Seed Germination of GA-Insensitive sleepy1 Mutants Does Not Require RGL2 Protein Disappearance in Arabidopsis

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We explore the roles of gibberellin (GA) signaling genes SLEEPY1 (SLY1) and RGA-LIKE2 (RGL2) in regulation of seed germination in Arabidopsis thaliana, a plant in which the hormone GA is required for seed germination. Seed germination failure in the GA biosynthesis mutant ga1-3 is rescued by GA and by mutations in the DELLA gene RGL2, suggesting that RGL2 represses seed germination. RGL2 protein disappears before wild-type seed germination, consistent with the model that GA stimulates germination by causing the SCFSLY1 E3 ubiquitin ligase complex to trigger ubiquitination and destruction of RGL2. Unlike ga1-3, the GA-insensitive sly1 mutants show variable seed dormancy. Seed lots with high seed dormancy after-ripened slowly, with stronger alleles requiring more time. We expected that if RGL2 negatively controls seed germination, sly1 mutant seeds that germinate well should accumulate lower RGL2 levels than those failing to germinate. Surprisingly, RGL2 accumulated at high levels even in after-ripened sly1 mutant seeds with 100% germination, suggesting that RGL2 disappearance is not a prerequisite for seed germination in the sly1 background. Without GA, several GA-induced genes show increased accumulation in sly1 seeds compared with ga1-3. It is possible that the RGL2 repressor of seed germination is inactivated by after-ripening of sly1 mutant seeds.

INTRODUCTION

The seed is the main mechanism of plant propagation, and the proper regulation of seed dormancy and germination is of critical importance to plant survival. Seeds that fail to germinate under favorable environmental conditions are said to be dormant (reviewed in Bewley and Black, 1994). Dormant seeds acquire the ability to germinate following a period of dry storage called after-ripening. Dormancy helps to ensure the survival of a species by allowing some ungerminated seeds to survive natural catastrophes. Seed dormancy is also important from the agronomic viewpoint because the absence of dormancy in several cereal grains causes preharvest sprouting when cool humid conditions persist close to harvest. There are two classes of seed dormancy: coat-imposed and embryo dormancy. In coat-imposed dormancy, radicle emergence is blocked by the physical barrier of the testa, aleurone, or endosperm layer. Seed coat–imposed dormancy, which is commonly found in Arabidopsis thaliana, can be overcome by cutting the seed coat (Debeaujon and Koornneef, 2000). Conversely, seeds with embryo dormancy will not germinate when the seed coat is cut (Bewley and Black, 1994). Germination of dormant seeds can also be stimulated by a period of cold imbibition called stratification prior to imbibition under conditions that stimulate germination.

Genetic analysis of seed dormancy is complicated by the fact that the degree of dormancy is dependent on both environmental and genetic factors. However, some genes influencing the degree of seed dormancy have been cloned. For example, quantitative trait loci analysis of a cross between highly dormant Arabidopsis ecotype Cvi (Cape Verde Islands) and the less dormant ecotype Landsberg erecta (Ler) has identified seven quantitative trait loci associated with seed dormancy (Alonso-Blanco et al., 2003). Loss of the COMATOSE gene encoding a putative ATP binding cassette transporter results in strong seed dormancy and reduced germination potential without affecting seed or plant morphology (Russell et al., 2000; Footitt et al., 2002). Loss of the DOF AFFECTING GERMINATION1 gene encoding a putative zinc finger transcription factor results in reduced seed dormancy as a maternal effect without affecting seed coat structure (Papi et al., 2000, 2002). Other examples of genes involved in the establishment and maintenance of seed dormancy include ABSCISIC ACID INSENSITIVE3, LEAFY COTYLEDON1 (LEC1), LEC2, and FUSCA3 (Ooms et al., 1993; Keith et al., 1994; Nambara et al., 1995; Parcy and Giraudat, 1997).

Seed dormancy is subject to hormonal control. The plant hormone abscisic acid (ABA) is needed to induce seed dormancy during embryo maturation, whereas the hormone gibberellin (GA) is needed to stimulate seed germination. Mutants with decreased ABA biosynthesis or sensitivity show decreased seed dormancy, whereas mutants with decreased ABA catabolism or increased ABA sensitivity show increased seed dormancy (reviewed in Finkelstein and Rock, 2002; Kusihro et al., 2004; Okamoto et al., 2006).

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Several lines of evidence demonstrate the importance of GA biosynthesis and signaling for stimulating seed germination in Arabidopsis. The role of GA in promoting various stages in Arabidopsis growth and development is demonstrated by the phenotypes of GA biosynthesis mutants like ga1-3, including severe dwarfism, male sterility, and strong coat-imposed seed dormancy (reviewed in Olszewski et al., 2002). GA application rescues all of these defects. GA does not appear to directly control dormancy per se but acts as an antagonist to ABA by stimulating radicle emergence (reviewed in Bewley, 1997). Many GA biosynthetic proteins are upregulated in seed tissues during seed germination and in the cold (Yamaguchi et al., 2001; Penfield et al., 2006). GA signaling is also required for efficient seed germination in Arabidopsis. Mutations in the SLEEPY1 (SLY1) GA signaling gene lead to increased seed dormancy, and a triple knockout of the GA receptors GID1A, GID1B, and GID1C leads to failure to germinate (Steber et al., 1998; Griffiths et al., 2006).

Our current understanding of the control of Arabidopsis seed germination by GA signaling is derived mostly from studies of the GA signaling genes SLY1 and the DELLA gene RGA-LIKE2 (RGL2) (Steber et al., 1998; Lee et al., 2002; Bassel et al., 2004; Tyler et al., 2004). DELLA proteins are a subfamily of the GRAS (for GA, RGA, and Scarecrow) family of putative transcription factors that are nuclear-localized and negatively regulate GA signaling (Peng et al., 1999; Pysh et al., 1999). There are five DELLA proteins in Arabidopsis with partially overlapping functions in GA signaling. Consistent with the negative role of DELLA proteins, loss-of-function mutations in DELLA genes result in a decreased requirement for GA in plant growth and development. Based on phenotypic analysis, the main negative regulator of stem elongation is the DELLA protein RGA, although GAI and RGL1 also function in stem elongation. DELLA proteins RGA, RGL2, and RGL1 are the main regulators of flowering (reviewed in Thomas et al., 2005). No function has yet been attributed to RGL2 protein is required for seed germination and that rapid disappearance of RGL2 protein requires the SCF SLY1 E3 ubiquitin ligase complex (Tyler et al., 2004). Based on genetic evidence, it is clear that RGL2 negatively regulates seed germination. The question that remains is whether the decreases in RGL2 transcript and protein levels are the sole mechanisms controlling GA-stimulated seed germination.

To address this question, this study examines whether the pattern of RGL2 protein accumulation in sly1 mutants is consistent with the current model for control of seed germination by RGL2. This study shows that the dormant seed phenotype of sly1 mutants is variable between different seed lots. Those seed lots showing high seed dormancy eventually after-ripen. Stronger alleles show a stronger seed germination phenotype and require more time to after-ripen. For example, the sly1-1 allele can require up to 2 years to fully after-ripen. Based on the current model, we expected that the level of RGL2 protein accumulation should be higher in those sly1 lines that show lower germination capacity. Surprisingly, we found that RGL2 protein accumulated at high levels in sly1 mutants regardless of the degree of seed dormancy. RGL2 protein accumulated to the same level in after-ripened and dormant sly1-2 seeds prior to radicle emergence.
Indeed, after-ripened sly1-2 seeds that were capable of 91% germination expressed high levels of RGL2 protein. This suggests that SCFSLY1 is the main E3 ubiquitin ligase controlling RGL2 protein accumulation and that in the sly1 mutant background, RGL2 protein disappearance is not a prerequisite for seed germination. The possibility that the RGL2 protein accumulating in after-ripened sly1 mutant seeds is either inactive or controlled by other factors is considered.

RESULTS

Isolation of a New SLY1 Allele

Here, we compare two new mutant alleles to the two recessive alleles of sly1 in the Ler background previously described (Figure 1A; Steber et al., 1998; Steber and McCourt, 2001; McGinnis et al., 2003). The sly1-2 allele is a 2-bp deletion causing a frame shift and the predicted loss of the last 40 out of 151 amino acids. The sly1-10 allele contains a 23-bp deletion followed by an ~8-kb insertion, causing the predicted loss of the last eight amino acids and the addition of 46 random amino acids (Figure 1A). The new allele sly1-DS in the Ler background resembles sly1-10 and contains a transposon insertion resulting in loss of the last 12 amino acids (Sundaresan et al., 1995). The new sly1-t2 allele in the Wassilewskija (Ws) background was identified in the Sussman lines (Krysan et al., 1999). It contains a T-DNA insertion within the F-box motif (at +219 bp) and is predicted to result in loss of the last 78 amino acids. This is the most severe lesion identified in the SLY1 gene thus far. The sly1-t2 allele showed severe defects in plant development, such as dwarfism and reduced fertility (see Supplemental Table 1 online). The effect of each allele on SLY1 mRNA levels was examined by RT-PCR using SLY1 gene-specific primers (Figure 1B). When the SLY1-specific primers were employed, no significant change in mRNA accumulation was observed in sly1-10 and sly1-2, whereas a marked decrease was seen in sly1-t2. These results indicate that SLY1 transcript accumulation is reduced by the T-DNA insertion in sly1-t2, whereas the other two alleles, sly1-10 and sly1-2, do not alter transcript levels compared with the wild type.

The sly1 Germination Phenotype Is Variable but Dependent on the Allele and the Age of the Seed

Because GA synthesis is required for seed germination, it was expected that GA signaling would also be required for seed germination. Consistent with this, mutants in the GA signaling gene SLY1 cause increased seed dormancy and increased sensitivity to ABA in seed germination (Strader et al., 2004). Subsequently, anecdotal evidence showed that the sly1 seed dormancy phenotype was variable. To characterize the sly1 seed dormancy phenotype, the percentage of germination of many independent sly1 seed lots of different ages were compared after 7 d of incubation at 22°C (Figure 2). While the germination capacity of independent seed lots varied significantly, it did correlate with allele and with the period of seed dry storage.

In general, sly1-2 seeds showed stronger dormancy than sly1-10 seeds. The sly1-t2 allele showed the highest degree of seed dormancy. However, the germination capacity of sly1-t2 seeds cannot be directly compared with sly1-2 and sly1-10 because it is in a different genetic background. In sly1-10, the germination rate of the 0.5-month-old seed lots ranged from 8.0 to 98.4% (Figure 2A). Less variation in germination rate was seen after seeds were subjected to 1 to 5 months (77.1 to 100%) and 12 to 31 months (96.3 to 100%) of room temperature dry storage. The sly1-2 allele showed more severe seed dormancy (Figure 2B). In sly1-2, the germination rate of the 0.5-month-old seed lots ranged from 2.0 to 43.0%. The germination rate increased following longer periods of dry storage for 1 to 5 months (22.0 to 56.1%), 12 to 20 months (64.2 to 75.4%), and 26 to 28 months (73.4 to 91.1%). Unlike sly1-10, sly1-2 seeds never reached 100% germination. The sly1-t2 seeds showed the most severe dormancy phenotype. No seed germination was observed in 16 independent seed lots stored for 8 months or less, and seeds stored for 11 to 18 months showed between 0 and 9.6% seed germination (Figure 2C; data not shown). It is not known whether sly1-t2 seeds ever fully after-ripen. These results indicate that the degree of seed dormancy is dependent on the severity of the sly1 allele. However, other factors, possibly environmental, may cause the variation in dormancy.

The data in Figures 2A and 2B show that both sly1-2 and sly1-10 seeds lose their capacity to germinate following long periods of dry storage. In sly1-10, seed lots stored for 49 to 54 months showed 32.9 to 72.3% seed germination, whereas sly1-2 seeds stored for 56 to 66 months showed 9.2 to 25.2% germination. It is possible that this loss of germination capacity is due to seed deterioration and that this loss of viability is more severe in the stronger allele.

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Figure 1. Structure of the SLY1 Gene and the SLY1 mRNA Expression Detected by RT-PCR in Three Mutant Alleles.

(A) The mutation sites are shown in three alleles. The sly1-10 mutant has an 8-bp insertion at +429 nucleotides from translational start. The sly1-2 mutant has a 2-bp deletion at the +333-nucleotide position. The T-DNA is inserted at the +219-nucleotide position in sly1-t2.

(B) The top panel shows the SLY1 expression in three mutant alleles and the wild type using the sly1-2f and sly1-2r primers (pair A) that flank the insertion in sly1-12 giving no product. The middle panel shows the expression using the 2-63f and sly1-r primers (pair B) that flank the sly1-10 rearrangement giving no product. The bottom panel shows the ACTIN2 control.
To clarify if dormancy or poor viability caused seeds to show low percentage of germination, intact seeds were stained with tetrazolium solution, a vital stain (Debeaujon et al., 2000). In sly1-2, a similar staining pattern was seen in a 0.5-month-old seed lot (#35) showing 2% germination as in a 26-month-old seed lot (#21) showing 91.1% seed germination (Figures 2A, 2B, and 3A). A similar staining pattern was also seen in Ler (wild-type), ga1-3, and sly1-10 seeds of comparable age (data not shown). By contrast, reduced staining (66.8% stained) was seen in sly1-2 seeds stored for 63 months (#7) and giving 10% germination (Figures 2B and 3A). By contrast, 63-month-old Ler seeds showed 100% germination and 84.3% tetrazolium-stained seeds. These data suggest that the low percentage of germination in 0.5-month-old sly1-2 seeds is due to seed dormancy, whereas the reduction in germination capacity of older sly1-2 seeds is due, at least in part, to loss of longevity. Consistent with this, germination of 0.5-month-old sly1-2 seeds is rescued by cutting the seed coat as is the germination of 0.5-month-old ga1-3 seeds and sly1-t2 (Figure 3B). Failure to achieve 100% germination is probably due to inadvertent damage to the embryo.

**sly1 Mutants After-Ripen Slowly**

The fact that sly1 seed lots subjected to longer periods of dry storage show better seed germination suggests that dormant sly1 seeds are able to after-ripen slowly (Figure 2). Ler (wild-type) seeds are fully after-ripened and show 100% germination after 0.5 months of dry storage (Figure 4). During an after-ripening time course, a single sly1-2 seed lot (#37) showed 18.7% germination.

**Figure 2.** Germination Rates of sly1 Mutant Seeds Varied but Were Dependent on Allele and Age of Seeds.

Seeds plated on MS-agar were incubated 3 d at 4°C followed by 7 d at 22°C. Percentage of germination was calculated based on cotyledon emergence for independent seed lots of: sly1-10 (A), sly1-2 (B), and sly1-t2 (C). Duration of dry storage or age of seed is indicated above the bars (months). N.G., none of the seeds germinated. Error bars show SE (n = 3). Seed lot numbers (#) are indicated on the x axis.

**Figure 3.** Determination of Seed Viability by Tetrazolium Staining and Germination Rescue by Cutting the Seed Coat.

(A) Seeds of the wild type (0.5, 26, and 63 months old), after-ripened sly1-2 (26 months old, seed lot #21), and dormant sly1-2 (0.5 and 63 months old, seed lots #35 and #7, respectively) were stained with 0.5% tetrazolium for 10 h. Fully stained seeds were counted as viable.

(B) To examine seed viability, dormant sly1-2 (seed lot #35, 0.5 months old) and sly1-t2 (seed lot #12, 0.5 months old) seed coats were nicked with forceps to stimulate seed germination. ga1-3 (0.5 months old) seeds were used as a positive control for seed germination rescue. Age of each seed stock is shown below the graph (months). Germination rates (G) are shown below the figure (%). Error bars show SE (n = 30).
after-ripen also appears to depend on the severity of the allele. The period required to reach 100% germination after 0.5 months of dry storage, 53.2% germination after 1 month (28 months old), sly1-10 (27 months old, #3), after-ripened (A) sly1-2 (26 months old, #21), dormant (D) sly1-2 (0.5 months old, #35), 1-month-old wild type (Col), sly1-12 rgl2-13 (1 month old), and sly1-12 (1 month old, crossed to wild-type Col) were used. Error bars show SE (n = 3).

(A) Percentage of radicle emergence based on radicle protrusion from seed coat.

(B) Percentage of cotyledon emergence based on appearance of green cotyledons outside the seed coat.

(C) Rescue of sly1-12 seed germination by the rgl2-13 mutation based on percentage of radicle emergence.

Figure 4. sly Mutant Seeds Germinate More Slowly Than the Wild Type, and Germination Is Rescued by a Mutation in RGL2.

Seeds were plated on MS-agar and the number of germinated seeds scored daily after transfer to 22°C. The wild type (28 months old), sly1-10 (27 months old, #3), after-ripened (A) sly1-2 (26 months old, #21), dormant (D) sly1-2 (0.5 months old, #35), 1-month-old wild type (Col), sly1-12 rgl2-13 (1 month old), and sly1-12 (1 month old, crossed to wild-type Col) were used. Error bars show SE (n = 3).

(A) Percentage of radicle emergence based on radicle protrusion from seed coat.

(B) Percentage of cotyledon emergence based on appearance of green cotyledons outside the seed coat.

(C) Rescue of sly1-12 seed germination by the rgl2-13 mutation based on percentage of radicle emergence.

after 0.5 months of dry storage, 53.2% germination after 1 month of dry storage, and 64.2% after 8 months of dry storage (Figure 2B; see Supplemental Figure 1 online). The period required to after-ripen also appears to depend on the severity of the allele. The sly1-10 seeds reached ~97.0% germination after 1 to 5 months after-ripening, whereas sly1-2 seeds reached 73.4 to 91.1% germination after 26 to 28 months after-ripening (Figure 2).

Segregation analysis was used to examine the dormant seed phenotype of sly1-12. The mutant was crossed with Ws (wild-type), and F2 progeny were sown to check the segregation ratio of germinated-to-ungerminated seeds. Of 133 F2 progeny, 29 failed to germinate, giving a 3:1 segregation ratio ($x^2 = 0.72; 1.0 < P < 0.5$). These results suggested that the seed dormancy phenotype in sly1-10 was caused by a single recessive mutation. When germination was rescued by cutting the seed coat, all of the ungerminated seeds gave rise to dwarf sly1 plants (data not shown). None of the 104 germinated seedlings gave rise to dwarves. The sly1-12 allele shows 0 to 9.9% seed germination following 18 months of after-ripening. Thus, this severe truncation of the SLY1 gene results in strong seed dormancy.

sly1 Seeds Germinate Slowly and Show Increased Sensitivity to Polyethylene Glycol and Paclobutrazol in Germination

After-ripened sly1 mutant seed lots showed reduced germination capacity compared with wild-type Ler seeds. This was first evaluated based on percentage of germination over time. To accurately examine the effect of the sly1 mutations on germination rate, both the percentage of radicle emergence (Figure 4A) and percentage of cotyledon emergence (Figure 4B) were determined over time comparing wild-type Ler with sly1-10 (#3) and dormant and after-ripened sly1-2 seeds (#35 and #21, respectively). Similar curves were obtained using percentage of radicle emergence and cotyledon emergence, except that cotyledon emergence occurred ~1 d (24 h) after radicle emergence. Wild-type Ler and Ws (data not shown) reached 100% radicle emergence within 2 d. Germination was considerably slower in sly1 mutants. The sly1-10 seeds required 5 d to reach 100% radicle emergence, whereas after-ripened sly1-2 seeds (#21) required 7 d to reach a final 85.6% radicle emergence.

Plating experiments using increasing concentrations of polyethylene glycol (PEG) 8000 were used to examine the effect of increasing media osmotic potential on sly1 mutant and wild-type seed germination. We have observed that when sly1-10 and sly1-2 seeds are sown on moistened soil, virtually no seeds germinated (data not shown). This observation suggested that the sly1 mutant seeds have difficulty imbibing water. To examine this, after-ripened wild-type and sly1 mutant seeds were sown on Murashige and Skoog (MS)–agar media containing various low concentrations of PEG 8000. This experiment showed that sly1 seeds were more sensitive to PEG 8000 than the wild type and that sly1-2 showed a more severe germination phenotype than sly1-10 (see Supplemental Figure 2 online). Next, the sensitivity of sly1 seeds to GA biosynthesis inhibitor paclobutrazol (PAC) was examined. After-ripened wild-type, sly1, and ga1-3 mutant seeds were first incubated in 100 μM PAC solution for 3 d at 4°C, and then these seeds were sown on different concentrations of GAs. This experiment showed that sly1 seeds were more sensitive to PAC than the wild type and that sly1-2 showed a more severe germination phenotype than sly1-10 (see Supplemental Figure 3 online).

A sly1-12 rgl2-13 double mutant was constructed to determine the effect of the rgl2-13 mutation on the sly1 seed germination phenotype. Although the germination of double mutant seeds was somewhat slower than the wild type, the 1-month-old sly1-12 rgl2-13 seeds reached 100% germination by day 4, whereas the 1-month-old sly1-12 seeds crossed into the Columbia (Col) background never germinated (Figure 4C). Thus, it appears...
that the RGL2 mutation is sufficient to rescue sly1-t2 germination. Similar suppression of the sly1-2 germination phenotype was seen in a sly1-2 rgl2-13 double mutant (data not shown). This result suggests that the reduced germination in sly1 mutants is due to overaccumulation of RGL2 protein. This is consistent with data of Tyler et al. (2004) showing that RGL2 protein disappears within 5 h of GA treatment in ga1-3 seeds but not in sly1 mutant seeds.

**RGL2 Does Not Disappear during sly1 Mutant Seed Germination**

This section examines the hypothesis that the decreased germination capacity of GA mutants is due to increased RGL2 protein accumulation. The expression of RGL2 protein was examined over a time course of seed imbibition in wild-type, ga1-3, and sly1 mutant seeds to determine whether RGL2 disappears prior to wild-type seed germination and to determine whether RGL2 protein levels correlate with the severity of the sly1 seed germination phenotype (Figure 5). This experiment was conducted using polyclonal antibody to RGL2 that was found to be specific for RGL2 protein isolated from seeds imbibed at 4°C.

Protein gel blot analysis (exposure time 5 to 10 min) of samples used were L. RGL2 protein was detected by protein gel blot analysis. (A) Ler seeds were incubated for 6 d at 4°C and then time points were at indicated intervals after transfer to 22°C (blot exposure >30 min). The correlation between RGL2 protein accumulation and radicle emergence (%) was determined. RGL2 protein disappeared within 12 h, before radicle emergence.

(B) Time-course analysis of RGL2 protein in wild-type and mutant backgrounds. Protein gel blot analysis (exposure time 5 to 10 min) of protein isolated from seeds imbibed at 4°C for 1, 3, 5, and 6 d and from seeds first imbibed for 3 d at 4°C then transferred to 22°C for indicated number of days. Protein from dry seeds was also examined. Seed samples used were Ler (28 months old), ga1-3 (25 months old), sly1-10 (27 months old, #3), after-ripened (A) sly1-1 (26 months old, #21), dormant (D) sly1-1 (0.5 months old, #35), Ws (0.5 months old), and sly1-12 (0.5 months old, #12). Asterisks indicate nonspecific bands.

Figure 5. Accumulation of RGL2 Protein during a Time Course of Seed Imbibition.

RGL2 protein was detected by protein gel blot analysis. (A) Ler seeds were incubated for 6 d at 4°C and then time points were at indicated intervals after transfer to 22°C (blot exposure >30 min). The correlation between RGL2 protein accumulation and radicle emergence (%) was determined. RGL2 protein disappeared within 12 h, before radicle emergence.

(B) Time-course analysis of RGL2 protein in wild-type and mutant backgrounds. Protein gel blot analysis (exposure time 5 to 10 min) of protein isolated from seeds imbibed at 4°C for 1, 3, 5, and 6 d and from seeds first imbibed for 3 d at 4°C then transferred to 22°C for indicated number of days. Protein from dry seeds was also examined. Seed samples used were Ler (28 months old), ga1-3 (25 months old), sly1-10 (27 months old, #3), after-ripened (A) sly1-1 (26 months old, #21), dormant (D) sly1-1 (0.5 months old, #35), Ws (0.5 months old), and sly1-12 (0.5 months old, #12). Asterisks indicate nonspecific bands.

It was expected that if RGL2 is a major negative regulator of seed germination, RGL2 protein should disappear well before wild-type radicle emergence. Following 6 d of imbibition at 4°C, Ler (wild-type) seeds were transferred to 22°C, and time points were taken for protein blot analysis using RGL2 antibody (Figure 5A; see Supplemental Figure 4C online). RGL2 protein was present at low levels immediately after transfer to 22°C, persisted at very low levels through 5 h of incubation, and disappeared by 12 h of incubation. At 12 h of incubation, only 2% of seeds showed radicle emergence. Thus, RGL2 protein disappeared well before completion of radicle emergence at 24 h incubation at 22°C. This is consistent with data from previous studies suggesting that RGL2 protein must disappear prior to wild-type seed germination (Tyler et al., 2004).

If the seed germination phenotype of GA mutants is due to overaccumulation of RGL2 protein, then we expect the severity of the germination phenotype to positively correlate with the level of RGL2 protein accumulation. To test this hypothesis, a time course was performed examining RGL2 protein accumulation during imbibition of wild-type, ga1-3, and sly1 mutant seeds (1) over 6 d of stratification at 4°C and (2) over 2 to 6 d of imbibition at 22°C following 3 d of stratification at 4°C (Figure 5B). In both Ler and Ws seeds, RGL2 protein accumulation was barely detected within 5 to 6 d of seed imbibition at 4°C (Figure 5; see Supplemental Figure 4C online). When wild-type seeds were transferred to 22°C following 3 d of stratification, RGL2 protein was not detected after 24 h of transfer. In the ga1-3 seeds, RGL2 protein was detected earlier and at higher levels than in Ler during stratification at 4°C and did not decrease during 6 d of incubation at 22°C. During this time, ga1-3 seeds did not germinate.

RGL2 protein accumulation in after-ripened sly1-10 (seed lot #3, 27 months old) and sly1-2 (seed lot #21, 26 months old) seeds known to show efficient seed germination were compared with dormant sly1-2 (seed lot #35, 0.5 months old) and sly1-12 seeds (seed lot #12, 0.5 months old) (Figures 2 and 5B). The percentage of radicle emergence for each time point in this experiment can be found in Figure 4A. When sly1 mutant seeds were stratified at 4°C, the RGL2 protein gradually accumulated to high levels similar to the pattern of expression seen in ga1-3 (Figure 5B). Following transfer of seeds to 22°C, RGL2 protein accumulated at higher levels in sly1 than in ga1-3 mutants, regardless of allele, degree of seed dormancy, or seed lot (Figure 5B; see Supplemental Figures 4B and 4D online). Interestingly, although the sly1-10 seeds showed 73.7% radicle emergence after 3 d of incubation at 22°C, RGL2 protein levels remained high. Rather than decreasing as germination approached, RGL2 protein levels gradually increased, reaching a maximum at day 3, and remained high after 100% radicle emergence was reached at day 5 (Figures 4 and 5B). It was expected that since sly1-2 shows a more severe germination phenotype than sly1-10, the RGL2 protein levels would be higher in sly1-10. However, the accumulation of RGL2 protein in the after-ripened sly1-2 seeds was similar to or less than that seen in sly1-10. It was expected that RGL2 protein would accumulate at higher levels in dormant sly1-2 seeds than after-ripened. However, almost no difference was seen between after-ripened and dormant sly1-2 through 4 d
of incubation at 22°C. At 5 and 6 d of incubation at 22°C, slightly higher RGL2 levels were seen in dormant sly1-2 than in after-ripened sly1-2. At day 6, dormant sly1-2 seeds showed 7.4% radicle emergence, whereas after-ripened sly1-2 seeds showed 85.6%. Thus, higher germination levels may partly correlate with reduced RGL2 protein levels in after-ripened sly1-2 seeds. However, since after-ripened sly1-2 reached near-maximum radicle emergence (71.2%; Figure 4) after 4 d at 22°C, it does not appear that decreased RGL2 protein levels are required prior to radicle emergence in the sly1-2 or sly1-10 genotype. The sly1-t2 allele had the strongest seed germination phenotype, showing 0% germination at the conclusion of the experiment. Compared with wild-type Ws, the sly1-t2 allele also showed increased RGL2 protein accumulation. After the seeds were transferred at 22°C, the RGL2 protein persisted throughout the time course.

Next we examined the expression of another DELLA protein, RGA, during seed imbibition. In a double mutant study examining four of the five DELLA genes, mutations in RGA rescued the germination of ga1-3 seeds better than any DELLA mutation but rgl2 (Cao et al., 2005). When we examined RGA protein accumulation during seed germination, RGA protein showed a very similar expression pattern to RGL2 (see Supplemental Figure 5C online). The RGA protein accumulated at high levels after 6 d of stratification and did not disappear after transfer to 22°C in ga1-3 and sly1 mutant seeds. Somewhat higher protein accumulation was found in after-ripened sly1-2 than in dormant sly1-2 after 4 d at 22°C (see Supplemental Figure 5D online). These results indicate that at least two major DELLA proteins involved in seed germination do not disappear during sly1 seed germination.

**sly1 Mutations Cause Increased RGL2 mRNA Accumulation**

The increased level of RGL2 protein in sly1 mutants could be either due to a requirement for SLY1 in triggering the disappearance of RGL2 protein or mRNA. It was expected that sly1 mutants act by stabilizing the RGL2 protein since SLY1 is the F-box subunit of an SCF E3 ubiquitin ligase required for DELLA protein disappearance (McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004). To determine if this is true, the effect of sly1 mutations on the accumulation of RGL2 mRNA was determined by RT-PCR analysis of the same seed lots and imbibition time points used to examine RGL2 protein expression in Figure 5B (Figure 6). Control RT-PCR reactions using the UBF11 primers are shown in Supplemental Figure 6A online. As previously shown, the RGL2 mRNA levels gradually increased during imbibition of wild-type and ga1-3 mutant seeds over 6 d at 4°C (Lee et al., 2002). A similar pattern of expression was seen during cold imbibition of sly1-10, sly1-t2, and dormant and after-ripened sly1-2 seeds. Next, RGL2 mRNA expression was examined following transfer to 22°C. RGL2 mRNA levels decreased within 1 d (24 h) of transfer to 22°C in Ler and Ws seeds, the time point corresponding to 72.5 and 81.6% radicle emergence, respectively. RGL2 mRNA levels never decreased during imbibition of ga1-3 seeds that failed to germinate.

In sly1 mutants, RGL2 mRNA levels appeared to decrease after radicle emergence. RGL2 mRNA levels decreased after 4 d of imbibition at 22°C in sly1-10 and after 5 d of imbibition in after-ripened sly1-2 seeds. These time points corresponded to 90.3 and 81.6% radicle emergence, respectively. Like ga1-3, the RGL2 mRNA levels remained high through 6 d of imbibition of dormant sly1-2 and sly1-t2 seeds showing 9.4 and 0% final radicle emergence, respectively (Figure 4). The decline in RGL2 mRNA levels during this time course occurred prior to the decrease in RGL2 protein levels (Figure 5B). In sly1-10, RGL2 mRNA levels decreased after 4 d of imbibition at 22°C, while RGL2 protein levels remained high at least through 5 d of imbibition. In after-ripened sly1-2 seeds, RGL2 mRNA levels decreased after 5 d of imbibition at 22°C, whereas RGL2 protein levels remained high through 6 d of imbibition. This suggests that the high levels of RGL2 protein accumulation following seed germination of sly1-10 and sly1-2 seeds are due at least in part to loss of SCFSLY1-stimulated proteolysis of RGL2 protein.

**GA Partially Rescues sly1 Germination without Causing Reduced RGL2 Protein Levels**

The current model is that GA stimulates germination via degradation of DELLA proteins, including RGL2, RGA, and GAI (Dill et al., 2004; Fu et al., 2004; Tyler et al., 2004). The strongest evidence of this is that RGL2 protein disappears within 5 h of adding GA to imbibing ga1-3 seeds but not sly1 mutant seeds. This result raised the question of whether GA treatment can stimulate sly1 mutant seed germination and the disappearance of RGL2 protein over a time course of imbibition. Previous work showed that GA treatment did not cause an increase in sly1-2 hypocotyl elongation (Steber and McCourt, 2001). As shown in Figure 7A, GA treatment increased the percentage of germination of all sly1 alleles tested.
even dormant sly1-2 and sly1-t2. Improvement of the germination was dependent on the concentration of GA applied, and the best recovery was found at 10 μM GA3 (Figure 7A). Next, we examined the RGL2 protein levels after GA treatment of the same seed lots used for the germination experiments. Seeds were plated either in the absence or presence of 10 μM GA3 and protein extracts prepared after the indicated incubation at 22°C following 3 d of stratification at 4°C. As expected, RGL2 protein disappeared after 60 h of incubation on GA in ga1-3 but not in the sly1 mutants (Figures 7B and 7C). Further time points showed that RGL2 protein levels did not decrease in sly1 mutants after 84 h of incubation on GA. When RGL2 mRNA levels were examined by RT-PCR analysis, the apparent level of RGL2 mRNA remained constant in dormant sly1-2 and sly1-t2 and decreased slightly after 84 h of incubation in the presence of GA in after-ripened sly1-2 and sly1-10 (Figure 7D).

Overall, these results suggest that GA treatment improves seed germination without reducing RGL2 protein levels in sly1-10, sly1-2, and sly1-t2 backgrounds and suggests the existence of an RGL2-independent pathway for stimulating seed germination.

GA-Regulated Genes Show Altered Expression in sly1 Seeds

The fact that sly1 mutant seeds can germinate in the presence of high levels of RGL2 protein suggested that RGL2 protein disappearance may not be required to derepress GA-dependent gene expression in the sly1 mutant background. To investigate this notion, the mRNA levels of GA-regulated genes were examined during the imbibition of sly1 seeds and ga1-3 after 60 h of incubation at 4°C in water followed by 24 h of imbibition at 22°C on MS-agar and then 12 h of imbibition at 22°C in the presence and

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**Figure 7.** GA Partially Rescued sly1 Germination without Reducing RGL2 Protein Levels. **(A)** Percentage of germination on indicated GA3 concentration in ga1-3 biosynthesis mutant (25 months old), sly1-10 (5 months old, #13), after-ripened (A) sly1-2 (26 months old, #21), dormant (D) sly1-2 (0.5 months old, #35), and sly1-t2 (0.5 months old, #12; 18 months old, #2). Germination was calculated based on cotyledon appearance. Age of the stock is shown below the line (months). Error bars show SE (n = 30 to 60). **(B)** Percentage of radicle emergence determined during time-course experiment examining RGL2 protein accumulation in the presence of 10 μM GA3 using the same seed lots used in (A) except for 0.5-month-old sly1-t2. Radicle emergence was determined at 60, 72, and 84 h at 22°C following 3 d of 4°C. **(C)** RGL2 protein expression in the absence and presence of 10 μM GA3. Protein was extracted from imbibing seeds at indicated time points at 22°C following 3 d at 4°C. **(D)** RGL2 mRNA accumulation in the absence and presence of GA3 during the same time-course experiment in (C). Left panel, RGL2 mRNA; right panel, ACTIN2 mRNA.
absence of GA. At this time, none of the seeds had germinated. We examined seven transcripts showing GA induction in ga1-3 based on previous reports (Herzog et al., 1995; Ogawa et al., 2003; Yamauchi et al., 2004; Cao et al., 2006). The mRNA accumulation in these seven genes was upregulated after GA was applied to ga1-3 seeds (Figure 8). Dormant sly1-2 seeds show reduced accumulation of GA-induced genes in the presence and sometimes in the absence of GA compared with ga1-3. This inability to induce GA-regulated genes is consistent with a GA-insensitive phenotype. Interestingly, the expression of many GA-induced genes is increased in after-ripened sly1-2 seeds compared with dormant seeds both in the presence and absence of exogenous GA. After-ripened seeds of the weaker sly1-10 allele did not appear to have as strong a GA-insensitive phenotype as sly1-2 since some GA-regulated transcripts showed GA-induced expression. In after-ripened seeds, four transcripts, α-EXPANSIN1 (EXP1), XYLOGLUCAN ENDOTRANSGLYCOSYLASE5 (XTH5), XTH31, and CYSTEIN PROTEINASE (CP), showed increased expression in the absence of GA in after-ripened sly1-2 and sly1-10 compared with ga1-3. Three transcripts, PECTIN METHYLESTERASE (PME), GIBBERELLIC ACID STIMULATED ARABIDOPSIS4 (GASA4), and SERINE CARBOXYPEPTIDASE S10 FAMILY PROTEIN (SCS10), showed higher expression in after-ripened sly1-2 than in ga1-3 in the absence of GA and showed lower accumulation in the presence of GA. These patterns of expression are consistent with the notion that the RGL2 protein accumulating in after-ripened sly1 seeds is not fully active as a repressor of GA-regulated gene expression.

**DISCUSSION**

**Mutations in SLY1 Result in Allele-Dependent Defects in Seed Germination**

The data presented here provide clear evidence that sly1 mutations cause increased seed dormancy, delayed after-ripening, and decreased germination potential (Figures 2 to 5). Even sly1 mutant seed lots showing 100% seed germination germinate more slowly and have increased sensitivity to inhibition of germination by ABA, PEG 8000, and PAC (Strader et al., 2004; Figure 4; see Supplemental Figures 2 and 3 online). In addition, alleles that caused loss of more of the SLY1 protein sequence also caused a more severe seed germination defect (Figures 2 to 4; see Supplemental Figures 2 and 3 online). For example, the sly1-2 mutation resulting in loss of the C-terminal 40 amino acids showed stronger seed dormancy and required more time to after-ripen than the sly1-10 mutation, resulting in loss of the last eight amino acids of the protein (Figures 1 and 2). These results also are consistent with the fact that sly1-2 shows more severe dwarfism and reduced fertility compared with sly1-10 (McGinnis et al., 2003; Dill et al., 2004; see Supplemental Table 1 online). The new sly1-t2 allele exhibited the strongest seed dormancy of the three alleles, suggesting that sly1-t2 may be a null allele. However, a direct comparison is not possible because different ecotypes show differences in seed dormancy (Debeaujon and Koornneef, 2000). Tetrazolium staining and after-ripening experiments showed that dormant sly1-2 seeds are viable. However, sly1-2 seeds stored for ~5 years show reduced viability, whereas Ler wild-type seeds of the same age show no decrease in germination or viability (Figure 3). The sly1-2 and sly1-10 seeds showed variability in the degree of seed coat–imposed dormancy (Figure 2). This variability is most likely due to environmental effects, possibly through differences in accumulation and sensitivity to other plant hormones, such as brassinosteroid, ethylene, and ABA (Koornneef and Karssen, 1994; Steber and McCourt, 2001; Okamoto et al., 2006). Both the sly1-2 and sly1-10 mutations result in increased sensitivity to inhibition of seed germination by ABA (Strader et al., 2004). Thus, it is possible that the variability in the sly1 germination phenotype is due to differences in ABA levels or signaling. It is interesting to note that transcript levels of GA-induced genes were lower in imbibing dormant sly1-2 seeds than in after-ripened sly1-2 seeds (Figure 8). It is possible that dormancy somehow represses GA-induced genes in sly1-2 seeds. This suggests that SLY1 contributes to, but is not essential for, the derepression of these genes during after-ripening.

The fact that sly1 mutants show increased sensitivity to PEG 8000 inhibition of seed germination compared with the wild type does suggest that sly1 mutations cause altered permeability to water. While light and transmission electron microscopy...
revealed no obvious morphological differences in the seed coat structure of wild-type and sly1 seeds (K.M. McGinnis and C.M. Steber, unpublished data), we cannot exclude the possibility that changes in the seed coat are involved. Many of the GA-induced genes that show decreased expression in dormant sly1-2 seeds, such as EXP1, XTH5, XTH31, and PME, are involved in cell wall loosening and may help to release the testa or endosperm barriers during seed germination (Ogawa et al., 2003; Yamauchi et al., 2004). Thus, it is possible that the sly1 mutations cause increased seed dormancy in part through effects on seed coat loosening.

**Mechanisms Underlying the sly1 Mutant Seed Germination Phenotype**

Analysis of RGL2 mRNA accumulation in sly1 mutants showed that the SLY1 gene is required for GA-regulated disappearance of RGL2 mRNA. Previous studies demonstrated that (1) RGL2 mRNA levels increase during cold imbibition of wild-type and ga1-3 seeds, (2) RGL2 mRNA levels decrease in wild-type seeds imbibed at 23°C during radicle emergence, (3) RGL2 mRNA levels persist at high levels in ga1-3-seeds imbibing at 23°C, and (4) GA treatment of ga1-3 seeds stimulates germination and triggers reduced accumulation of RGL2 mRNA (Lee et al., 2002; Bassel et al., 2004). Bassel et al. (2004) showed that RGL2 mRNA did not fully disappear prior to radicle emergence, indicating that RGL2 mRNA disappearance is not required for seed germination. This work further suggested that RGL2 activity might be controlled by multiple mechanisms. Here, we demonstrated that RGL2 mRNA overaccumulated in both dormant and after-ripened sly1 seeds imbibed at 22°C in a manner similar to that seen in ga1-3 seeds (Figure 6). Unlike ga1-3 seeds, however, sly1 seeds showed no significant decrease in RGL2 mRNA levels after 60 h of GA treatment (Figure 7), suggesting that SLY1 is required for GA-stimulated disappearance of RGL2 mRNA. RGL2 mRNA accumulation did decrease after germination of sly1-10 and after-ripened sly1-2 seeds (Figures 6 and 7). This suggests that RGL2 mRNA accumulation is subject to SLY1-independent downregulation after seed germination. RGL2 mRNA decreased markedly when wild-type Ler reached 72.5% radicle emergence (day 1), whereas RGL2 transcript levels remained quite high when sly1-10 (73.7%) and sly1-2 (71.2%) showed comparable radicle emergence at 3 and 4 d, respectively (Figures 4 and 6). Thus, RGL2 mRNA appears to be downregulated during seedling establishment in sly1 mutants.

Previous studies postulated that GA stimulates seed germination by causing the SCF<sub>SLY1</sub> complex to trigger the proteolysis of the RGL2 protein via ubiquitination (Tyler et al., 2004). This model predicted that the degree of seed dormancy should directly correlate with the level of RGL2 protein in the seeds. The sly1 mutants that showed variable seed dormancy and that required a long period of after-ripening provided an excellent tool for examining this prediction. This study showed that sly1 mutants accumulate high levels of RGL2 and RGA protein during imbibition regardless of the sly1 allele or the dormancy status of the seed (Figure 5B; see Supplemental Figure 5 online). What this result does show is that the degree of sly1 dormancy is due to factors other than the absolute level of RGL2 protein accumulation. Since mutations in RGL2 rescue the increased dormancy and reduced germination capacity of dormant sly1 mutant seeds, it appears that these phenotypes are due to RGL2 overaccumulation. The increased germination of sly1 seeds due to after-ripening may be due to inactivation of RGL2 or to competition with parallel pathways. Based on previous work, we considered the possibility that other E3 ubiquitin ligases, such as the SLY1 homolog SNEEZY and the PHOR1 U-box E3 ubiquitin ligase, could replace SLY1 in GA signaling and lead to SLY1-independent fluctuations in RGL2 levels during seed germination (Monte et al., 2003; Fu et al., 2004; Strader et al., 2004). No such fluctuations were seen, suggesting that SCF<sub>SLY1</sub> is the major E3 ubiquitin ligase transmitting the GA signal under the imbibition conditions used. RGL2 protein does not disappear in GA-treated sly1 mutant seeds, but GA treatment does somewhat stimulate seed germination (Figure 7). This suggests that GA may stimulate sly1 mutant seed germination via an RGL2-independent pathway, either through a gene that stimulates seed germination or a gene that controls RGL2 activity (Figure 9A). This notion that there is an RGL2-independent mechanism for GA regulation of
Seed germination is consistent with a recent microarray study showing that there are GA-regulated genes with DELLA-independent expression (Cao et al., 2006).

Mechanisms Controlling RGL2 Function

The fact that loss-of-function mutations in DELLA genes rescue the defects of the ga1-3 GA biosynthesis mutant forms the genetic basis for the model that DELLA proteins act as negative regulators of GA signaling and that GA acts via negative regulation of DELLA proteins (Dill and Sun, 2001; King et al., 2001; Cheng et al., 2004). Previous work has also shown that DELLA proteins disappear in response to GA and that this disappearance requires the SCFSLY1 E3 ubiquitin ligase (Silverstone et al., 2001; McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004; Tyler et al., 2004). Prior to this study, it was known that GA treatment of ga1-3 seeds causes RGL2 protein to disappear prior to the disappearance of RGL2 mRNA (Tyler et al., 2004). These data together with the evidence that RGL2 persists at high levels after GA treatment of sly1 mutants are evidence that the RGL2 DELLA protein is also subject to GA-stimulated destruction via the SCFSLY1 and ubiquitin-proteasome pathway (Figure 5).

This article and others have revealed that RGL2 function in seed germination must be regulated at multiple levels, including transcript and protein accumulation (Lee et al., 2002; Bassel et al., 2005; Figures 5 to 7). This study suggests that RGL2 functions as a repressor of seed germination in dormant sly1 seeds and that the RGL2 repressor is deactivated after-ripening either due to effects of dry storage itself or due to posttranslational regulation. Transcript levels of GA-induced genes are higher in imbibing after-ripened sly1 seeds than in ga1-3 seeds in the absence of GA (Figure 8). These data suggest that the RGL2 protein accumulating in after-ripened sly1 mutant seeds is either less active as a repressor of GA-induced genes or is competing with a transcripational activator of GA-induced genes (Figure 9A). One possibility is that RGL2 activity is controlled by posttranslational regulation. It appears that RGL2 is phosphorylated and that phosphorylation stabilizes the protein (Hussain et al., 2005). Possibly a kinase either activates or inactivates the RGL2 repressor (Figure 9A). It is also known that the SPINDLY O-Glc–N-ac transferase acts as a negative regulator of GA signaling during seed germination (Jacobsen and Olszewski, 1993). It is possible that O-Glc–N-ac modification is needed for RGL2 to act as a repressor and that the RGL2 protein accumulating in sly1 mutants is incompletely modified. Accumulation of unmodified RGL2 in sly1 mutants may be due to overaccumulation of the protein or to indirect effects of the sly1 mutation. Alternatively, RGL2 activity may be regulated by protein–protein interaction. For example, RGL2 alone may act as a negative regulator of GA signaling, while RGL2 bound to the GA receptor GID1 may act as a positive regulator of GA signaling (Figure 9B). Positive regulation of GA signaling by GA–GID1–RGL2 in sly1 mutants may compete with negative regulation by RGL2, leading to an intermediate germination phenotype in spite of the fact that RGL2 is present at high levels. This may also account for the fact that the sly1 phenotypes in plant height and in fertility are less severe than those of ga1-3 (Steber et al., 1998).

METHODS

Plant Materials and Growth Conditions

Seeds of wild-type Arabidopsis thaliana (accessions Ler and/or Ws), sly1-10, sly1-2, sly1-DS, and sly1-t2 were cultivated according to McGinnis et al. (2003). The sly1-t2 line was isolated by searching the Sussexs Basta lines by PCR using the T-DNA left border primer JL202 (5′-CAT-TTTATAAAGCGCTGGCGACATC-3′) and SLY1-specific primer sly1T-DNAr (5′-ACTTTGGATTACGATTGTTCCAGGTA-3′) to obtain a 2.0-kb product (L. Strader and C.M. Steber, unpublished data; Krysan et al., 1999). Homozygous mutant plants were identified by PCR using the primers above and the SLY1-specific primers 2-62f (5′-AAGGCATCT-GAGAAACC-3′) and sly1T-DNAr that do not amplify the large T-DNA insertion in sly1-t2. The sly1-DS line was obtained from ABRC (stock No. CS101918; Sundaresan et al., 1995). The sly1-t2 rgl2-13 double mutant was made by crossing the sly1-t2 allele in the Ws ecotype to rgl2-13 in the Col ecotype. For comparison in germination experiments, we also crossed sly1-t2 once to Col wild type. Failure to germinate was seen in all independent isolates of sly1-2 recovered from the cross to Col (data not shown).

Germination Experiments

For germination experiments, 30 to 60 seeds were sterilized with 10% bleach for 15 to 20 min and plated per repetition on 0.5× MS salts (Sigma-Aldrich)/0.8% agar (MSagar). Percentage of germination was determined following 3 d of incubation at 4°C followed by 7 d of incubation under constant fluorescent light at 22°C. The average germination rate was calculated using three independent experiments. After harvest, seeds were stored in open tubes for 2 weeks at room temperature and then stored at room temperature in closed tubes in a dessication cabinet. For viability tests, seeds were soaked in 0.5% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) solution at 37°C in darkness for 10 h. Fully red-stained seeds were counted as viable (Debeaujon et al., 2000). For preparation of the PEG 8000-supplemented media, a sterile 40% stock solution of PEG 8000 was prepared and then mixed into the MS-agar medium to give 4, 6, and 8% PEG 8000 plates. Seeds from homozygous sly1-10, after-ripened sly1-2, and wild-type Ler were plated on this PEG-containing media. After 3 d of incubation at 4°C, they were transferred to 22°C under constant light, and the germination rate was determined over 7 d. For experiments in which GA was applied, seeds from homozygous ga1-3, sly1-10, after-ripened sly1-2, dormant sly1-2, and wild-type (Ler or Ws) of similar age were used. Seeds were sown on MS-agar plates containing 0, 0.1, 1, and 10 μM of GA_s. After 3 d of incubation at 4°C, they were transferred to 22°C under constant light, and germination rate was determined at 60, 72, and 84 h. For PAC experiments, homozygous ga1-3, sly1-10, after-ripened sly1-2, and the wild type (Ler) were used. First, these seeds were incubated in 100 μM PAC solution at 4°C for 3 d, washed five times with distilled water, and sown on MS-agar plates containing 0, 0.1, 1, and 10 μM of GA_s. The MS-agar plates were then incubated at 22°C under constant light, and germination rate was determined after 7 d.

RT–PCR Analysis of mRNA Expression

RT–PCR analysis was used to analyze the accumulation of SLY1 mRNA expression in different mutant alleles. Total RNA was isolated from flower buds using an RNA easy kit (Qiagen), and genomic DNA contamination was removed using the DNA-Free RNA kit (ZYM0 Research). cDNA was generated from 5 μg total RNA using a first-strand cDNA synthesis kit (GE Healthcare). cDNA was diluted and then used as a template for PCR amplification with the SLY1-2f (5′-CTCGGTCTGATGTTCTCCTT-3′) and
SLY1-2r (5'-CTCTCAGAGATTTTCTACGC-3') primers to obtain a 147-bp product and with the 2-63f and sly1-r primers to obtain a 213-bp product. SLY1-2f and SLY1-2r primers detect mRNA in sly1-10, sly1-2, and the wild type but not in sly1-t2 because they flank the T-DNA insertion. 2-63f and sly-r primers detect transcript in sly1-t2, sly1-2, and the wild type but not in sly1-10 because these primers flank the DNA rearrangement in this allele. ACTIN2 was amplified as a loading control using actin2-F (5'-CTGGATTCTGGTGATGGTGTGTC-3') and actin2-R (5'-CTTTTGTCTATACGGTCAGGC-3') primers (An et al., 1996). PCR was performed with Takara Ex Taq polymerase (Takara Shuzo) for 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 59°C, and extension for 1 min at 72°C, followed by final extension for 5 min at 72°C. PCR reaction for ACTIN2 amplification was performed for 23 cycles.

RGL2 mRNA accumulation was analyzed by RT-PCR using total RNA extracted from whole seeds imbibed as indicated in Figure 7 using a phenol-sodium dodecyl sulfate extraction method (Sambrook et al., 1989). Genomic DNA contamination was removed, and cDNA strand synthesis was performed as described above. To determine the transcript level of RGL2, PCR was performed on cDNA using RGL2int-F (5'-CACCAGGAGAATCTCAAATAGGC-3') and RGL2orf3R (5'-GCCGAGTTCCACGCCCAGG-3') primers. The ubiquitin gene was amplified as a constitutive control as described by Tyler et al., (2004). Primer specificity was confirmed using the 2-63f and sly1-r primers to obtain a 213-bp product. SLY1-2f and SLY1-2r primers detect mRNA in 147-bp product and with the 2-63f and sly1-r primers to obtain a 213-bp product. Genomic DNA contamination was removed, and cDNA strand synthesis was performed as described above. To determine the transcript level of RGL2, PCR was performed on cDNA using RGL2int-F (5'-CACCAGGAGAATCTCAAATAGGC-3') and RGL2orf3R (5'-GCCGAGTTCCACGCCCAGG-3') primers. The ubiquitin gene was amplified as a constitutive control as described by Tyler et al., (2004). Primer specificity was confirmed using the 2-63f and sly1-r primers to obtain a 213-bp product.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Table 1.** Phenotypes of Different sly1 Mutant Alleles.

**Supplemental Table 2.** Primer Sequences of GA-Regulated Genes for RT-PCR.

**Supplemental Figure 1.** Time-Course After-ripening Experiment in Dormant sly1-2.

**Supplemental Figure 2.** sly1 Mutant Seed Germination Is More Sensitive to PEG 8000.

**Supplemental Figure 3.** sly1 Mutant Seed Germination Is More Sensitive to PAC.

**Supplemental Figure 4.** RGL2 Protein Detection by Protein Gel Blot Analysis.

**Supplemental Figure 5.** RGA Protein Detection by Protein Gel Blot Analysis.

**Supplemental Figure 6.** Ubiquitin Control RT-PCR Reaction and Confirmation of Amplification in RGL2 in Different Arabidopsis Ecotypes.

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**REFERENCES**


Cao, D., Cheng, H., Wu, W., Meng Soo, H., and Peng, J. (2006). Gibberellin mobilizes distinct DELLA-dependent transcriptomes to...


