell Elongation through the Action of *AtEXPA1*

Bioinformatical analysis revealed that *AtGASA6* is coexpressed with *AtEXPA1* and *AtEXPA8* genes (Supplemental Fig. S7; http://atted.jp/cgi-bin/locus.cgi?loc=At1g74670), which encode cell wall loosening EXPA proteins. *AtGASA6* encodes a small polypeptide (101 amino acids) that shares common structural features with other GASA family proteins: an N-terminal putative signal peptide sequence, a variable hydrophilic intermediate region, and a highly conserved 60-amino acid C-terminal domain containing 12 conserved Cys residues, named the GASA domain (Supplemental Fig. S8). The subcellular localization of the AtGASA6 protein was determined by analysis of the AtGASA6-enhanced GFP (EGFP) fusion protein and transiently and stably expressed in onion (*Allium cepa*) epidermal and Arabidopsis root cells, respectively, under the regulation of the cauliflower mosaic virus 35S promoter. GFP fluorescence in the cell periphery was observed in both cell types, whereas a control soluble GFP protein was uniformly localized to the nucleus and cytoplasm (Supplemental Fig. S2C), indicating that AtGASA6 is mainly localized in the cell wall as the eFP Browser suggested (Supplemental Fig. S8D; Winter et al., 2007). In addition, our results suggested that *AtGASA6* accelerates seed germination by promoting hypocotyl cell expansion. Thus, we speculated that the AtGASA6 protein somehow functions with AtEXPA1 and/or AtEXPA8 in cell expansion. To investigate their relationship, we first analyzed whether the two *EXPA* genes share the same responses to exogenous stimulus with *AtGASA6*. The expression of *AtEXPA1* was higher in the *AtGASA6-OE*

lines and lower in the *AtGASA6* RNAi lines (Fig. 8A). In addition, we observed that the expression of *AtGASA6* was not changed in the *AtEXPA1* mutants (Fig. 8B). Notably, the expression of *AtEXPA1* in response to the addition of Glc, GA, and ABA is similar to that of *AtGASA6* (Fig. 8C). These results suggest that *AtEXPA1* functions downstream of *AtGASA6* action.

We further investigated the potential function of *AtEXPA1* in seed germination through the use of transgenic plants with reduced or elevated *AtEXPA1* expression (Supplemental Fig. S9). Our results indicate that *AtEXPA1* indeed functions in the seed germination process in a similar fashion to *AtGASA6*, because it promotes seed germination under Glc, PAC, or ABA treatment. Specifically, the germination rate of the *AtEXPA1-OE* seeds was greater than that of wild-type seeds after 6% (w/v) Glc, PAC, or ABA treatments, whereas the germination rate of seeds from the *expa1* knockout line was significantly less (Fig. 9). Additionally,
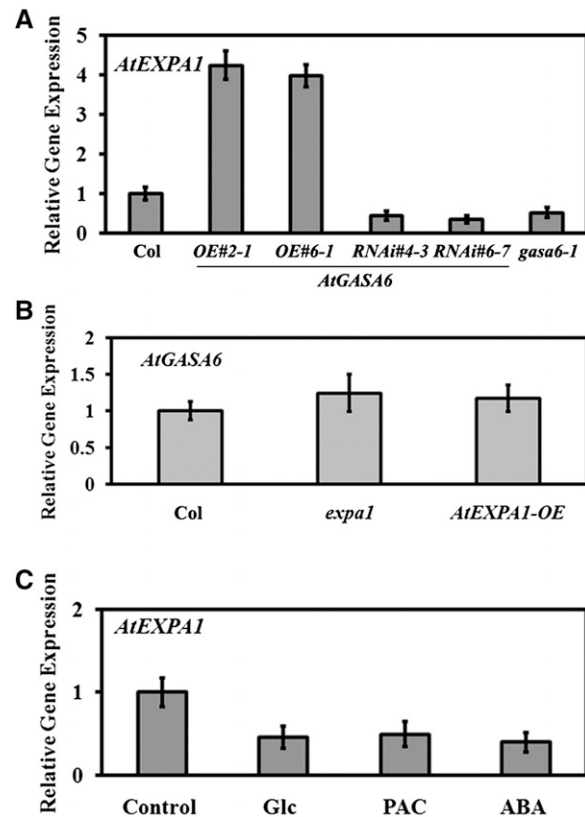

Figure 8. *AtGASA6* induces *AtEXPA1* expression in germinating seeds. A, Relative transcript levels of *AtEXPA1* in germinating wild-type (Col-0), *AtGASA6-OE* (*OE#2-1* and *OE#6-1*), RNAi (*RNAi#4-3* and *RNAi#6-7*), and *gasa6-1* seeds. B, Relative transcript levels of *AtGASA6* in germinating wild-type (Col-0), *expa1*, and *AtEXPA1-OE* seeds. C, Relative transcript levels of *AtEXPA1* in response to 6% (w/v) Glc, 0.5 μM PAC, or 1 μM ABA in germinating Col-0 seeds. The transcript levels of gene expression were normalized to *UBQ1* gene expression. Results are averages $\pm$ SE ($n = 3$). All experiments were repeated at least three times with similar results.
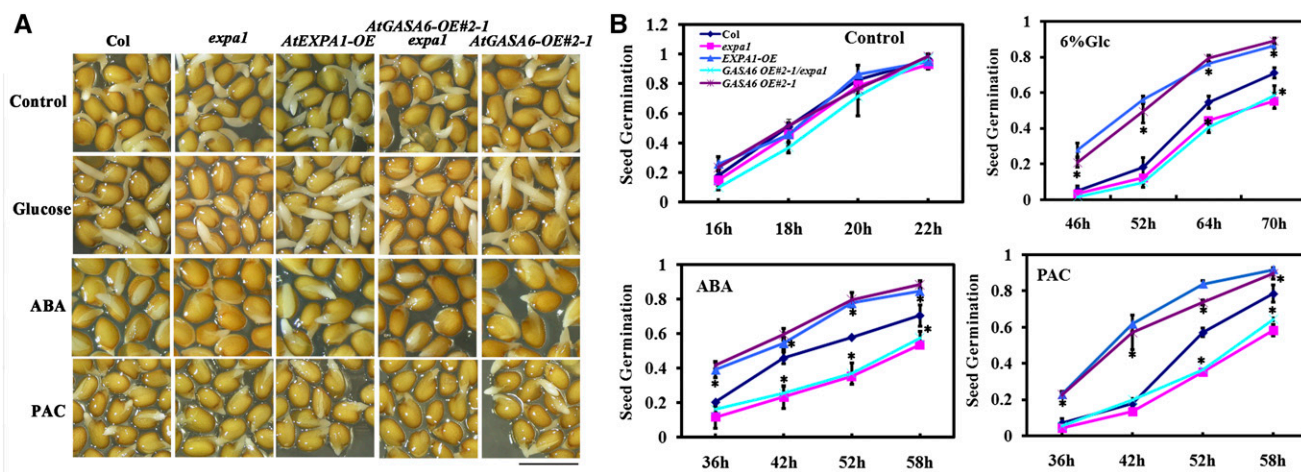
Figure 9. *AtEXPA1* is necessary for *AtGASA6* function in response to GA, ABA, and Glc during seed germination. A, Photographs of germination phenotypes of wild-type (Col-0), *expa1*, *AtEXPA1-OE*, *AtGASA6-OE#2-1*, and *AtGASA6-OE#2-1/expa1* seeds untreated or treated with 6% (w/v) Glc, 1 μM ABA, or 0.5 μM PAC. Bar = 2 mm. B, Seed germination assays of wild-type (Col-0), *expa1*, *AtEXPA1-OE*, *GASA6-OE#2-1*, and *AtGASA6-OE#2-1/expa1* seeds untreated or treated with 6% (w/v) Glc, 1 μM ABA, or 0.5 μM PAC. The transcript levels were normalized to *UBQ1* gene expression. Results are averages $\pm$ SE ($n = 3$). All experiments were repeated at least three times with similar results. *, One-way ANOVA: $P < 0.01$.

the embryonic axis and cell lengths of *AtEXPA1-OE* lines were greater than for wild-type seeds but less than for the *expa1* seeds after Glc, ABA, and PAC treatments (Fig. 10).

Given the similar expression patterns and function of *AtEXPA1* and *AtGASA6* in the process of seed germination as well as the fact that *AtEXPA1* expression was affected by *AtGASA6*, we crossed the *expa1* mutant with *AtGASA6-OE* plants to elucidate the genetic relationship between these genes. As expected, the faster germination rate and greater cell length of the *AtGASA6-OE* plants were abolished by the *AtEXPA1* knockout mutation (Figs. 9 and 10), suggesting that the *AtGASA6*-mediated seed germination under 6% (w/v) Glc, ABA, or PAC treatments is dependent on the action of the *AtEXPA1* gene.

DISCUSSION

Numerous studies show that a typical GA response is the promotion of seed germination. The DELLA protein RGL2 is involved in integrating phytohormones and environmental factors controlling seed germination (Lee et al., 2002; Ariizumi and Steber, 2007; Piskurewicz et al., 2008; Ariizumi et al., 2013). However, downstream events of RGL2 are not well understood. The *GAST1*-like family, members of which encode small proteins with a conserved Cys-rich domain, is known as one of the few identified GA-induced gene families (Shi et al., 1992; Roxrud et al., 2007; Zhang and Wang, 2008; Lin et al., 2011). Although many studies have attempted to elucidate their roles in plant development, GA responses, in particular, are still unclear. Here, we provide evidence supporting the role of AtGASA6 protein in mediating not only GA responses but also,

ABA and Glc responses in regulating the timing of seed germination and suggest that GASA family members function on a multiple phytohormones network.

AtGASA6 Integrates the GA, ABA, and Glc Signaling Pathways

Seed germination is known to be governed by two major counteracting phytohormones, ABA and GA, in response to various environmental factors (Finkelstein et al., 2008). More signaling components of these two phytohormones pathways, however, need to be found in regulating seed germination. *AtGASA6* expression was induced by GA and repressed by PAC and ABA in both germinating seeds and seedlings (Fig. 3; Supplemental Fig. S4). Expressions of several other *AtGASA6* homologous genes are also induced by GA and inhibited by ABA (Zhang and Wang, 2008; Lin et al., 2011), indicating that GASA family members are some important factors involve in GA and ABA signaling network. However, *gasa6-1* seeds did not show significant germination phenotype under the normal condition, probably because of functional redundancy; this problem is also reported on *AtGASA4*, the homologous gene of *AtGASA6* (Rubinovich and Weiss, 2010). Overexpression of *AtGASA6* can effectively overcome the inhibiting effects of PAC and ABA (Fig. 2), whereas the difference in seed germination rates under ABA treatment between *AtGASA6-OE* and RNAi seeds was eliminated by exogenous GA (Fig. 3D), indicating that *AtGASA6* operates in both GA and ABA signaling pathways during seed germination. To identify the specific position of *AtGASA6* in the GA and ABA signaling network, we examined *AtGASA6* expression in DELLA mutants *rga24*, *gai-t6*, and *rgl2-1* and ABI
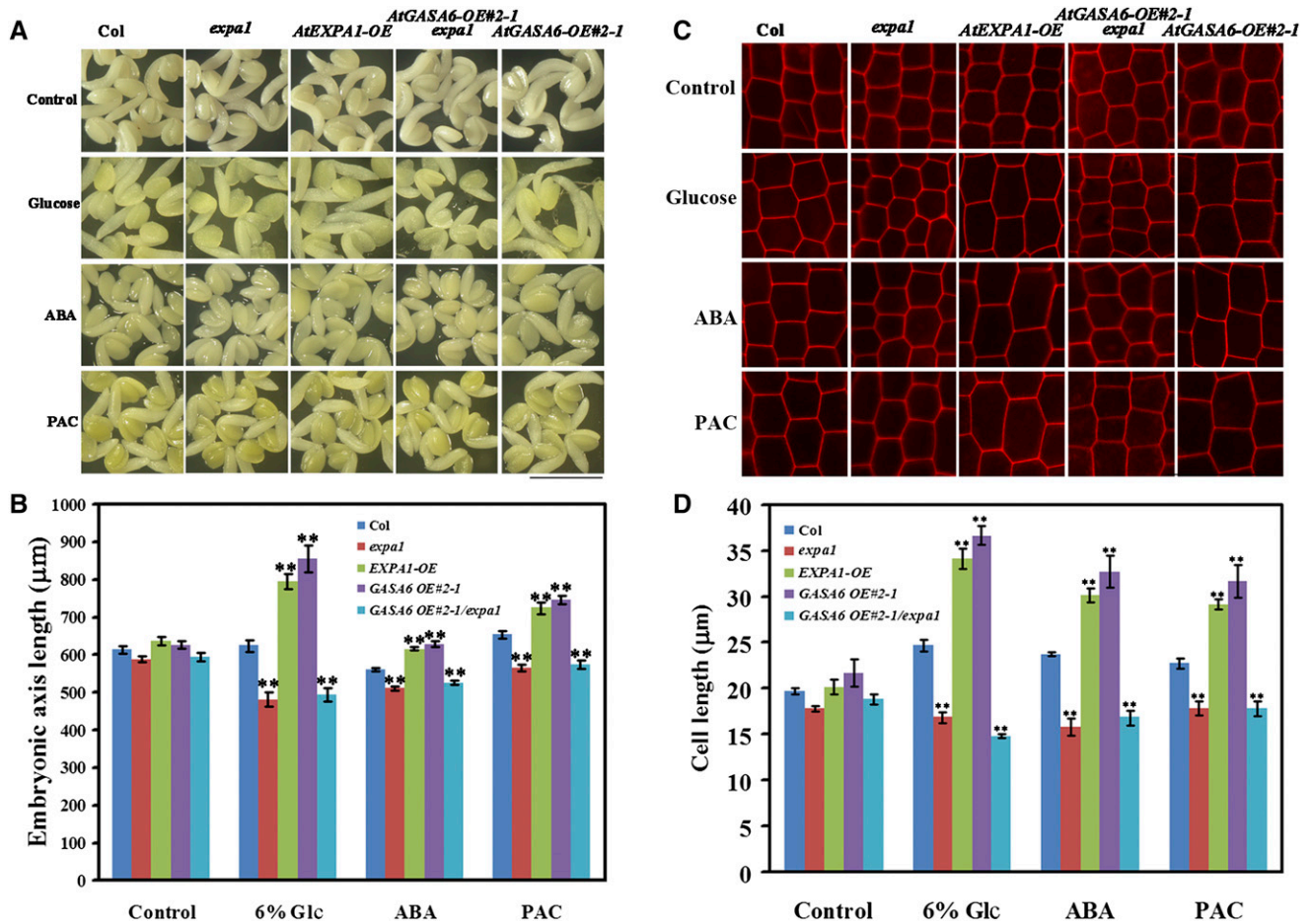
Figure 10. *AtGASA6* promoting cell elongation requires the participation of *AtEXPA1*. A, Photographs of the embryonic axis of wild-type (Col-0), *expa1*, *AtEXPA1-OE*, *GASA6-OE#2-1*, and *GASA6-OE#2-1/expa1* seeds untreated or treated with 6% (w/v) Glc, 1 μM ABA, or 0.5 μM PAC; minimal medium was used as control. Bar = 2 mm. B, Embryonic axis lengths of wild-type (Col-0), *expa1*, *AtEXPA1-OE*, *GASA6-OE#2-1*, and *GASA6-OE#2-1/expa1* seeds untreated or treated with 6% (w/v) Glc, 1 μM ABA, or 0.5 μM PAC; minimal medium was used as control. Similar results were obtained in three independent experiments. C, Photographs of cell size change of wild-type (Col-0), *expa1*, *AtEXPA1-OE*, *GASA6-OE#2-1*, and *GASA6-OE#2-1/exp1* seeds untreated or treated with 6% (w/v) Glc, 1 μM ABA, or 0.5 μM PAC; minimal medium was used as control. Bar = 2 μm. D, Hypocotyl cell lengths of wild-type (Col-0), *expa1*, *AtEXPA1-OE*, *GASA6-OE#2-1*, and *GASA6-OE#2-1/expa1* seeds untreated or treated with 6% (w/v) Glc, 1 μM ABA, or 0.5 μM PAC. The transcript levels were normalized to *UBQ1* gene expression. Results are averages ± SE (n = 3). One-way ANOVA was used to analyze the significant difference. All experiments were repeated at least three times with similar results. *, $P < 0.05$; **, $P < 0.01$.

mutants *abi3*, *abi4*, and *abi5*. Results showed that all of the tested DELLA proteins down-regulate *AtGASA6* expression and that RGL2 is the main protein modulating *AtGASA6* expression in germinating seeds (Fig. 1). This expression pattern is consistent with the finding that *AtGASA6* expression is repressed in the *RGL2*-mediated seed germination transcriptome (Stamm et al., 2012). Our results show that, under condition, with added PAC, the highest seed germination rates of *rgl2-13* were reduced by the loss of *AtGASA6* function (Fig. 4, C and D), suggesting that AtGASA6 is an important factor for RGL2-mediated seed germination under PAC treatment. Interestingly, *AtGASA4* may also function downstream of RGL2 proteins in seed germination (Ariizumi et al., 2013). In addition,

several *GASA* family genes have been reported to function downstream of the DELLA proteins (Zhang and Wang, 2008; Sun et al., 2013). These findings show us that GA mediates GASA protein function in different plant developmental processes through the regulation of DELLA proteins. In the absence of *ABI5*, which acts downstream of *ABI3* and represses seed germination in response to ABA and GA (Lopez-Molina et al., 2002; Piskurewicz et al., 2008), up-regulation of *AtGASA6* occurs, and the repression of *AtGASA6* by PAC is diminished (Fig. 4B). *AtGASA6* expression was also up-regulated in *abi3* and *abi4* mutants (Fig. 4A). These results suggest that *AtGASA6* acts downstream of ABI5 to mediate the interaction between GA and ABA. When GA levels are low, *RGL2*

promotes ABA biosynthesis by stimulating the expression of *XERICO*, which encodes an REALLY INTERESTING NEW GENE-H2 zinc finger factor (Ko et al., 2006; Zentella et al., 2007; Piskurewicz et al., 2008). In turn, elevation of endogenous ABA levels promotes expression of *RGL2* and *ABI5* and a subsequent increase in protein levels (Piskurewicz et al., 2008). Presumably, RGL2 allows *AtGASA6* to respond to both the GA and ABA pathways by generating a single signal output that does not permit germination under unfavorable environmental conditions.

To date, at least three types of Glc signal transduction mechanisms have been found in plants, of which the HXK-dependent pathway may play a leading role (Xiao et al., 2000). Glc-induced ABA accumulation has been suggested to be essential for HXK-mediated Glc responses (Arenas-Huertero et al., 2000), and inhibition of seed germination by Glc has been shown to be caused by a slowing of the decline in endogenous ABA levels through modulating the transcription of ABA biosynthesis and perception genes (Price et al., 2003); however, the role of GA in sugar signaling and the issue of whether the HXK-dependent sugar signaling pathway is affected by GA is not well understood. In this work, *AtGASA6* expression was substantially down-regulated by 6% (w/v) Glc in both germinating seeds and seedlings (Fig. 3; Supplemental Fig. S4), and *AtGASA6-OE* lines displayed enhanced tolerance of Glc-induced inhibition seed germination (Fig. 2) and cotyledon greening (Supplemental Fig. S10). Consistent with these results, *AtGASA6* has also been characterized as an in vivo sugar marker gene (Gonzali et al., 2006) and is severely repressed by Arabidopsis *Tandem Zinc Finger1*, which serves as a positive regulator of ABA/sugar and a negative regulator of GA responses (Lin et al., 2011). These findings indicate that *AtGASA6* plays important roles in the sugar signaling pathway and may participate in the cross talk of sugar/GA and sugar/ABA signaling. The expressions of *AtGASA6* were clearly higher in the *gin2* and *gin1* mutants (Fig. 4A), and the Glc-inhibited *AtGASA6* expression was absent in *gin2* (Fig. 4B). Additionally, the reduction of *AtGASA6* expression in the *gin2* mutant resulted in decreased *gin2* seed tolerance to 6% (w/v) Glc (Fig. 4F), indicating that *AtGASA6* functions downstream of *GIN2*. Notably, the inhibition of *AtGASA6* expression by the 6% (w/v) Glc treatment, was abolished by GA application (Fig. 3C) along with the Glc-induced inhibition of *AtGASA6* RNAi seed germination (Fig. 3E). These results indicate that GA signaling affects the Glc signaling pathway and that *AtGASA6* may integrate their cross talk. Among the GA signaling components, only the *RGL2* gene is found to be up-regulated by Glc, and the inhibitory effect of Glc on seed germination has been suggested to be accomplished through the inactivation of the GA signaling pathway through *RGL2* (Yuan and Wysocka-Diller, 2006). Interestingly, the expression of *GIN2* was lower in germinating *rgl2-1* mutant seeds (Supplemental Fig. S11), indicating that

HXK-dependent Glc signaling may be regulated by *RGL2*, although the regulatory mechanisms of *RGL2* in Glc signaling are unclear. Genetic analysis revealed that *AtGASA6* also plays an important role downstream of *RGL2* in Glc signal-inhibited seed germination, because reduction of *AtGASA6* altered the tolerance of *rgl2* seeds to the Glc signal (Fig. 5).

AtGASA6 Regulates Cell Elongation at the Embryonic Axis through *AtEXPA1*

The completion of seed germination is marked by the appearance of the radicle through the surrounding endosperm and testa (Bewley, 1997; Bentsink and Koornneef, 2008; Weitbrecht et al., 2011). Physiologically, rupture of the endosperm and testa is associated with cell division and enlargement, which require cell wall loosening (Cosgrove, 2000a). Some cell wall-localized proteins are suggested to be involved in cell expansion/elongation in seed germination (e.g. EXPAs; Chen and Bradford, 2000; Chen et al., 2001; Yan et al., 2014), and several cell wall-localized *GASA* proteins from different plant species are involved in cell division and elongation (de la Fuente et al., 2006; Zhang et al., 2009), indicating that GASAs may play a role in cell wall extensibility modification. AtGASA6 is localized mainly in the cell wall (Supplemental Fig. S2C), and
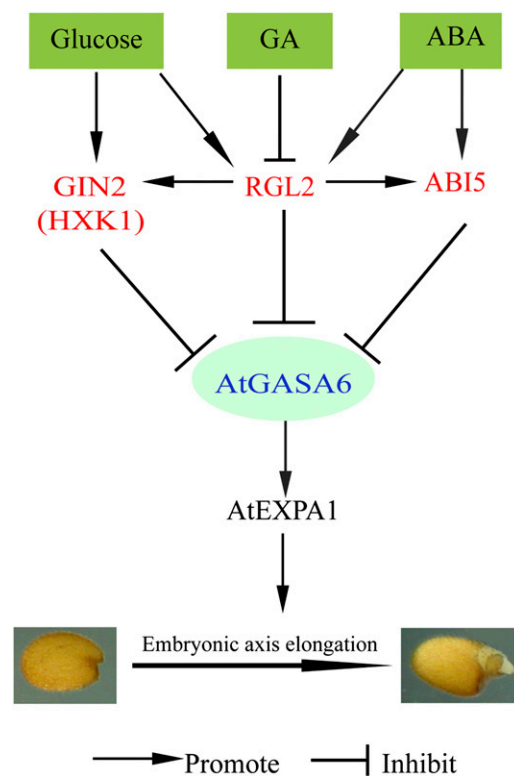

Figure 11. Proposed model of *AtGASA6* function integrating GA, ABA, and Glc signals to regulate seed germination.

AtGASA6 was specifically expressed in the hypocotyl-radicle transition zone of embryonic axis in germinating seeds (Fig. 6), suggesting that *AtGASA6* regulates seed germination, possibly by mediating embryonic axis elongation. Interestingly, further results showed that *AtGASA6* accelerates cell expansion within the hypocotyl-radicle region during seed germination under Glc, ABA, and PAC treatment (Fig. 7). However, *AtGASA6* does not play roles in the mannitol-mediated seed germination inhibition. Under mannitol treatment, all of the seeds showed reduced seed germination rate and shorter embryonic axis length but slightly larger cell expansion compared with the normal condition (Figs. 2E and 7, B and D); this implies that the relationship between cell expansion and embryonic axis length is complex under mannitol treatment and that the cell division is needed to be taken into consideration to elucidate the relationship among cell expansion, embryonic axis length, and seed germination under mannitol treatment in future work. Combined with the observation that *AtGASA6* coexpresses with several cell wall-loosening genes (Supplemental Fig. S7), it is reasoned that the function of AtGASA6 protein may contribute to cell wall loosening by association with EXPA action. EXPAs are thought to induce cell wall extension by disrupting noncovalent load-bearing linkages between cellulose microfibrils and the cross-linking matrix glycans (Cosgrove, 2005). Different EXPAs perform diverse tissue- or organ-specific roles (Cosgrove, 2000b), including endosperm rupture, seed germination (Chen and Bradford, 2000; Chen et al., 2001; Yan et al., 2014), and leaf development (Pien et al., 2001; Goh et al., 2012). Tomato *EXPA4* (*LeEXP4*) is expressed specifically in the micropylar endosperm cap region and associated with endosperm cap weakening (Chen and Bradford, 2000). In contrast, *LeEXP8* mRNA is localized to the radicle cortex of the embryo, whereas *LeEXP10* mRNA is expressed throughout the embryo during seed germination (Chen et al., 2001). Our data show that the expression of *AtEXPA1* was markedly higher in *AtGASA6-OE* seeds and lower in RNAi seeds, whereas *AtGASA6* expression did not change in *AtEXPA1* mutants (Fig. 8). In addition, *AtEXPA1* can also promote seed germination by regulating cell elongation at the embryonic axis, similar to *AtGASA6* (Figs. 9 and 10). The faster germination rates and greater cell length phenotypes of *AtGASA6-OE* seeds were abolished by *expa1* (Figs. 9 and 10). Taken together, these results suggested that the function of *AtGASA6* on cell expansion depends on the actions of *AtEXPA1*. Therefore, we hypothesized the existence of a direct physical interaction between AtGASA6 and AtEXPA1, but this was not supported by bimolecular fluorescence complementation and yeast (*Saccharomyces cerevisiae*) two-hybrid analyses (data not shown). In the *RGL2*-regulated seed germination transcriptome, several *EXPA* genes, including *AtEXPA1*, *AtEXPA3*, *AtEXPA8*, and *AtEXPA9*, are repressed by *RGL2* (Stamm et al., 2012). *AtEXPA2* is also involved in seed germination and abiotic stress responses, and its expression levels

in germinating seeds are repressed by expression of *DELLA* genes, including *RGL1*, *RGL2*, *RGA*, and *GAI* (Yan et al., 2014). Whether the *DELLA* genes repress *EXPA* genes directly or indirectly to regulate seed germination is not known; however, our results indicate that *AtGASA6* influences seed germination by mediating a signal between DELLA proteins and EXPAs.

CONCLUSION

In conclusion, our results suggest a role for *AtGASA6* as a positive regulator of seed germination. We propose a model (Fig. 11) where GA promotes and Glc and/or ABA inhibit *AtGASA6* expression, the cumulative effects of which govern GA- and ABA-mediated seed germination. In addition, *AtGASA6* promotes seed germination through *AtEXPA1* action by promoting cell elongation and consequently, embryonic hypocotyls length. However, the functional mechanism of *AtGASA6* in regulating seed germination and cell elongation and the working relationship between *AtGASA6* and *AtEXPA1* and even the other EXPAs need to be elucidated in the future with more evidence.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used in the generation of all transgenic plants and as the experimental control in this study unless specifically indicated. The mutant lines *gin2-1*, *abi3-1*, *gai-t6*, *rgl2-1*, *rga24*, and *gai-t6/rga24* are in the L*er* background and were provided by Xiangdong Fu (Institute of Genetics and Developmental Biology of the Chinese Academy of Sciences); *rgl2-13* is in the Col-0 background (provided by Ling Li at South China Normal University). *gasa6-1* (SALK_072904), *expa1* (SALK_010506C), *AtEXPA1-OE* (provided by Xuechen Wang at China Agriculture University), and *abi4-102* are in the Col-0 background; *gin1* and *abi5* are in the Wassilewskija (Ws) background. All mutant seeds were bought from the Arabidopsis Biological Resource Center Stock Center of Ohio State University unless otherwise indicated. PCR was utilized on genomic DNA using gene-specific and T-DNA border-specific primers to obtain plants homozygous for the T-DNA insertion. The double-mutant lines utilized in this study were created by cross pollination between the relevant mutants, and the genotypes were verified by resistance and PCR.

Plant Growth Conditions and Seed Germination Assay

Plants were grown at 22°C under long days (16-h-light/8-h-dark cycle), and light was supplied by cool and warm white fluorescent bulbs, reaching an intensity of approximately 100 μmol m^{-2} s^{-1} on the shelf surface. Seeds were surface sterilized with 70% (v/v) ethanol for 10 s and then 1% (v/v) sodium hypochlorite for 10 min, and they were rinsed four to five times with sterile distilled water. Sterilized seeds were subsequently sown on basal MS (Sigma-Aldrich) containing 0.8% (w/v) agar (MBCHEM) and 1.5% (w/v) Suc at pH 5.7. Plates were kept at 4°C in darkness for 3 d for stratification and then transferred to a plant culture room set at 22°C with a 16-h-light/8-h-dark photoperiod. For the germination assay, at least 80 seeds for each genotype were sterilized and sown on basal MS supplemented with or without 6% (w/v) Glc, 6% (w/v) mannitol, 1.5% (w/v) Suc, 1 μM GA$_3$, 0.5 μM PAC, or 1 μM ABA; basal MS as control; and 6% (w/v) mannitol as osmotic control. The radical tip emergence was defined as the first sign of seed germination. Seeds were scored daily until seed germination rate reached over 98%. The germination results were calculated based on at least three independent experiments. For seedling genotype assay, at least 80 seeds for each genotype were sterilized and sown on basal MS

with 6% (w/v) Glc and 6% (w/v) mannitol, and at least 60 growing similar seedlings for each genotype were measured based on at least three independent experiments; MS was used as control. For real-time PCR assays of different treatments, seeds were collected at 4 h after stratification and sown on basal MS supplemented with or without 6% (w/v) Glc, 10 μM GA$_3$, 0.5 μM PAC, or 10 μM ABA for 6 h. For the control, seeds were collected at 10 h after seeds were transferred into light at 22°C from dark at 4°C. The GA$_3$, PAC, and ABA stock solutions were made with 100% (v/v) alcohol and stored frozen at −20°C in the dark until use depending upon the experiment.

Plasmid Construction and Plant Transformation

The *GASA6* gene, its promoter (1,200 bp before the 5′ untranslated region), and the coding sequence (CDS) were PCR amplified using high-fidelity DNA polymerase (*Pfu* Ultra DNA Polymerase) from Arabidopsis genomic DNA and complementary DNA (cDNA) and cloned into a T-Vector (Takara Bio, Inc.). The sequence of the cloned *AtGASA6* gene, promoter, and CDS was verified by DNA sequencing and subcloned into pBI101 and modified pBin19 binary vectors. For overexpression experiments, the cauliflower mosaic virus 35S promoter was used to drive the expression of the *GASA6* CDS. For promoter analysis, the promoter was subcloned into pBI101 to drive expression of the GUS gene (*pGASA6::GUS*). For RNAi experiments, the hydrophobic region fragment was subcloned into pRNAi-35S vector (provided by Yaoguang Liu at South China Agriculture University). The fusion of *GASA6* and EGFP was made by cloning GASA6 (with the stop codon removed) in frame to the N terminus of the GFP protein of the modified pBin19 binary vectors. The 35S promoter was used to drive expression of the fusion protein. Plants were transformed with *Agrobacterium tumefaciens* by the floral dipping method (Clough and Bent, 1998). Transformants were selected by 50 μg mL^{-1} kanamycin and verified by PCR using specific primers. RNAi seeds were selected by using 25 μg mL^{-1} Hygromycin B. All transformed plants were selected for two more generations, and homozygous transgenic plants (T3) were used for further characterization.

GUS Staining and GFP Localization

T3 transgenic lines carrying the *pGASA6::GUS* construct were assayed for GUS staining. The samples were immersed into the GUS staining solution (50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% [v/v] Triton X-100, 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 2 mM ferricyanide, and 2 mM ferrocyanide) and incubated at 37°C for 12 h or overnight. Samples were washed in 70% (v/v) ethanol several times until the material was bleached. Finally, the samples were observed and photographed using an Olympus BX Microscope (Olympus Corporation). For GASA6-EGFP localization analysis, the roots of 4-d-old young seedlings were observed using a confocal laser-scanning microscopy combination system (LSM510/ConfoCor2; Zeiss). The recombinant sequences were incubated with gold particle, and the constructed gold-coated DNA particles were delivered into onion (*Allium cepa*) epidermal cells using the PDS-1000/He Particle Bombardment System (Bio-Rad). The samples were observed using a confocal laser-scanning microscopy combination system or a fluorescence microscope (Nikon TS-100) and photographed using a Nikon 5400 Camera (Nikon).

Genomic DNA Extraction and T-DNA Insertion Mutant Screening

Homozygous T-DNA insertion mutants were identified following the protocol described at the SALK Insertion Sequence Database (http://signal.salk.edu/tabout.html) by PCR. Approximately two to three leaves of 4-week-old seedlings growing in soil were collected from individual plants. Genomic DNA was extracted using a quick cetyltrimethyl ammonium bromide method (Rogers and Bendich, 1985) and used for PCR reactions with the primers recommended in the SALK protocols.

Semiquantitative RT-PCR

Total RNAs from tissues were isolated using the total RNA isolation kit with genomic DNA removed according to the manufacturer's instructions (Takara Bio, Inc.). The cDNAs were synthesized from 1 μg of total RNA using the Prime Script First-Strand cDNA Synthesis Kit (Takara Bio, Inc.), and 1 μL of cDNA was used for subsequent PCR. All PCR reactions were performed in a total

volume of 20 μL using approximately 22 to 30 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. PCR products were subject to 1% (w/v) agarose gel electrophoresis, photographed using the Bio Imagine System (Gene Company Ltd.), and analyzed with Gene Tools software (Gene Company Ltd.). Gene expressions were normalized to the expression of *Actin1*. Primer sequences were used as previously reported (Zhang et al., 2009).

Real-Time PCR

Total RNA from germinating seeds or 2-week-old seedlings was extracted using the total RNA isolation kit with genomic DNA removed according to the manufacturer's instructions and reverse transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA). Quantitative real-time PCR was performed using the Promega Real-Time PCR Kit (Promega). Gene expression was normalized to the expression of *ubiquitin1* (*UBQ1*). Primer sequences used were as previously reported (Jiang et al., 2012).

Hypocotyl Length Assay

For hypocotyl length assay, seeds were germinated on basal MS with 0.8% (w/v) agar with or without GA$_3$, PAC, ABA, Glc, or mannitol; stratified for 3 d in the dark at 4°C; and then grown at 22°C under light conditions. Hypocotyl length was quantified with Image J software (http://rsbweb.nih.gov/ij/) after photography of seeds collected at the corresponding time points.

Observation of Embryonic Axis and Cotyledon Epidermal Cells

For embryonic axis epidermal cells observation, seeds were collected in the corresponding time points under stress conditions, stained with 100 μM propidum iodide solution for approximately 10 to 30 s, rinsed with water three times, and photographed. For cotyledon epidermal cells observation, the cotyledons of 6-d-old young seedlings were collected and immersed in 75% (v/v) ethanol until the tissue was bleached. The samples were stained with 100 μM propidium iodide for 30 min, subsequently rinsed with water three times, and photographed. All of the pictures were photographed by using a commercial laser-scanning microscope (LSM510/ConfoCor2) combination system (Zeiss).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL database under the following accession numbers: *ABI3*, At3g24650; *ABI4*, At2g40220; *ABI5*, At2g36270; *Actin1*, At2g37620; *AtEXP1*, At1g69530; *GAI*, At1g14920; *AtGASA6*, At1g74670; *GIN1*, At1g52340; *GIN2*, At4g29130; *RGA*, At2g01570; *RGL2*, At3g03450; and *UBQ1*, At3g52590. All of the primers are listed in Supplemental Table S1.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Coexpression analysis of *AtGASA6*, *AtEXPA1*, and *RGL2* genes.

Supplemental Figure S2. *AtGASA6* gene expression pattern and AtGASA6 subcellular localization.

Supplemental Figure S3. Transcript levels of *AtGASA6* and its homologous genes in *AtGASA6-RNAi* plants.

Supplemental Figure S4. *AtGASA6* expression levels in response to GA, Glc, and ABA in early seedling development.

Supplemental Figure S5. Seed germination rates of different *AtGASA6* genotype plants under GA treatment.

Supplemental Figure S6. *AtGASA6* expression levels in L*er*/*AtGASA6-RNAi* and *gin2*/*AtGASA6-RNAi* plants.

Supplemental Figure S7. Coexpression gene analysis of *AtGASA6*.

Supplemental Figure S8. Bioinformatical analyses of AtGASA6 protein.

Supplemental Figure S9. *AtEXPA1* expression levels in the different *EXPA1* genotype seeds.

Supplemental Figure S10. *AtGASA6* regulates cotyledon greening in response to 6% (w/v) Glc in early seedling development.

Supplemental Figure S11. *GIN2* expression level in the *rgl2-1* mutant.

Supplemental Table S1. Gene-specific primer sequences.

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