The Regulator of G-Protein Signaling Proteins Involved in Sugar and Abscisic Acid Signaling in Arabidopsis Seed Germination

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The regulator of G-protein signaling (RGS) proteins, recently identified in Arabidopsis (Arabidopsis thaliana; named as AtRGS1), has a predicted seven-transmembrane structure as well as an RGS box with GTPase-accelerating activity and thus desensitizes the G-protein-mediated signaling. The roles of AtRGS1 proteins in Arabidopsis seed germination and their possible interactions with sugars and abscisic acid (ABA) were investigated in this study. Using seeds that carry a null mutation in the genes encoding RGS protein (AtRGS1) and the α-subunit (AtGPA1) of the G protein in Arabidopsis (named rgs1-2 and gpa1-3, respectively), our genetic evidence proved the involvement of the AtRGS1 protein in the modulation of seed germination. In contrast to wild-type Columbia-0 and gpa1-3, stratification was found not to be required and the after-ripening process had no effect on the rgs1-2 seed germination. In addition, rgs1-2 seed germination was insensitive to glucose (Glc) and sucrose. The insensitivities of rgs1-2 to Glc and sucrose were not due to a possible osmotic stress because the germination of rgs1-2 mutant seeds showed the same response as those of gpa1-3 mutants and wild type when treated with the same concentrations of mannitol and sorbitol. The gpa1-3 seed germination was hypersensitive while rgs1-2 was less sensitive to exogenous ABA. The different responses to ABA largely diminished and the inhibitory effects on seed germination by exogenous ABA and Glc were markedly alleviated when endogenous ABA biosynthesis was inhibited. Hypersensitive responses of seed germination to both Glc and ABA were also observed in the overexpressor of AtRGS1. Analysis of the active endogenous ABA levels and the expression of NCED3 and ABA2 genes showed that Glc significantly stimulated the ABA biosynthesis and increased the expression of NCED3 and ABA2 genes in germinating Columbia seeds, but not in rgs1-2 mutant seeds. These data suggest that AtRGS1 proteins are involved in the regulation of seed germination. The hyposensitivity of rgs1-2 mutant seed germination to Glc might be the result of the impairment of ABA biosynthesis during seed germination.

Many physiological and biochemical responses of plants to extracellular stimuli are mediated by the heterotrimeric guanine nucleotide-binding proteins (G proteins) signaling pathway, which is considered a widespread mechanism of signal transduction in eu-karyotic organisms (McCudden et al., 2005). The extracellular signal molecules (i.e. ligands) interact specially with G-protein-coupled receptors (GPCRs), which results in changes in the conformation of GPCR structure and thus initiates intracellular signaling by dissociating the G-protein α-subunit from the βγ-subunit complex and facilitating the exchange of GTP for GDP on the G-protein α-subunit. As a consequence, the activated GTP-bound α-subunit and βγ-subunit complexes interact separately with a variety of downstream effectors. The duration that Ga-subunits remain in active GTP-bound form is limited because the Ga-subunit itself possesses an intrinsic GTPase activity. Once the Ga-subunit hydrolyzes GTP to GDP, the heterotrimer reforms and signaling terminates (Gudermann et al., 1997; Ford et al., 1998; Li et al., 1998; Wall et al., 1998). Thus, heterotrimeric G proteins act as molecular switches, which coordinate the transfer of information from environmental signals to the cell interior.

However, more rapid desensitization of G-protein-mediated signaling has been observed in many cases of cells, which cannot be fully explained by the intrinsic GTPase activity of G-protein α-subunit both in vivo and in vitro. It has long been suggested that there exist many other mechanisms to inhibit the G-protein signaling, including the G-protein-coupled receptor kinase (GRK) and β-arrestin proteins-mediated rapid inactivation of signaling (Kohout and Lefkowitz, 2003), and the mechanisms involved in phosducin and its homologs (phosducin-like proteins) that binds to G-protein βγ complexes and affects G-protein activities (Muller et al., 1996). The discovery of the regulator of G-protein signaling (RGS) proteins provides a new...
way to regulate G-protein signaling (Koelle and Horvitz, 1996; Siderovski et al., 1996). RGS proteins accelerate the intrinsic activity of the Ga-subunit and thus return the Ga-subunit GTP-bound state to its basal GDP-bound state (Berman et al., 1996; Tesmer et al., 1997).

In contrast to mammalian cells, where many kinds of RGS proteins have been identified and extensive studies have been carried out on their functions, plant RGS proteins have been reported only recently when Chen et al. (2003) identified a RGS-like protein in Arabidopsis (Arabidopsis thaliana; called AtRGS1). This protein, unlike all other known RGS proteins, has a predicted seven-transmembrane structure domain in the N terminus, which is similar to a GPCR, as well as a RGS box in the C terminus with a GTPase-accelerating activity. Biochemical analysis has shown that a RGS protein has a strong interaction with Ga-subunit and activates the GTPase of Ga-subunits (Chen et al., 2003). Further studies have shown that AtRGS1 protein may be involved in the regulation of cytokinesis and proliferation of some cell types (Chen et al., 2003).

Recently, Chen and Jones (2004) used different kinds of sugars and their analogs to study their effects on seedling development and found that seedling development of Atgrs1 mutants was less sensitive to high concentrations of Glc, and this insensitive response to Glc was not due to osmotic stress because Atgrs1 mutants and AtRGS1 overexpressors had wild-type responses to the same concentration of D-mannitol (6%). They suggested that AtRGS1 is involved in the regulation of seedling development responses to sugar signaling. Furthermore, AtRGS1 likely functions in a hexokinase (HXK)-independent Glc-signaling pathway because sugar metabolism and phosphorylation by HXK are not required for AtRGS1-mediated signaling (Chen and Jones, 2004).

Seed germination is a key developmental process in the plant life cycle and may be involved in many signal transduction pathways and their interactions, including phytohormone-, sugar-, and G-protein-mediated signaling (Koornneef et al., 2002). Biochemical and pharmacological studies have shown that G proteins participate in the early events in this signaling pathway. Several recent studies have shown that heterotrimeric G proteins are involved in the response to either seed germination or seedling development (Perfus-Barbeoch et al., 2004). The Arabidopsis GPA1 protein-null mutants appear to be hypersensitive to abscisic acid (ABA) as well as to sugars, hyposensitive to gibberellins, and insensitive to brassinosteroids (Ullah et al., 2002; Iwasaki et al., 2003; Lapik and Kaufman, 2003). Epistasis analyses using double and triple mutants of gcr1 and G-protein α- and β-subunit genes indicate that GCR1 can act independently of heterotrimeric G protein in response to brassinosteroids and gibberellins in Arabidopsis seed germination (Chen et al., 2004). Overexpression of GCR1 abolishes seed dormancy and enhances the expression of germination-associated genes (Colucci et al., 2002).

In rice (Oryza sativa), Gα-antisense insert transformants show dwarf phenotype and small seeds (Fujisawa et al., 1999), and the d1 mutant, which is defective in the Gα protein, partially impairs the GA signaling pathway and reduces the disease resistance (Ueguchi-Tanaka et al., 2000; Suharsono et al., 2002).

Apparently, the roles of AtRGS proteins in regulating plant growth and development are complex and remain to be clarified. If AtRGS proteins are involved in seed germination, the mechanism is not known. Our major objectives in this study focused on the roles of...
AtRGS protein in the responses of seed germination to sugars and ABA and possible interactions between the two signaling pathways. Two Arabidopsis mutants, rgs1-2 and gpa1-3, for the genes encoding RCS protein (AtRGS1) and the α-subunit (AtGPA1) of the G protein, respectively, were used and results confirmed the hypothesis that sugar sensing in seed germination requires the biosynthesis of endogenous ABA.

RESULTS

Stratification Is Not Required for the Seed Germination of the rgs1-2 Mutant

Generally, Arabidopsis seeds require either after-ripening process and/or cold stratification (moist prechilling at 4°C) for facilitating germination (Bewley, 1997). We first compared the germination of wild-type Columbia (Col) and rgs1-2 seeds after different times of harvesting and with or without the stratification (Fig. 1). One week after harvest, nearly 80% of the seeds germinated for rgs1-2, in contrast to less than 20% for Col seeds. More than 90% of the germination rate was recorded for rgs1-2 seeds after 2 weeks of harvest, as compared with about 40% of germination for Col seeds (Fig. 1A). In the absence of stratification, rgs1-2 seeds showed a much higher germination than that of Col seeds, although stratification treatment had a positive influence on the seed germination for both Col and rgs1-2 seeds, especially for Col seeds (Fig. 1, B and C).

Seed Germination of the rgs1-2 Mutant Is Less Sensitive to Sugars

The response of rgs1-2 mutant germination to several mono- and disaccharides, as compared to those of gpa1-3 mutant and wild-type Col-0, was quantified. Figure 2 showed that, when Glc concentration was less than 3% and Suc concentration less than 4%, there were no obvious differences in seed germination among Col, gpa1-3, and rgs1-2. Higher sugar concentrations had inhibitory effects on seed germination and different genotypes had different sensitivities. When Glc concentration was more than 6%, the germination of gpa1-3 was almost completely arrested, whereas about 50% of the rgs1-2 mutant seeds still germinated (Fig. 2A). Similar results were also observed for the effect of Suc on seed germination (Fig. 2B), although the inhibitory effect was less than that of Glc. No differences in seed germination were observed among the different genotypes when treated with various concentrations of mannitol and sorbitol (Fig. 2, C and D), suggesting that the insensitivities of rgs1-2 seed germination to Glc and Suc were due to sugar signaling rather than osmotic stress sensing.

Figure 2. Effects of different kinds of sugars on seed germination. Matched seed lots of Col, rgs1-2, and gpa1-3 were sterilized, stratified, and sown on plates containing the indicated concentrations of Glc (A), Suc (B), mannitol (C), and sorbitol (D) under continuous white light at 22°C. The germination was scored after 5 d. Values presented are the mean germination rate from three separate experiments, and the error bar indicates ±ss.
Seed Germination of the rgs1-2 Mutant Has an Altered Sensitivity to ABA

To clarify if the insensitivity of rgs1 mutant seed germination to Glc and Suc observed in this experiment operated via the ABA signaling pathway, seeds were pretreated with the ABA biosynthesis inhibitor fluridone (100 μM) and/or exogenously applied ABA between 0 and 8 μM. Seed germination of all genotypes was suppressed by ABA treatment, but less sensitivity of rgs1-2 seed germination to ABA was observed as compared with those of Col and gpa1-3 (Fig. 3, A and B). Fifty-percent germination occurred at approximately at 3.5, 3, and 1.5 μM of exogenous ABA for rgs1-2, Col, and gpa1-3, respectively (Fig. 3A). When seeds were germinated on plates supplemented with 2 μM ABA for different times, the germination rate increased with the increment of incubation time. On

Table 1. Effects of fluridone and Glc treatments on endogenous ABA levels in germinating seeds

Seeds were treated with deionized water or 100 μM fluridone for 48 h at 4°C, immersed in 0.5 × MS medium plus or minus 6% Glc. After being incubated in light at 22°C for 5 d, the seeds were rinsed and lyophilized for ABA quantification. Small and capital letters represent significant difference at P < 0.05 and P < 0.01 levels of probability, respectively.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>ABA Level (nmol g⁻¹ dry weight)</th>
<th>Col-0</th>
<th>rgs1-2</th>
<th>gpa1-3</th>
<th>35S-RGS1</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>6.13 ± 0.23B</td>
<td>4.45 ± 0.29BC</td>
<td>6.38 ± 0.15B</td>
<td>8.47 ± 0.23B</td>
<td></td>
</tr>
<tr>
<td>Fluridone</td>
<td>4.94 ± 0.37C</td>
<td>3.88 ± 0.20C</td>
<td>5.06 ± 0.31C</td>
<td>6.09 ± 0.20C</td>
<td></td>
</tr>
<tr>
<td>6% Glc</td>
<td>7.70 ± 0.31A</td>
<td>5.30 ± 0.20A</td>
<td>8.16 ± 0.36A</td>
<td>10.63 ± 0.22A</td>
<td></td>
</tr>
<tr>
<td>Fluridone + 6% Glc</td>
<td>5.03 ± 0.19C</td>
<td>4.76 ± 0.28AB</td>
<td>5.42 ± 0.38BC</td>
<td>6.88 ± 0.15BC</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Altered sensitivities to ABA in rgs1-2 mutant seed germination. Matched seed lots were pretreated with deionized water or 100 μM fluridone for 48 h at 4°C before being placed at 22°C under continuous white light for germination. A, Germination of wild-type Col, rgs1-2, and gpa1-3 mutant seeds in the presence of the indicated concentrations of ABA at 5 d after stratification. B, Germination of wild-type Col, rgs1-2, and gpa1-3 mutant seeds in the presence of 2 μM ABA over time (days after stratification). C, Seeds pretreated with deionized water (no fluridone treatment, No Flu) or 100 μM fluridone (Flu) were washed and sown on plates with or without 2 μM ABA. D, Seeds pretreated with deionized water or 100 μM fluridone were washed and sown on plates containing 6% Glc. Values presented are the mean germination rate from three separate experiments, and the error bar indicates ±SE. Data in A and B were statistically analyzed with t test. Single asterisk indicates significance at P < 0.05 level and double asterisk indicates significance at P < 0.01 level.
day 8 of incubation, about 94% of rgs1-2 seeds germinated, but only 67% of gpa1-3 mutant seeds germinated (Fig. 3B). When seeds were pretreated with 100 μM of fluridone, the inhibition of exogenous ABA on seed germination was largely alleviated (Fig. 3C). Furthermore, fluridone pretreatment significantly reduced the inhibitory effect of Glc on seed germination and the difference of Glc-induced inhibition of seed germination among genotypes largely diminished (Fig. 3D). These results suggest that the rgs1-2 seed germination is less sensitive to ABA and the different responses of seed germination among the three genotypes to exogenous ABA or Glc are largely due to the different levels of endogenous ABA. This hypothesis was confirmed further by measuring the endogenous ABA level. As compared with those of control treatments (grown on normal culture medium), fluridone pretreatment reduced endogenous ABA levels by 19.4%, 12.8%, and 20.7% for the Col, rgs1-2, and gpa1-3, respectively. In contrast, 6% Glc treatment increased ABA levels by 25.6%, 19.1%, and 27.9%, respectively, for Col, rgs1-2, and gpa1-3 (Table I).

Overexpressors of AtRGS1 Proteins Are Hypersensitive to Glc and ABA in Seed Germination Process

The overexpression approach was used to examine the in vivo responses of AtRGS1 in seed germination to Glc and ABA. The coding region of AtRGS1 was fused to the 35S promoter of Cauliflower mosaic virus and transformed to Arabidopsis (Col-0) plants (see “Materials and Methods”). After preliminary analysis, transgenic lines with higher RGS1 expression level were selected for more detailed analysis. As shown in Figure 4A, seed germination of wild-type Col on the control culture medium was almost identical to that of overexpressors. However, in the presence of either Glc (6%) or ABA (2 μM), seed germination of overexpressors (35S) was almost completely inhibited (less than 20%), suggesting an increased sensitivity of 35S RGS1. When ABA concentration was more than 1 μM, seed germination of overexpressor was nearly completely arrested, whereas those of the wild-type Col and rgs1-2 continued to maintain at a high level (Fig. 4B), confirming that AtRGS1 protein is required for the Glc and ABA sensing during seed germination.

The Stimulation of ABA Biosynthesis Is Responsible for Glc-Induced Inhibition of Seed Germination

To investigate whether RGS1 protein is related to ABA biosynthesis in the seed germination, we also looked at the effects of Glc treatment on the expression of two key genes encoding two enzymes that catalyze ABA biosynthesis, i.e. NCED3 and ABA2, in wild-type Col, rgs1-2, and gpa1-3 mutants and 35S-RGS1 transgenic plants. Earlier evidence indicates that 9'-cis-epoxycarotenoid dioxygenase (NCED) catalyzes the oxidative cleavage from 9'-cis-neoxanthin to xanthoxin, which is a key step controlling ABA biosynthesis (Iuchi et al., 2001), and ABA2 encodes xanthoxin oxidase, which catalyzes xanthoxin oxidation to form abscisic aldehyde (Schwartz et al., 1997). In this experiment, seeds were germinated either on control medium without Glc or on medium supplemented with 6% Glc, and germinating seeds were harvested on day 5 after sowing. By both reverse transcription (RT)-PCR and real-time quantitative PCR, lower expression of NCED3 and ABA2 was observed in rgs1-2 mutants and overexpression of RGS1 significantly enhanced the transcription level of NCED3 and ABA2 genes, as compared with their wild-type counterparts. Glc (6%) treatment significantly induced expression of NCED3 and ABA2 for Col and ABA2 for 35S-RGS1, but there was little effect of Glc on the expression of NCED3 for
DISCUSSION

In metazoans, heterotrimeric G proteins couple stimulus perception by GPCRs with numerous downstream effectors. In the human genome, at least 800 GPCRs, 17 Ga, five Gβ, and 12 Gγ genes have been found. However, in Arabidopsis, only one GCR1, one canonical Ga gene, one Gβ, and two candidates for Gγ genes have been identified (for review, see Jones and Assmann, 2004). Despite the apparent simplicity of G-protein-mediated signaling elements in plants, G proteins have been involved in the regulation of many physiological processes, including seed germination (Ullah et al., 2002), the responses to light (Okamoto et al., 2001; Jones et al., 2003), phytohormones (Hooley, 1999; Ullah et al., 2002; Lapik and Kaufman, 2003), stresses (Coursol et al., 2003), and cell division and elongation (Ullah et al., 2001). Furthermore, G-protein signaling is itself regulated at different levels with different mechanisms. One of the most effective regulatory elements at the level of heterotrimeric G protein is the RGS proteins (McCudden et al., 2005). RGS proteins act as an effective desensitizer of G-protein-mediated signaling by accelerating the deactivation of G-protein α-subunits from G-protein GTP form to G-protein GDP form (Neubig and Siderovski, 2002). More recently, Chen et al. (2003) have identified a RGS-like protein in Arabidopsis (called AtRGS1). Genetic and biochemical evidences have shown that AtRGS1 strongly interacts with Ga-subunit (AtGPA1) and has a GTPase activity, implying that AtRGS1 protein may be involved in the G-protein-mediated signaling (Chen et al., 2003). Further studies have shown that AtRGS1 modulates plant cell proliferation and is involved in sugar signaling (Chen and Jones, 2004). However, many aspects of the roles of AtRGS1 protein in regulating physiological processes in plants have yet to be elucidated. In this study, using seeds that carry the null mutation in the gene encoding RGS1 (AtRGS1) and the α-subunit (AtGPA1) of the G protein in Arabidopsis (named rgs1-2, gpa1-3, and the wild-type Col-0), we presented genetic and molecular evidence to show that AtRGS1 protein plays important roles in Glc- and ABA-mediated signaling during seed germination.

Low Endogenous ABA Level May Be Responsible for the Lack of After-Ripening and Stratification Process for Seed Germination of the rgs1-2 Mutant

Seed germination is a complicated process and regulated by many factors, such as sugars (for review, see Sheen et al., 1999; Gibson, 2005), phytohormones (Ullah et al., 2002; Chen et al., 2004), as well as germination conditions (Chen and Jones, 2004). Usually, full germination capacity cannot be realized until finishing an after-ripening process (several weeks after harvest) and satisfying stratification conditions (light and chilling). In this study, we demonstrate that stratification appears not to be necessarily required for seed germination and after-ripening process has no obvious influence on this process for the rgs1-2 mutant, as compared with those of Col and gpa1-3 (Fig. 1), although the exact mechanism is still to be explored. It has been well documented that ABA plays important roles in controlling seed maturation and germination (Koornneef et al., 2002). During seed maturation, ABA accumulates in all seed tissues, either as a result of transportation from the mother plant through the phloem (Zeevaart and Boyer, 1984; Zhong et al., 1996) or synthesized in seeds. ABA level decreases during seed desiccation and is relatively low in mature seeds. During seed germination, endogenous ABA level decreases further as a consequence of ABA breakdown. So, it is suggested that active ABA level is the major modulator controlling seed germination. It is reasonable to hypothesize that the absence of requirement of stratification and after-ripening processes for rgs1-2 seed germination may be the result of low endogenous ABA level. To test this hypothesis, we measured the endogenous active ABA levels in Col and rgs1-2 mutant seeds. The endogenous ABA level of Col seeds (6.31 nmol g⁻¹ dry weight) is 18.4% higher than that of rgs1-2 seeds (data not shown). Of course, the active endogenous ABA level is not only determined by ABA synthesis, but also by ABA catabolism and translocation. So, detailed studies are needed and a better knowledge of ABA catabolism and translocation would be valuable for the understanding of ABA action in seed germination.

The Insensitivity of rgs1-2 Seed Germination to Glc Is Due to the Lower Endogenous ABA Level

It had been shown recently that AtRGS1 seedling growth is insensitive to Glc and less sensitive to Fru and Suc, implying that AtRGS1 protein mediates sugar signaling in Arabidopsis (Chen and Jones, 2004). We provide here further evidence that the seed germination of rgs1-2 is insensitive not only to Glc but also to Suc and the insensitivities were not due to the osmotic effect because the germination of rgs1-2 mutant seeds...
had the same responses to the same concentrations of mannitol and sorbitol as those of gpa1-3 mutants and wild-type Col-0 (Fig. 2). Furthermore, the germination of overexpressors of AtRGS1 was shown to be hypersensitive to Glc. All these results suggest that AtRGS1 is involved in the sugar signaling in the process of seed germination. However, it is still unclear how AtRGS1 protein mediates the seed germination responses to Glc. Using different mono- and disaccharide sugars as well as sugar analogs, Chen et al. (2004) demonstrated that sugar metabolism and phosphorylation by HXK are not required for AtRGS1-mediated sugar signaling.

Currently, our understanding of the sugar signaling is that it is not simply linear but composed of a complex signaling network. The cross talk between sugar and phytohormones during seed germination has received intensive attention. It has been proposed that the sugar inhibition of seed germination is due to the increase in the active endogenous ABA level (Arenas-Huertero et al., 2000). The results that ABA-biosynthetic and -insensitive mutant seeds are insensitive to Glc provide additional genetic evidence to support the above hypothesis (Huijser et al., 2000; Laby et al., 2000). Recently, Ullah et al. (2002) has shown that reduction of endogenous ABA by ABA biosynthesis inhibitor can eliminate the hypersensitivity of gpa1 mutant germination to Glc, implying that G-protein-mediated sugar signaling is operating, at least to some extent, via ABA signaling. In other experimental systems, evidence has been accumulated that G proteins participated in early events in ABA signaling. The loss of function for the Go gene (GPA1) exhibits insensitivity to ABA inhibition of stomatal opening and altered ABA responsiveness of guard cell inward K\(^+\) channels and slow anion channels (Wang et al., 2001).

It is reasonable to assume that AtRGS1-mediated germination responses to sugars may also be involved in the ABA signaling. To confirm this hypothesis, two methods were used in this study. First, we compared the seed germination in the media containing different concentrations of exogenous ABA. Second, we reduced the active endogenous ABA level using the ABA biosynthetic inhibitor, fluridone. We measured the active endogenous ABA levels of germinating seeds of the different genotypes investigated and the results showed that the rgs1-2 mutant had lower ABA level than that of Col-0. Fluridone treatment significantly reduced Glc-induced ABA biosynthesis (Table I) and markedly alleviated the inhibitory effects of Glc on seed germination (Fig. 3D). Moreover, fluridone treatment obviously diminished the differences of the seed germination responses to Glc among gpa1-3, Col, and rgs1-2 genotypes (Fig. 3D). These results provide strong experimental evidence that Glc-induced inhibition of seed germination is largely through increasing endogenous ABA level and the insensitivity of rgs1-2 seed germination to Glc is due to the lower endogenous ABA level in the presence of Glc, as compared with that of Col-0.

We now have a better understanding of ABA biosynthesis and its regulation in the vegetative tissues, especially under the stress conditions. However, limited progress has been made on the regulation of ABA biosynthesis in seeds. To date, only a few ABA biosynthetic steps have been described in detail (Finkelstein et al., 2002; Nambara and Marion-Poll, 2003; Xiong and Zhu, 2003). NCED, a key enzyme regulating ABA biosynthesis in vegetative tissues of many species, has been reported to control ABA level in seeds, and overexpression of NCED genes increases seed ABA content and seed dormancy, and delays seed germination (Thompson et al., 2000; Qin and Zeevaart, 2002). We also analyzed the expression of NCED3 and ABA2 genes by real-time quantitative PCR. Lower and higher expression of NCED3 and ABA2 was observed in rgs1-2 mutants and overexpressors of RGS1, respectively, as compared with that in Col. Treatment of 6% Glc had no obvious influences on the expression of NCED3 and ABA2 of the rgs1-2 mutant, but markedly induced expression of NCED3 and ABA2 in Col (Table II), which was consistent with the different ABA contents in

### Table II. Relative expression of NCED3 and ABA2 genes in wild-type Col, rgs1-2 mutant, and 35S-RGS1 transgenic seedlings in response to 6% Glc treatment

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Genotype</th>
<th>NCED3</th>
<th>ABA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Col</td>
<td>$1 \pm 0.34^{30c}$</td>
<td>$1 \pm 0.37^{38c}$</td>
</tr>
<tr>
<td></td>
<td>rgs1-2</td>
<td>$0.16 \pm 0.23^{30c}$</td>
<td>$0.24 \pm 0.29^{30c}$</td>
</tr>
<tr>
<td>6% Glc</td>
<td>Col</td>
<td>$17.29 \pm 0.75^{38c}$</td>
<td>$5.12 \pm 0.41^{38c}$</td>
</tr>
<tr>
<td></td>
<td>rgs1-2</td>
<td>$0.27 \pm 0.61^{30c}$</td>
<td>$0.44 \pm 0.50^{30c}$</td>
</tr>
<tr>
<td>35S-RGS1</td>
<td>$90.59 \pm 0.79^{38a}$</td>
<td>$95.27 \pm 0.96^{38a}$</td>
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</table>

*NCED3 and ABA2 gene expression levels are presented as relative expression values compared with the wild-type Col-0 (Fig. 2).*
different genotypes investigated (Table I). These results provide additional evidence that the increase in seed endogenous ABA level induced by Glc is likely to rely partly on the stimulation of the expression of NCED3 and ABA2 genes, and AtRGS1 might act as a positive component in this signaling event.

In conclusion, based on the results presented in this study, we conclude that AtRGS1 protein is involved in the regulation of seed germination, and the hyposensitivity of rgs1-2 mutant seed germination to Glc might be the result of the impairment of ABA biosynthesis during seed germination (Fig. 6). Understandably, seed germination is a very complicated process and complex interactions between various signaling pathways would be expected. Thus, further work is required in this direction.

MATERIALS AND METHODS

Germination Assay

Germination rates were compared between seed lots that were produced, harvested, and stored under identical conditions. Before planting, seeds were surface sterilized with 70% ethanol for 1 min, then with 2% hypochlorite for 5 min, and rinsed five times with sterile deionized water. Fifty to 100 seeds from wild type (Col-0), rgs1-2, gpa1-3, and 35S::AtRGS1 seeds were stratified at 4°C for 48 h and planted in petri dishes on half-strength Murashige and Skoog (MS) 0.8% phytoagar medium lacking Suc and Gamborg vitamins at 22°C under continuous white light in triplicate. Seeds were considered germinated when the radicles completely penetrated the seed coat.

Hormonal and Sugar Treatment

The effects of ABA on seed germination were studied by determining the endogenous ABA level induced by Glc is likely to rely partly on the expression of the target gene relative to reference gene (ACTIN2) in a test sample compared with an untreated Col sample.

RNA Isolation, RT-PCR, and Real-Time PCR

Total RNA was prepared from plants by using the RNeasy plant minikit (Qiagen). Two micrograms of RNA was used as a template for first-strand cDNA synthesis using the SuperScript II first-strand synthesis system for RT (Invitrogen). PCR amplification was performed using Taq DNA polymerase (Invitrogen).

Real-time quantitative PCR was run on an ABI 7500 real-time PCR system (Applied Biosystems) according to the manufacturer’s recommendations. Real-time quantitative PCR reaction contained 25 μL 2 × SYBR Premix Ex Taq (TaKaRa), 2 μL primer mix, 1 μL ROX Reference Dye II, 4 μL cDNA, and 18 μL deionized water to make a total volume of 50 μL. After setting the amplification conditions, experiments were repeated twice. The primers were as follows: ABA2 (At1g52340), 5′-ccagtgtttggtcattgc-3′ and 5′-ccagtttccaccccctt-3′; N Ced3 (At3g14440), 5′-ccagttggtgcgcaacaaac-3′, and 5′-ccacgctcctccaagag-3′; and Actin2 (At3g18780), 5′-gctgaggactcagactcctc-3′ and 5′-cacaagtttggtcctgtcagag-3′. For relative quantification the method of Pfaffl (2001) was used to determine the relative expression ratio. This determines the expression of the target gene relative to reference gene (ACTIN2) in a test sample compared with an untreated Col sample.

ABA Extraction and Quantification by ELISA

Sterilized seeds were immersed in deionized water or fluridone and kept in darkness at 4°C for 2 d and moved to plates with or without 6% Glc. After being cultured in light at 22°C for 5 d, the seeds and seedlings were lyophilized. Samples were ground in an ice-cooled mortar in 4 mL of 80% (v/v) methanol extraction medium containing 1 mM butylated hydroxytoluene as an antioxidant. The extract was incubated at 4°C for 24 h and centrifuged at 7,000 rpm for 15 min at the same temperature. The supernatant was passed through Chromosep C18 columns (C18 Sep-Pak Cartridge; Waters) and prewashed with 10 mL of 100% (w/v) methanol, respectively. Two milliliters of hormone fractions eluted from the columns were dried under N2 and dissolved in 0.5 mL phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5) for ABA analysis by competitive ELISA.

The antigens (ABA hapten-carrier protein), mouse monoclonal antibodies against ABA, and IgG hors eradish peroxidase used in ELISA were produced at the Phytohormones Research Institute (China Agricultural University). ELISA was performed on a 96-well microplate. Each well on the plate was coated with 100 μL coating buffer (1.5 g L−1 Na2CO3, 2.93 g L−1 NaHCO3, and 0.02 g L−1 Na2SO3, pH 9.6) containing 0.25 μg mL−1 antigens. The coated plates were incubated for 30 min at 37°C, and then kept at room temperature for 3 to 4 min. After washing three times with PBS-Tween 20 buffer (0.1% (v/v) Tween 4), each well was filled with 50 μL of either extracts or ABA standards (0–2000 ng mL−1 dilution range), and 50 μL of 20 μg mL−1 ABA antibodies. The plate was incubated for 30 min at 37°C, and then washed as above. One-hundred microliters of 1.25 μg mL−1 IgG horseradish peroxidase substrates was added to each well and incubated for 30 min at 37°C. The plate was rinsed four times with the above PBS-Tween 20 buffer, and 100 μL color-appearing solution containing 1.5 mg mL−1 orthophenylenediamine and 0.008% (v/v) hydrogen peroxide was added to each well. The reaction was stopped by adding 50 μL 4 M H2SO4 per well when the 2000 ng mL−1 standard had a pale color and the 0 ng mL−1 standard had a deep color in the wells. Color development in each well was detected using a Microplate Reader (model EL310, Bio-TEK) at optical density A490. The results are the means ± se of at least three replicates.

Constructs and Arabidopsis Transformation

The open reading frame of AtRGS1 was amplified by PCR (primers 5′-ccaggtggagggagtatgtc-3′ and 5′-actcctaccccggactcctc-3′) from a cDNA library made from seedlings grown in light for 2 weeks and cloned into the pENTR/D-TOPO vector (Invitrogen), subcloned into Gateway plant transformation destination vector pGWB2 (Research Institute of Molecular Genetics) by an LR recombination reaction, and transformed into Arabidopsis (Arabidopsis thaliana; Col-0 ecotype) according to the vacuum infiltration method (Bechtold and Pelletier, 1998) using Agrobacterium tumefaciens strain GV3101. In this vector, expression of RGS1 was driven by the 35S promoter of the Cauliflower mosaic virus. For the phenotypic investigation, T3 or T4 homozygous lines were used.

Statistical Analysis

The significance analyses were made using the SAS statistical analysis package (version 6.12; SAS Institute). Multiple comparisons were made among different treatments and genotypes. Small and capital letters represent significant difference at P < 0.05 and P < 0.01 levels, respectively (Tables I and II).

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