DELLA Proteins and Gibberellin-Regulated Seed Germination and Floral Development in Arabidopsis[1[w]]

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RGA (repressor of *ga1-3*) and GAI (gibberellin insensitive) are negative regulators of plant hormone gibberellin (GA) signaling in Arabidopsis. The GA-deficient mutant *ga1-3* is a nongerminating, extreme dwarf that flowers late and produces male-sterile flowers. The *rga* and *gai* null alleles interact synergistically to rescue vegetative growth and floral initiation in *ga1-3*, indicating that RGA and GAI are major repressors for these processes. However, *rga* and *gai* in combination cannot rescue seed germination or floral development in *ga1-3*. RGA and GAI belong to the DELLA subfamily within the GRAS family of plant regulatory proteins. Three additional DELLA proteins RGL1, RGL2, and RGL3 are present in Arabidopsis. Previous studies provided evidence that RGL2 and possibly RGL1 control seed germination. To investigate further the function of the *RGL* genes, we examined the expression profiles of all 5 DELLA protein genes by real-time PCR. *RGA* and, to a lesser extent, *GAI* mRNAs were expressed ubiquitously in all tissues, whereas *RGL1*, *2*, and *3* transcripts were present at high levels only in germinating seeds and/or flowers and siliques. Using the newly isolated *rgl1*, *rgl2*, and *rgl3* T-DNA insertion mutants, we demonstrated that RGL2 is the major repressor in seed germination. We further provided evidence that RGA, RGL1, and RGL2 are all involved in modulating floral development. Interestingly, *RGL2* expression is regulated not only at the transcript level. We showed that RGL2 protein in imbibed seeds is rapidly degraded by GA treatment and that the F-box protein SLY1 is required for RGL2 degradation to occur.

Bioactive gibberellins (GAs) are phytohormones that are essential for many processes throughout the life of a plant. Seed germination, vegetative growth, and floral and seed development, for example, all require GAs (Davies, 1995). The importance of GAs is clearly illustrated by the Arabidopsis *ga1-3* mutant. This mutant contains a large deletion in the *GA1* gene, which encodes *ent*-copalyl diphosphate synthase, the enzyme catalyzing the first committed step in GA biosynthesis (Sun and Kamiya, 1994). The large reduction in bioactive GAs in *ga1-3* leads to a GA-deficient phenotype characterized by dark green leaves and severe dwarfism (Koornneef and van der Veen, 1980; Silverstone et al., 2001). The *ga1-3* plant also exhibits defects in all the developmental processes regulated by GAs: This mutant is impaired in root growth and trichome initiation, has reduced apical dominance, and fails to flower under short-day conditions (Wilson et al., 1992; Chien and Sussex, 1996; Silverstone et al., 1997; Fu and Harberd, 2003). Under long-day conditions, floral initiation in *ga1-3* is delayed, and the mutant flowers are male-sterile (Koornneef et al., 1983; Wilson et al., 1992). Also, *ga1-3* seeds cannot germinate without the application of GA (Koornneef et al., 1983).

Loss-of-function mutations in *RGA* (repressor of *ga1-3*) and *GAI* (GA insensitive) can suppress some of the effects of GA deficiency, suggesting that RGA and GAI negatively regulate a subset of GA responses in Arabidopsis (Peng et al., 1997; Silverstone et al., 1997). Fusions of either RGA or GAI to the green fluorescent protein (GFP) are localized to the nuclei of cells in transgenic Arabidopsis (Silverstone et al., 2001; Fleck and Harberd, 2002). Thus, RGA and GAI may function as transcriptional regulators that directly or indirectly repress the expression of GA-induced genes. Through studies of the *rga-24* and *gai-t6* null alleles in a *ga1-3* background, RGA and GAI proteins were demonstrated to have overlapping functions in repressing many vegetative growth processes (e.g. leaf expansion, apical dominance, abaxial trichome development, and stem elongation) as well as floral initiation (Dill and Sun, 2001; King et al., 2001). Although both RGA and GAI regulate these processes, the contribution of RGA is greater than that of GAI (Dill and Sun, 2001). Interestingly, seed germination and floral development are not restored by removing both RGA and GAI functions in the *ga1-3* background,

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suggesting that neither RGA nor GAI plays a major role in controlling these two GA-dependent processes (Dill and Sun, 2001).

GAI and RGA belong to the DELLA subfamily within the GRAS family of plant regulatory proteins (Pysh et al., 1999). GAI and RGA are distinguished from other GRAS family members by an N-terminal DELLA domain (Peng et al., 1997; Silverstone et al., 1998). This domain is involved in modulating the activity of the RGA and GAI proteins in response to GA (Peng et al., 1997; Dill et al., 2001). Deleting 17 amino acids (named the DELLA motif) in the N-terminal region of either GAI or RGA confers a GA-unresponsive dwarf phenotype in Arabidopsis (Koornneef et al., 1985; Peng et al., 1997; Dill et al., 2001). It has also been demonstrated that GA de-represses its signaling pathway by inducing the degradation of RGA and GAI (Silverstone et al., 2001; Dill et al., 2004) and the DELLA motif in RGA is essential for its GA-dependent proteolysis (Dill et al., 2001). Deletion of the DELLA motif in RGA stabilizes the mutant rga protein (rga-Δ17), converting it into a GA-unresponsive, constitutively active repressor of GA signaling (Dill et al., 2001).

Recent studies have indicated that GA-induced degradation of RGA and GAI requires the SLEEPY1 (SLY1) protein, a positive regulator of GA signaling in Arabidopsis (McGinnis et al., 2003; Dill et al., 2004). The loss-of-function *sly1-10* mutant is a GA-insensitive dwarf (Steber et al., 1998) that accumulates high levels of RGA and GAI proteins (McGinnis et al., 2003; Dill et al., 2004). Furthermore, a combination of *rga* and *gai* null alleles completely suppresses the *sly1-10* dwarf phenotype. Cloning of *SLY1* revealed that this gene encodes an F-box containing protein, which is likely a component of the ubiquitin E3 ligase SCF complex (McGinnis et al., 2003). In response to the GA signal, SCF^{SLY1} may target RGA for ubiquitination and subsequent degradation by the 26S proteasome.

With the sequencing of the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000), three other members of the DELLA subfamily were identified: *RGA-LIKE 1*, *2*, and *3* (*RGL1*, *RGL2*, and *RGL3*; Dill and Sun, 2001; Lee et al., 2002; Wen and Chang, 2002). While RGA and GAI exhibit approximately 82% identity at the amino acid level, RGL1 (At1g66350), RGL2 (At3g03450), and RGL3 (At5g17490) are 53% to 62% identical to RGA and GAI (Dill and Sun, 2001; Lee et al., 2002; Wen and Chang, 2002). Because of the presence of the DELLA domain, the *RGL* genes have been hypothesized, like *GAI* and *RGA*, to encode negative regulators of the GA signaling pathway. It has been further proposed that one or more of the *RGL* genes may be responsible for regulating GA responses such as seed germination and flower development, which are not dramatically affected by *RGA* or *GAI* (Dill and Sun, 2001).

To date, two groups studying *RGL1* and *RGL2* have provided evidence for the roles of these genes in GA signaling, although some of their conclusions are not the same. Wen and Chang (2002) showed that transgenic Arabidopsis lines expressing a dominant *35S:rgl1* transgene (containing the DELLA motif deletion) exhibit a GA-unresponsive dwarf phenotype, similar to that of plants carrying the dominant *gai-1* or *rga*-Δ*17* allele with the DELLA motif deletion. This finding suggested that endogenous RGL1 may modulate vegetative growth in wild-type plants, although ectopic expression of the dominant *rgl1* gene by the constitutive 35S promoter may contribute to some of the transgenic plant phenotype. This study also suggested that *RGL1* regulates seed germination because seeds of a transgenic line in which endogenous *RGL1* expression is silenced germinate in the presence of the GA biosynthesis inhibitor paclobutrazol (PAC; Wen and Chang, 2002). In contrast, Lee et al. (2002) studied *Ds* insertion alleles of *RGL1* and *RGL2* and determined that *RGL2*, but not *RGL1*, is important in regulating seed germination. The discrepancy between these two studies could be explained by the following possibilities. The PAC-resistant phenotype in germinating seeds of the silenced *rgl1* line could be due to cosuppression of the *RGL2* and *RGL3* genes because expression of these genes was not examined in the mutant seed (Wen and Chang, 2002). Alternatively, the *rgl1* allele with a Ds insertion might be a leaky allele because the Ds insertion is located 68 bp upstream of the ATG translational start codon (Lee et al., 2002). However, the unpublished RNA blot data suggest that this Ds insertion eliminates the *RGL1* gene expression (Lee et al., 2002). Therefore, the role of RGL1 in seed germination needs to be re-examined. Also, the role of RGL3 in GA signaling and the DELLA protein(s) that are responsible for modulating flower development have not been determined.

In this study, we employed quantitative PCR (qPCR) to examine the developmental expression profiles of all 5 DELLA protein genes. We also isolated new knockout alleles of *RGL1, RGL2,* and *RGL3* from T-DNA insertion mutant pools to investigate further the function of the *RGL* genes. Our data indicated that RGL2 is the major DELLA protein controlling seed germination. In addition, by generating multiple *rga/rgl* mutant combinations in the *ga1-3* background, we provided strong evidence that RGL1 and RGL2, in combination with RGA, contribute to the regulation of male fertility in developing flowers.

RESULTS

Developmental Expression Profiles of *RGA, GAI, RGL1, RGL2,* and *RGL3*

RGA and GAI are the major repressors controlling GA-stimulated vegetative growth and the transition to the reproductive phase (Dill and Sun, 2001; King et al., 2001). However, removal of RGA and GAI function does not appear to affect seed germination or flower development significantly. Recent studies provided evidence that two other DELLA proteins, RGL2 and perhaps RGL1, play a role in repressing GA signaling

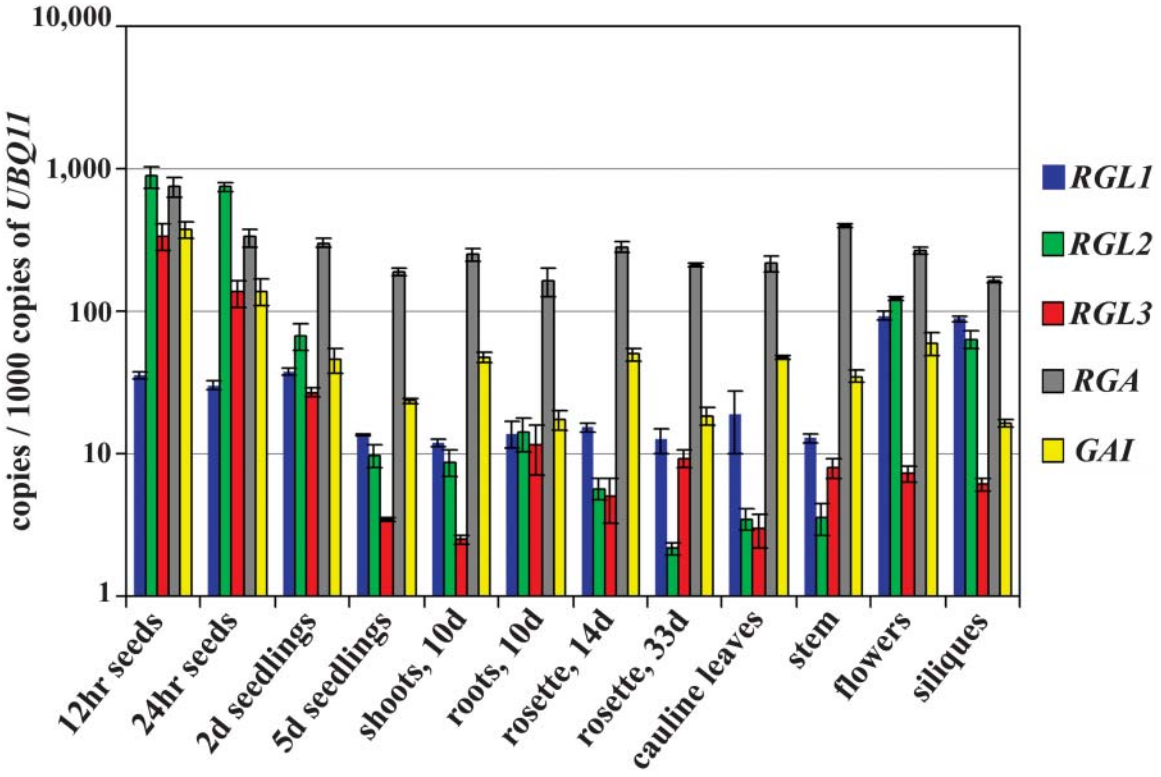

Figure 1. Transcript levels of *RGL1, RGL2, RGL3, RGA,* and *GAI* throughout development. The level of gene expression in each tissue sample, as determined by qPCR, is shown as the number of cDNA copies per 10^3 copies of *UBQ11* cDNA. The tissues and developmental stages tested were, from left to right in the graph, 12-h- and 24-h-imbibed seeds, 2-d-old and 5-d-old seedlings, 10-d-old shoots, roots of 10-d-old seedlings, 14-d-old rosettes, and the following tissues from 33-d-old plants: rosette leaves, cauline leaves, stems, flower clusters, and siliques. The means of three experiments ±SE are shown. Although *UBQ11* mRNA levels are constant in most tissues, they are approximately 3- to 4-fold higher in 33-d-old rosettes, flowers, stems, and cauline leaves (Supplemental Table I). Therefore, the expression levels of the DELLA protein genes in this figure are slightly underestimated in these tissues.

during seed germination (Lee et al., 2002; Wen and Chang, 2002). Although *RGA* and *GAI* mRNAs are detected in all tissues throughout plant development (Silverstone et al., 1998; Lee et al., 2002), expression of *RGL1*, *RGL2,* and *RGL3* appears to show tissue specificity (Lee et al., 2002; Wen and Chang, 2002). However, most of the expression data for *RGA*, *GAI,* and *RGL* genes came from RNA-blot analyses or RT-PCR, and only limited expression patterns for *RGL1* and *RGL2* were obtained using in situ hybridization or a promoter-GUS transgene. Furthermore, some of the results from previous studies are not consistent with each other. Therefore, a more detailed analysis of the expression pattern of these genes is necessary to identify their roles in modulating GA response in a given tissue during development.

We employed the sensitive, real-time PCR technique using gene-specific primers to quantify transcript levels of *RGA*, *GAI,* and the *RGLs* in different tissues and organs at various developmental stages. Among several housekeeping genes examined, only the expression of the *UBIQUITIN11* gene (*UBQ11)* remained fairly constant in most of the tissues across a wide range of developmental stages, when compared to *18S* rRNA (Supplemental Table I, available at www.plantphysiol.org). A few tissues did show small variations in *UBQ11* transcript levels: they are approximately 3- or 4-fold higher in 33-d-old rosette, stems, flowers, and cauline leaves. We decided to use *UBQ11* instead of *18S* rRNA as a control to normalize the samples because we found that the real-time qPCR using cDNA as a template gave more reproducible results than the qRT-PCR using the total RNA as a template. Figure 1 and Supplemental Table II show the cDNA copy number of each gene per 10^3 copies of *UBQ11* cDNA in different tissue samples. Consistent with previous RNA-blot data (Silverstone et al., 1998), *RGA* was ubiquitously expressed and was present at more than 160 copies/10^3 copies of *UBQ11* in all tissues examined (Fig. 1). *GAI* was moderately expressed (approximately 50 copies) in most tissues, with highest mRNA levels in 12-h-imbibed seeds (>300 copies) and low levels in 5-d-old seedlings, roots, 33-d-old rosette leaves, and siliques (16–24 copies). *RGL1*, *2*, and *3*

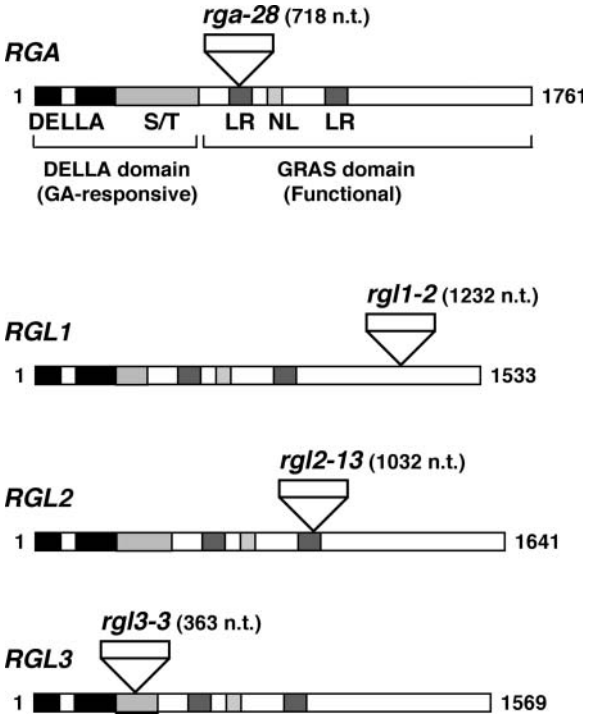

Figure 2. Locations of the T-DNA insertion sites in the new alleles of *rga* and *rgls*. The schematic diagram of each gene shows the encoded functional domains and motifs as labeled: DELLA, DELLA motif; S/T, polymeric Ser and/or Thr; LR, Leu heptad repeat; NL, nuclear localization signal. The numbers refer to the nucleotide length of the coding region of each gene and the insertion site of each T-DNA. n.t., nucleotide.

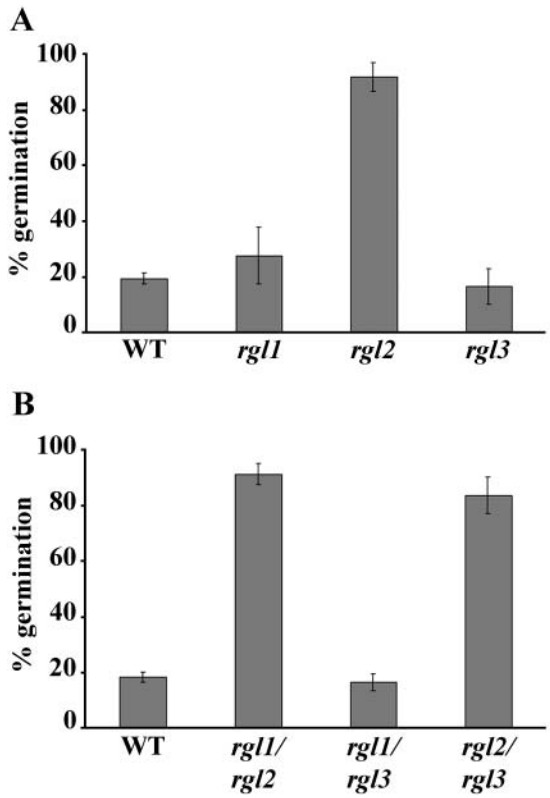

Figure 3. Germination of *rgl2* seeds is resistant to PAC. Seeds of wild-type plants (WT) and of homozygous single (A) and double (B) *rgl* mutants were treated with 120 μM PAC. The germination percentages in A and B are the means of three separate experiments. Error bars indicate the SE of the mean. For each experiment, approximately 100 to 160 seeds were scored per genotype.

genes were expressed at higher levels in germinating seeds, young seedlings, and/or flowers and siliques but only produced low amounts of transcripts in most vegetative tissues (Fig. 1). *RGL1* transcripts were present at approximately 30 copies in 12-h- and 24-h-imbibed seeds and 2-d-old seedlings and approximately 90 copies in flowers and siliques. *RGL2* mRNA accumulated at approximately 800 copies in germinating seeds, an extremely high level compared to other tissues, and was also high in 2-d-old seedlings (68 copies), flower clusters (123 copies), and siliques (64 copies). *RGL3* was only expressed highly in 12-h- and 24-h-imbibed seeds (337 and 136 copies). The expression profiles of these genes are consistent with the

Table I. *Flowering time, rosette size, and height of homozygous mutants and wild type*

Genotype	Days to Flower	Maximum Rosette Radius	Final Height
		mm	*cm*
ga1-3	42.4 ± 1.5	16.1 ± 0.4	1.50 ± 0.16[a]
ga1-3/rgl1	38.6 ± 1.1	16.4 ± 0.5	2.31 ± 0.23[a]
ga1-3/rgl2	43.8 ± 1.9	16.0 ± 0.2	1.92 ± 0.25[a]
ga1-3/rgl1/rgl2	38.8 ± 1.3	15.5 ± 0.5	2.32 ± 0.26[a]
ga1-3/rga-28	27.0 ± 0.5	21.9 ± 0.5	18.21 ± 0.31
ga1-3/rga-28/rgl1	29.1 ± 0.4	24.6 ± 0.9	20.18 ± 0.30
ga1-3/rga-28/rgl2	29.8 ± 0.4	19.7 ± 0.6	17.08 ± 0.32
ga1-3/rga-28/rgl1/rgl2	29.5 ± 0.5	23.8 ± 1.4	17.13 ± 0.36
Wild type	20.2 ± 0.5	27.8 ± 0.8	32.32 ± 0.59

The values shown are means ± SE for 11–18 plants per genotype. [a]Height 105 d after planting, rather than the final height. Unlike the mutants homozygous for *rga-28*, which all began bolting immediately after flowering, these plants did not begin bolting until day 65 or later. Only a fraction of the plants (35%, 75%, 38%, and 76% for *ga1-3, ga1-3/rgl1, ga1-3/rgl2,* and *ga1-3/rgl1/rgl2,* respectively) had bolted by the end of the experiment.

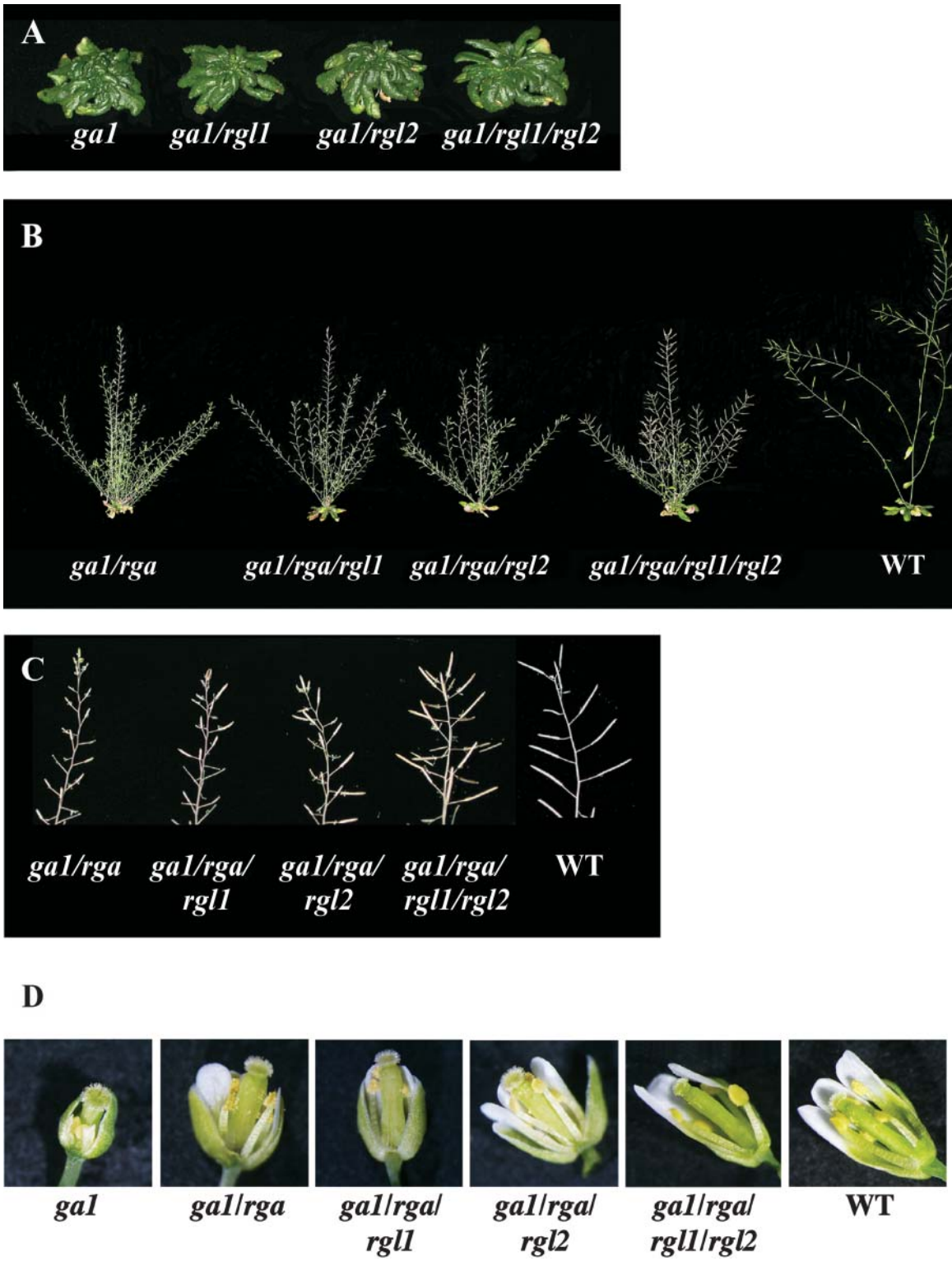

Figure 4. Phenotypic effects of loss-of-function *rga* and *rgl* mutations in a GA-deficient background. A, *ga1-3* and *rgl* mutant combinations without *rga-28*. B, *ga1-3*, *rgl*, and *rga-28* mutant combinations and a wild-type control. C, Primary inflorescence stems of mutants and wild type, as labeled. D, Flowers of mutant plants and wild type, as labeled. The front-most floral organs (sepal, petal, and in some cases stamen) were removed to expose the interior of the flower. Wild-type and homozygous mutant plants were grown on soil under long-day conditions. The plants shown in panels (A) and (B) are 73 d old, except for wild type, which is 44 d old.

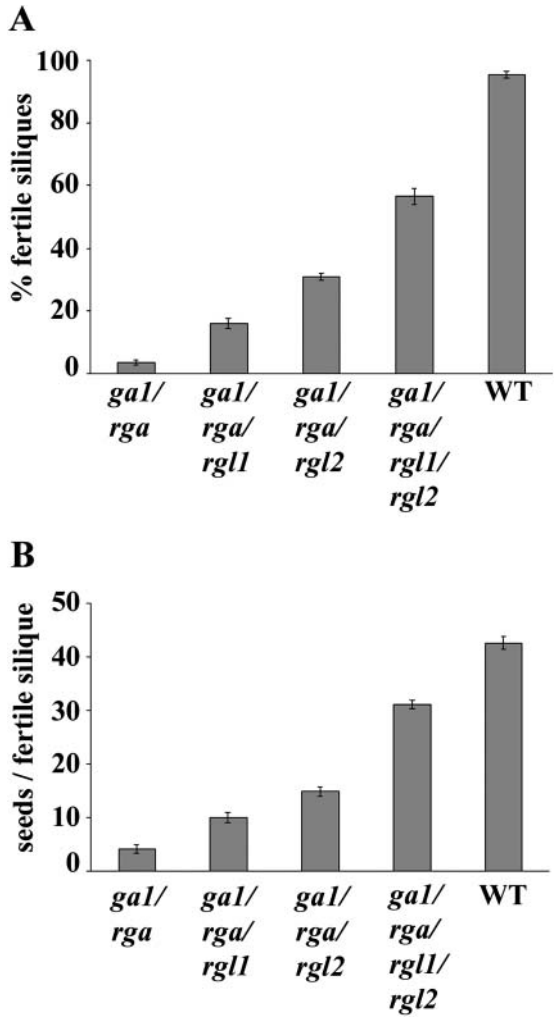

Figure 5. Effect of the *rga, rgl1,* and *rgl2* mutations on fertility in a *ga1-3* background. A, Percent of fertile siliques on the primary inflorescence. B, Number of seeds per fertile silique on the primary inflorescence. Seeds were counted for a minimum of 17 fertile siliques for each wild-type plant and for all possible fertile siliques for each mutant plant. Both A and B show the means ± SE for 14 to 16 plants per genotype.

previous finding that RGA and GAI are the major repressors in vegetative growth. The fact that *RGA* is expressed more highly than the other DELLA protein genes in vegetative tissues also supports the previous conclusion that RGA plays a predominant role in repressing GA signaling. All of the DELLA protein genes, except *RGL1*, were expressed at high levels (>100 copies) in imbibed seeds, suggesting that these four genes may be involved in regulating germination. In flower clusters and siliques, *RGL1*, *RGL2,* and *RGA* transcripts were present at high amounts (most are >90 copies). *GAI* transcript also accumulated to a moderate 59 copies in flowers. Therefore, RGL1, RGL2, RGA, and perhaps GAI may be important for flower and silique development.

Isolation of New T-DNA Insertion *rga, rgl1, rgl2,* and *rgl3* Alleles

To help define the role of each *RGL* gene in regulating GA signaling and plant development, we isolated *rgl1-2*, *rgl2-13*, *rgl3-3,* and a new *rga-28* allele by screening the T-DNA insertion mutant collection (Columbia [Col-0] background) from the Salk Institute (La Jolla, CA) using a PCR-based approach (Alonso et al., 2003). DNA sequence analysis indicated that in *rgl1-2*, the T-DNA insertion includes 116 extra unknown nucleotides and is located 1,232 nucleotides after the translational start site in *RGL1* (Fig. 2). The T-DNA insertions in *rgl2-13*, *rgl3-3,* and *rga-28* are 1032, 363, and 718 nucleotides, respectively, after the translational start site in each gene and include no extra sequence. *RGL1, RGL2*, *RGL3*, and *RGA* do not contain any introns, and their ORFs are 1533, 1641, 1569, and 1761 nucleotides, respectively (Fig. 2). Analysis of the previously isolated *rga* alleles indicated that the C-terminal GRAS domain is essential for the function of RGA as a GA signaling repressor (Silverstone et al., 1998; A.L. Silverstone and T.-p. Sun, unpublished data). The GRAS domain of the DELLA proteins is also highly conserved (Wen and Chang, 2002; Lee et al., 2002), suggesting that this region is important for the function of all DELLA proteins. The newly isolated *rga* and *rgl* alleles should not encode any functional proteins because the T-DNA is inserted in the middle of the coding sequence (Fig. 2). No wild-type transcript was detected in these mutants by RT-PCR using primers spanning the T-DNA insertions, although some transcripts were detected when using primers that amplified the 5′ sequences upstream of the insertion (data not shown). We will refer to the three new knockout *rgl* alleles as *rgl1*, *rgl2,* and *rgl3* in the rest of the paper. The homozygous single mutants, *rga-28*, *rgl1*, *rgl2,* and *rgl3*, have a wild-type-like phenotype (data not shown). This result is consistent with the previous finding that *rga-24* or *gai-t6* single null mutants have nearly wild-type morphology due to functional redundancy of the DELLA protein family (Peng et al., 1997; Dill and Sun, 2001).

rgl2, But Not *rgl1* or *rgl3*, Confers PAC Resistance during Germination

To study the role of the RGLs in seed germination, we tested the germination percentage of *rgl1*, *rgl2,* and *rgl3* in the presence of 120 μM PAC. PAC inhibits GA-dependent germination by blocking GA biosynthesis (Takahashi et al., 1991). Figure 3A shows that only *rgl2*, but not *rgl1* or *rgl3,* conferred PAC resistance during seed germination. We have generated a set of homozygous double mutants by genetic crossing among *rgl1*, *rgl2,* and *rgl3* and examined whether there is a synergistic interaction among these alleles in seed germination as observed between *rga* and *gai* null alleles in vegetative growth (Dill and Sun, 2001). However, the *rgl1*/*rgl2* and *rgl2*/*rgl3* double mutants had a similar germination percentage as that of *rgl2*

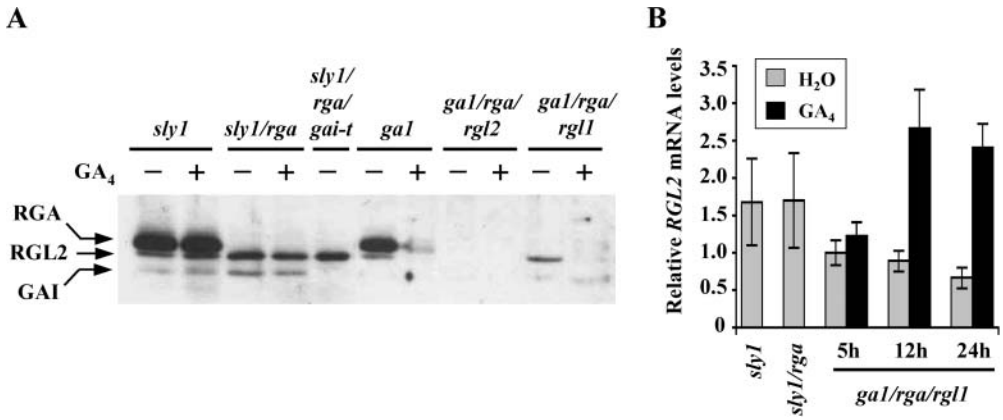

Figure 6. SLY1 regulates GA-induced RGL2 degradation. A and B, protein or RNA was isolated from imbibed seeds of various homozygous mutants as labeled. Before harvesting the tissues, the seeds were imbibed in water for 2 d, and then treated for 5, 12, or 24 h with 10 μM GA_4 (+) or water (–). A, The protein blot contains 60 μg total proteins from imbibed seeds of homozygous mutants (±GA for 5 h) and was probed with polyclonal anti-RGA antibodies from rat. B, Relative *RGL2* mRNA levels determined by qRT-PCR. At least three reactions were performed for each sample, and the *RGL2* mRNA level was normalized using *18S* rRNA. The means ± SE are shown. The value of *RGL2* mRNA in 5-h water-treated *ga1-3/rga/rgl1* is arbitrarily set to 1.0.

(Fig. 3B), indicating that *RGL2* is the primary GA signaling repressor during seed germination.

A loss-of-function mutation in *RGL2* was shown to rescue the germination defect of the *ga1-3* mutant seed (Lee et al., 2002). We therefore introduced the *ga1-3* allele into single *rgl1*, *rgl2*, *rgl3*, and *rga-28* mutants. Because the original *ga1-3* mutation is in the Landsberg *erecta* (L*er*) ecotype, we first made a *ga1-3* line that had been crossed for six generations into Col-0. We found that only *rgl2* rescued the germination defect of *ga1-3*, whereas *rgl1*, *rgl3*, or *rga* individually did not promote *ga1-3* germination (data not shown). These results are consistent with the finding of Lee et al. (2002), showing that RGL2 plays a major role in repressing seed germination.

Regulation of Floral Development by RGA, RGL1, and RGL2

The *ga1-3* mutant is male-sterile with poorly developed stamens and petals (Koornneef et al., 1983), indicating that GA is essential in controlling flower development in Arabidopsis. However, the appropriate level of GA signaling is important because wild-type plants overdosed with GA also have reduced fertility (Jacobsen and Olszewski, 1993). The flowers produced by the *rga-24/gai-t6* double null mutant have lower fertility than wild type, although a combination of *rga* and *gai* null mutations was unable to suppress the male-sterility of *ga1-3* (Dill and Sun, 2001). The reduced fertility of the *rga/gai* double null mutant mimics the GA overdose phenotype, suggesting that RGA and GAI play only a minor role in flower development (Dill and Sun, 2001). The high levels of *RGL1* and *RGL2* transcripts in flower clusters and siliques (Fig. 1) suggest that these two *RGL* genes may be important for floral and seed development. If any of the RGLs is the major repressor in GA-regulated floral development, we would expect to observe a reduced fertility phenotype when the given gene is mutated. We found that none of the single *rgl* mutants showed floral defects (data not shown). Because of functional redundancy, the role of the *RGL* genes may be better revealed in multiple mutant backgrounds. However, none of the double or triple homozygous *rgl* mutants showed any visible floral or fertility defect (data not shown), indicating that

Table II. *Interaction of RGLs and RGA with SLY1 and sly1-d in yeast two-hybrid assays*

LexA DB Fusion	Gal4 AD Fusion	His^- Media + 3-AT[a]	β-gal Units[b]
		mM	
SLY1	RGL1	2	1.5 ± 0.2
SLY1	RGL2	0	0.1 ± 0.1
SLY1	RGL3	5	1.4 ± 0.2
SLY1	RGA	2	0.7 ± 0.1
sly1-d	RGL1	5	7.1 ± 1.3
sly1-d	RGL2	30	43.0 ± 5.9
sly1-d	RGL3	30	34.0 ± 1.5
sly1-d	RGA	60	118.7 ± 16.1
LexA	RGL1	1	1.0 ± 0.0
LexA	RGL2	0	0.1 ± 0.1
LexA	RGL3	0	0.1 ± 0.0
LexA	RGA	0	0.1 ± 0.1
SLY1	Gal4	−[c]	Nd[d]
sly1-d	Gal4	−	Nd

[a]The relative growth on His^- plates containing 3-aminotriazole (3-AT) at 0, 1, 2, 5, 10, 30, and 60 mM. 3-AT is a competitive inhibitor of the His3 enzyme. The experiment was repeated twice. [b]β-gal activity (units). For each sample, at least three independent enzyme assays were performed and the means ± SE are shown. [c]No growth on His^- plates at 0 mM 3-AT. [d]Nd, Not determined.

RGA and/or GAI also must be inactivated to affect flower development.

The phenotypic effects of *rga* and *gai* null mutations are more evident in the *ga1-3* background, indicating that RGA and GAI are more active in repressing GA signaling in GA-deficient conditions (Dill and Sun, 2001; King et al., 2001). If this is also true for the RGLs, we would expect to reveal the physiological function of the RGLs more readily in the *ga1-3* background. We therefore analyzed a number of GA-regulated traits in single and multiple *rga-28* and *rgl* mutants in the GA-deficient *ga1-3* (Col-0) background. These included rosette leaf size (rosette radius), flowering time, final stem height, flower morphology, and fertility (average number of seeds per silique). The *rgl3* allele was not included in this study because *RGL3* mRNA was present at a very low level in reproductive tissues (Fig. 1; Supplemental Table II).

In the Col-0 background, the *ga1-3* mutant exhibited a dwarf and sterile phenotype, similar to that seen in the L*er* background. The only difference was that this mutant did bolt slightly, with a long delay between flowering and bolting (Table I). The phenotype of *ga1-3* (Col-0) is similar to another knockout *ga1* allele in the Col-0 background, which was identified from the T-DNA insertion mutant collection at the Salk Institute (M. Goellner-Mitchum and T.-p. Sun, unpublished data). The slightly bolting phenotype of *ga1-3* in the Col-0 background is consistent with the previous observation that the *erecta* (*er*) mutation in L*er* enhances a GA-related dwarf phenotype (Fridborg et al., 2001). Figure 4A and Table I show that *rgl1*and *rgl2*—singly or in combination—did not rescue the extreme dwarfism and sterility of *ga1-3*. This is consistent with our observation that the *RGL1* and *RGL2* transcripts are present at very low levels in leaves and stems (Fig. 1) and also with previous findings that RGA and GAI are the major repressors in vegetative growth (Dill and Sun, 2001; King et al., 2001). In the L*er* ecotype background, loss-of-function *rga* mutations partially rescue vegetative growth and floral induction, but not fertility of *ga1-3* (Silverstone et al., 1997). We found that in the Col-0 background, the knockout *rga-28* allele has a similar effect in restoring vegetative growth and floral initiation of *ga1-3* (Fig. 4B; Table I). Unexpectedly, *rga-28* also partially rescued the floral defect. In contrast to the flowers of *ga1-3*, the *ga1-3/rga-28* mutant flowers contained well-developed petals and more elongated stamen filaments and produced some viable pollen (Fig. 4D). A few (3%) of the flowers on the primary inflorescence stem of *ga1-3/rga-28* did produce fertile siliques with very few seeds (an average of four seeds/fertile silique) (Fig. 5, A and B). The homozygous triple mutants *ga1/rga/rgl1* and *ga1/rga/rgl2* produced more fertile siliques (16% and 31%, respectively) in comparison with the double mutant *ga1/rga* (Figs. 4C and 5). The homozygous quadruple mutant *ga1/rga/rgl1/rgl2* showed the highest fertility among all the mutant combinations (57% fertile siliques with an average of 31 seeds/fertile silique; Fig. 5, A and B). When comparing the stamen filaments among these mutant flowers, the quadruple mutant had the longest stamen filaments that reached the stigma surface, whereas the filaments of the double (*ga1/rga*) and triple (*ga1/rga/rgl1* and *ga1/rga/rgl2)* mutants were of intermediate length, but longer than in *ga1-3* (Fig. 4D). These results indicated that there is an additive interaction among *rga*, *rgl1,* and *rgl2* in restoring stamen development and fertility. As predicted by the expression patterns of *RGL1* and *RGL2* (Fig. 1), *rgl1* and *rgl2* mutations have little effect on the vegetative growth processes examined. The rosette radius, flowering time, and final stem height of the triple and quadruple mutants are similar to those of the double mutant *ga1/rga* (Fig. 4B; Table I).

GA-Induced RGL2 Degradation

Our qPCR data revealed that expression of the DELLA protein genes, especially for *RGL1*, *2,* and *3*, is regulated developmentally at the transcript level (Fig. 1). In the case of RGA and GAI activity, another regulatory mechanism appears to be essential. The endogenous RGA and GAI proteins are degraded rapidly in response to GA treatment, and the proteolysis of these proteins requires a functional SLY1, which is likely a component of the ubiquitin E3 ligase complex SCF^{SLY1} (Silverstone et al., 2001; McGinnis et al., 2003; Dill et al., 2004). To examine whether RGL2 protein stability is affected by GA and the loss-of-function *sly1-10* allele, we first analyzed RGL2 protein levels in imbibed seeds by immunoblot analysis. Imbibed seeds were tested because the *RGL2* mRNA level is high in this tissue and RGL2 is the major regulator during germination. Furthermore, seeds with the *ga1-3* mutant background were chosen for this analysis, because RGA protein levels are elevated under GA-deficient conditions (Silverstone et al., 2001; McGinnis et al., 2003). If GA affects RGL2 protein stability, RGL2 would accumulate to a higher level in this GA-deficient background. Using polyclonal anti-RGA antibodies, we detected a protein of approximately 61 kD in addition to RGA (64 kD) in *ga1-3* (Fig. 6A). A protein with the same mobility was also present in the *ga1-3/rga-28/rgl1* mutant (–GA sample) but was missing in *ga1-3/rga-28/rgl2* (Fig. 6A), suggesting that this protein is RGL2. Interestingly, this protein (presumably RGL2) disappeared after 5 h of GA treatment in both *ga1* and *ga1/rga/rgl1* seeds. In addition, in the *sly1-10* mutant background (*sly1-10*, *sly1-10/rga,* and *sly1-10/rga/gai-t6*), RGL2 accumulated at higher levels and was unaffected after GA treatment. These results suggest that RGL2, like RGA and GAI, is targeted for GA-induced degradation by SLY1. The effects of GA and *sly1-10* on the RGL2 protein levels are not due to changes in the amounts of the *RGL2* transcript (Fig. 6B).

Figure 6A also shows that the RGL2 signals detected by the polyclonal anti-RGA antibodies were significantly lower than those of RGA in imbibed seeds. Using

recombinant His-tagged RGA and RGL2 proteins in immunoblot analysis, we found that the affinity of the polyclonal anti-RGA antibodies to RGA is approximately 3-fold of the affinity to RGL2 (data not shown). Therefore, RGL2 is present at a lower level than RGA in imbibed seeds, even though RGL2 plays a major role in repressing seed germination. In addition, the amounts of RGL2 were elevated when RGA protein was absent (*sly1-10* versus *sly1-10/rga*, *ga1-3* versus *ga1-3/rga/rgl1;* Fig. 6A), suggesting that the cell has a regulatory mechanism to sense and modulate different DELLA protein levels to achieve proper levels of GA signaling. Alternatively, the changes in RGL2 protein levels may be due to differences in developmental stages caused by the absence of RGA.

Interaction of RGLs and SLY1 in Yeast Two-Hybrid Assays

Previous studies demonstrated that RGA and GAI directly interact with SLY1 in yeast two-hybrid assays and that a dominant mutation (*sly1-d*) affecting the C-terminal region of SLY1 enhances the interaction with RGA and GAI (Dill et al., 2004). We tested whether RGLs also interact with SLY1 and sly1-d in this yeast two-hybrid system, where SLY1 and sly1-d were expressed as fusion proteins with the LexA DNA binding domain (DB), and RGL1, RGL2, RGL3, and RGA were fused to the Gal4 transcription activation domain (AD). Expression of two reporter genes (*His-3* and *LacZ*) was assayed as previously described (Dill et al., 2004). We found that RGL1 and RGL3 showed a weak interaction with SLY1, similar to RGA (Table II). However, we were unable to detect an interaction between RGL2 and SLY1. Interestingly, sly1-d interacted much more strongly with RGL1, RGL2, and RGL3 than SLY1 did (Table II), consistent with the earlier finding that the *sly1-d* mutation allows for a greater interaction between sly1 and RGA (Dill et al., 2004). These results support the idea that SLY1 recruits RGL1, RGL2, and RGL3 for GA-induced degradation.

DISCUSSION

To study the potential function of the *RGL* genes and to identify the specific DELLA protein genes that control floral development in Arabidopsis, we surveyed the developmental expression profiles of all 5 DELLA protein genes and isolated and characterized new knockout *rga*, *rgl1*, *rgl2,* and *rgl3* mutants. Our results demonstrate that RGA, RGL1, and RGL2 together are important for modulating GA-regulated floral development, while RGL2 plays a major role during seed germination in Arabidopsis.

In the case of RGA and GAI, transcript levels do not necessarily reflect protein levels or activity, and ubiquitin-mediated proteolysis serves as a crucial mechanism for the regulation of RGA and GAI activity. However, our qPCR-generated expression data provided a starting point for the investigation of the potential function of each *RGL* gene and supported the idea of overlapping functions for DELLA subfamily members. Imbibed seeds contained high levels of *RGL2*, *RGL3*, *RGA,* and *GAI* transcripts, suggesting that they all might regulate germination. However, *rga* and *gai* null alleles—singly or in combination—cannot rescue the germination defect of *ga1-3* (Silverstone et al., 1997). Using the newly isolated knockout *rgl* mutants, we demonstrated that RGL2 plays the most important role (among the DELLA proteins) for regulating seed germination. Our results support the previous data of Lee et al. (2002) showing that only *rgl2*, but not *rgl1,* affects germination of *ga1-3* seed. However, GA or PAC dose response curves for seed germination need to be performed to determine whether other DELLA proteins may also contribute to this process. It is possible that additional DELLA proteins do play some roles in modulating germination, especially under different environmental conditions that may affect seed dormancy. It is interesting that *RGA*, *GAI,* and *RGL3* transcripts—like *RGL2* transcripts—accumulate to high levels in imbibed seeds (Fig. 1), and RGA protein is also relatively abundant in comparison to RGL2 (Fig. 6A). These results suggest that RGL2 may have a higher specific activity than RGA in repressing the germination of imbibed seeds. Alternatively, RGL2 and RGA may express in different tissue- or cell-types, and therefore may regulate different developmental aspects of germinating seed.

In this study, we found that the *rga-28* mutation in the Col-0 background restored petal development and also slightly rescued the stamen defect and male infertility of *ga1-3*. In contrast, *rga* alleles in the L*er* background have no effect on the floral defect or male sterility of *ga1-3* (Silverstone et al., 1997; Dill and Sun, 2001). The *er* mutation in L*er* enhances dwarf phenotypes of GA-deficient or GA-insensitive mutants (Fridborg et al., 2001), although the molecular mechanism involved has not been elucidated. A loss-of-function *rga* mutation may more readily suppress the floral defect of *ga1-3* in the Col-0 ecotype (*ER*) than in the L*er* background, because Col-0 does not contain the *er* mutation. Alternatively, additional modifier gene(s) may be present in different ecotype backgrounds. Our mutant analysis also revealed that *rgl1* and *rgl2*, in combination with *rga*, significantly increased the stamen filament growth, anther development, and fertility of *ga1-3* flowers. However, the quadruple mutant *ga1/rga/rgl1/rgl2* still did not reach wild-type levels of fertility (Fig. 5). Thus, there is a high degree of functional redundancy in controlling flower development. It remains to be determined whether *GAI* and/or *RGL3* also contribute to this regulation. Our qPCR data would predict that *GAI*, but not *RGL3,* is more likely to be involved because *RGL3* mRNA is present only at a very low level in flowers and siliques. Recently, GA has also been shown to affect pollen tube growth (Singh et al., 2002). Future studies will be

needed to elucidate the potential role of all the DELLA protein genes in this process. Therefore, in contrast to germination and vegetative growth, which are mainly controlled by 1 or 2 DELLA protein genes, floral development appears to have more complex regulation.

Studies of RGA and GAI orthologs in several crops indicate that the function of these DELLA proteins in repressing GA signaling is highly conserved between dicots and monocots (Richards et al., 2001; Olszewski et al., 2002; Gomi and Matsuoka, 2003). Interestingly, unlike Arabidopsis, only one functional ortholog of DELLA protein is present in rice (SLR1; Ikeda et al., 2001) and one in barley (SLN1; Chandler et al., 2002). Consequently, GA-independent stem growth is achieved by removing only SLR1 or SLN1, respectively, in these species. Similar to RGA, SLN1 and SLR1 are responsive to GA-induced proteolysis (Gubler et al., 2002; Itoh et al., 2002). The transcript levels of *RGA* and *SLR1* are slightly increased by GA, probably due to a feedback mechanism (Silverstone et al., 1998; Itoh et al., 2002).

Recent studies showed that *RGL2* mRNA levels in imbibed *ga1-3* seeds are reduced after GA treatment for 48 h at 23°C (Lee et al., 2002). This result led to the conclusion that GA promotes germination by reducing the amount of *RGL2* transcript. However, we found that in imbibed *ga1-3* seeds, RGL2 protein disappeared after only 5 h of GA treatment, and that the loss-of-function *sly1* mutation resulted in a high RGL2 protein level (Fig. 6A). In contrast, 5- to 24-h GA treatment or the *sly1* mutation did not significantly alter the *RGL2* transcript levels. Moreover, SLY1 and RGL2 interacted in a yeast two-hybrid assay. These data supported the idea that GA induces germination by causing proteolysis of RGL2 and that SLY1 targets RGL2 for degradation. The reduction of *RGL2* mRNA levels following a prolonged GA treatment seen in the previous study (Lee et al., 2002) may be a secondary effect of GA-induced germination. Our interpretation is also based on the observation that during wild-type seed germination, *RGL2* mRNA levels are elevated following imbibition but reduced when germination occurs (Lee et al., 2002; Fig. 1). After treating the imbibed *ga1-3* seeds with GA for 48 h at 23°C, germination would have occurred. In summary, *RGL2* gene expression is regulated at both transcript and protein levels during seed germination. Prior to the germination of wild-type imbibed seeds, elevation of endogenous GA biosynthesis (Ogawa et al., 2003) followed by GA-dependent rapid proteolysis of RGL2 (and other DELLA proteins) may be required to promote seed germination.

It is not clear why Arabidopsis contains five DELLA proteins whereas only one DELLA protein is present in rice and in barley. Nevertheless, results from our study and previous studies are beginning to reveal the complex regulation of these functionally overlapping genes for fine-tuning GA-regulated development in Arabidopsis. Isolation and characterization of DELLA proteins in additional species will allow us to determine whether complex developmental control of dicots requires multiple DELLA proteins. Alternatively, the differences in the DELLA-protein copy numbers may simply be the consequence of gene duplication events that occurred after the divergence of eudicots and monocots.

MATERIALS AND METHODS

Plant Lines

For all of the mutant characterization experiments, the Columbia-0 (Col-0) genetic background was used as the wild type. The *rgl1-2*, *rgl2-13*, *rgl3-3*, and *rga-28* lines were isolated in this study by PCR screening of a collection of T-DNA insertion lines generated in the Col-0 background at the Salk Institute (Alonso et al., 2003; see below). Crossing *ga1-3*, originally in Landsberg *erecta* (L*er*), to Col-0 six successive times yielded the *ga1-3* line (*ER* homozygous) used in this study. This *ga1-3* line was then used to generate double, triple, and quadruple mutants with the *rga-28* and *rgl* alleles. Homozygous mutants were identified by PCR using allele-specific primers (Supplemental Table III). For protein-blot analysis, several homozygous single, double, and triple mutant lines (*sly1-10*, *sly1-10/rga-24* and *sly1-10/rga-24/gai-t6;* McGinnis et al., 2003; Dill et al., 2004) that are in the L*er* background were also included.

Identification of T-DNA Insertion Mutant Lines

The *rga-28*, *rgl1-2*, *rgl2-13*, and *rgl3-3* mutants were isolated by screening the multidimensional DNA pools of T-DNA insertion mutant populations using a PCR-based method, as described previously (Alonso et al., 2003). The T-DNA left and right border primers (Alonso et al., 2003) and degenerate primers (primer 355 for *rgl* mutants and primer 244 for *rga* mutants) were used for PCR (Supplemental Table III). Mutant lines were identified by analyzing PCR products by DNA-blot analyses using gene-specific cDNA probes for each gene of interest, and the location of each T-DNA insertion in each mutant was determined by DNA sequence analysis of the PCR products. Homozygous mutant plants were identified by PCR using allele-specific primers (Supplemental Table III). To detect any remaining transcripts produced in each mutant, RT-PCR was performed using primers that span the T-DNA insertion (Supplemental Table III) or are specific for sequences upstream of the insertion.

Plant Growth Conditions

Plants were grown on MetroMix 200 soil (Scotts-Sierra Horticultural Products, Marysville, OH) at 22°C under long-day conditions (16 h light/8 h darkness). To promote the germination of GA-deficient seeds, all seeds for the characterization study (except wild type) were treated with 50 μM GA_4 at 4°C for 7 d and rinsed thoroughly with water before planting. Wild-type seeds were incubated in water at 4°C for 3 d prior to planting. The first clear appearance of a flower bud that was visible to the naked eye was taken to mark the flowering time. The length of the longest rosette leaf was considered to be the maximum rosette radius.

Quantitative, Real-Time PCR

Total RNA was extracted from approximately 150 to 200 mg samples of the following wild-type tissues: seeds imbibed in water for 12 or 24 h under continuous light; 2- and 5-d-old seedlings; shoots and roots of 10-d-old seedlings; entire aerial portions (rosettes) of 14-d-old plants; and rosette leaves, cauline leaves, stem tissue, flower clusters, and siliques from 33-d-old plants. Seeds (for 12 and 24 h imbibed samples) were washed in 0.02% Triton X-100 and rinsed with sterile water before plating on filter paper for imbibition under continuous light. At the 24 h time-point, most of the seeds had begun to germinate, as evidenced by a cracked seed coat or protruding radicle. Seedlings for the 2-, 5-, and 10-d time points were grown on plates of 1× Murashige and Skoog medium containing 2% sucrose and 0.8% agar. Seed-

lings for the 10-d time point were grown vertically to facilitate the recovery of root tissue. The stem tissue included only internodes, without the nodes. Flower clusters and siliques were at various stages of development.

RNA was extracted from seeds using the RNAqueous kit plus Plant RNA Isolation Aid (Ambion, Austin, TX). An RNeasy Plant Mini kit (QIAgen, Valencia, CA) was used for the remaining samples, with buffer RLT for all samples except siliques, for which buffer RLC was employed. DNA was removed via either an on-column DNase treatment for the QIAgen kit or a separate treatment with RQ1 DNase (Promega, Madison, WI) for the samples extracted with the Ambion kit. RT-PCR was first performed using primers that span an intron in *AtGA3ox1* (Supplemental Table III) to confirm that each RNA sample was free of genomic DNA contamination. For real-time PCR, the First Strand cDNA Synthesis kit (Roche, Diagnostics, Mannheim, Germany) was used to make cDNA from 1 μg of RNA in a 20 μL reaction volume. Each cDNA sample was diluted 1:20 in water, and 2 μL of this dilution was used as template for qPCR. Half-reactions (10 μL each) were performed with the Lightcycler FastStart DNA Master SYBR Green I kit (Roche) on a Roche LightCycler real-time PCR machine, according to the manufacturer's instructions. *UBIQUITIN11* (*UBQ11*, At4g05050) was used as a control in qPCR, because its transcript levels remained similar across the tissues and developmental stages tested when compared with the *18S* rRNA by quantitative RT-PCR (qRT-PCR) using the LightCycler RNA amplification kit SYBR Green I (Roche; Supplemental Table I). Gene-specific primers for detecting transcripts of *UBQ11*, *18S*, *RGL1*, *RGL2*, *RGL3*, *RGA*, and *GAI* are listed in Supplemental Table III. Primers were used at a final concentration of 0.5 μM each, and the annealing temperature was 55°C in all cases. The qRT-PCR for *18S* and *UBQ11* contained 5 pg and 50 ng of RNA template, respectively, as well as a final $MgCl_2$ concentration of 6 mM. The qPCR reactions for *RGL1* included a final $MgCl_2$ concentration of 3 mM. Reactions for *RGL3* were performed with 5 mM $MgCl_2$, and the remaining qPCR reactions (for *RGL2*, *RGA*, *GAI*, and *UBQ11*) included 4 mM $MgCl_2$. PCR products for *RGL1*, *RGL2*, *RGL3*, *RGA*, and *GAI* and a linearized plasmid for *UBQ11* were used to generate standard curves. The number of gene-specific cDNA copies was determined for each sample, normalized using the *UBQ11* cDNA level, and averaged over three replicates.

Germination Assays

For germination inhibition assays, a stock of 50 mM PAC (PhytoTechnology Laboratories, Shawnee Mission, KS) in 95% ethanol was diluted to 120 μM PAC in 0.01% Tween 20. Seeds were washed with 0.02% Triton X-100, rinsed with sterile water, and spread onto filter paper saturated with the 120 μM PAC solution, in petri dishes. As a negative control (–PAC), the seeds were incubated with 0.01% Tween 20 and 0.23% ethanol. After 7 d of incubation at 22°C under 16 h light (100 μE)/8 h darkness, radicle protrusion was scored as germination. Without PAC treatment, seeds of all genotypes germinated. Only seeds harvested at the same time were used for each experiment, because the percent germination could vary significantly depending on the age of the seeds.

To test the effect of *rga* or *rgls* in restoring *ga1-3* germination, the seeds were rinsed once with sterile water and spread onto filter paper saturated with sterile water. Germination was scored after 4 d of incubation under the same conditions as in the PAC assays.

Analysis of RGL2 Protein and Transcript Levels by Immunoblot Analysis and Real-Time RT-PCR

Seeds of homozygous mutants *ga1-3*, *ga1-3/rga-28/rgl1-2*, *ga1-3/rga-28/rgl2-13*, *sly1-10*, *sly1-10/rga-24*, and *sly1-10/rga-24/gai-t6* were sprinkled onto filter paper that was saturated with sterile water in petri dishes and imbibed for 2 d under continuous light of 100 μE at 22°C. Imbibed seeds were then treated with 10 μM GA_4 (+) or water (–) for 5 h (all genotypes) and 12 h or 24 h (only for *ga1-3/rga-28/rgl1*) before harvesting. Under these conditions, none of the seeds (±GA for 5 or 12 h) in the *ga1-3* background germinated, except *ga1-3/rga/rgl2*. After 24 h GA treatment, approximately 20% of *ga1-3/rga/rgl1* seeds germinated. For seeds in the *sly1* background, only a small fraction of the seeds germinated, and we only harvested ungerminated seeds for protein and transcript analysis. Total proteins were extracted from the 5 h ± GA samples and analyzed by immunoblot analysis using anti-RGA antibodies from rat (DUR18) as described previously (McGinnis et al., 2003). Ponceau staining was used to confirm equal loading.

RNA was extracted from the imbibed *ga1-3/rga-28/rgl1-2* seeds (±GA for 5 h, 12 h, and 24 h), *sly1-10* and *sly1-10/rga-24* seeds (–GA, 5 h) as described in the "Quantitative Real-Time PCR" section. The *RGL2* transcript levels were analyzed by qRT-PCR using a Roche LightCycler and the LightCycler RNA Amplification kit SYBR Green I (Roche) according to the manufacturer's instructions. Gene-specific primers for *RGL2* and *18S* rRNA (Supplemental Table III) were used in the qRT-PCR with the annealing temperature at 55°C in 7 mM $MgCl_2$ for *RGL2*, and 6 mM $MgCl_2$ for *18S*. 5 pg or 25 ng of total RNA was used as a template in each qRT-PCR reaction (10 μL volume) for *18S* rRNA and *RGL2* RNA, respectively. A no-template control was routinely included to confirm the absence of DNA or RNA contamination. Relative transcript levels of *RGL2* in all samples were normalized using *18S* rRNA because *UBQ11* expression is affected by the GA treatment. The reactions were performed three times using freshly diluted RNA samples in each set of reactions.

Yeast Two-Hybrid Assays

Yeast two-hybrid assays were performed using *Saccharomyces cerevisiae* strain L40 as described previously (Dill et al., 2004). The bait and prey protein fusions were expressed as LexA DNA-binding domain (DB) and Gal4 activation domain (AD) fusions using the yeast plasmid expression vectors pLexA-NLS (Vojtek et al., 1993) and pACTII (Li et al., 1994), respectively. pLexA-SLY1 and pLexA-sly1-d were made previously (Dill et al., 2004), and pGal4-RGL1 (in pACT) was a gift from Dr. Caren Chang (Wen and Chang, 2002). The Gal4-RGL2 and Gal4-RGL3 constructs were made by amplifying the coding regions of *RGL2* and *RGL3* from Col-0 genomic DNA with PCR primers that incorporate *Nco*I and *Bam*HI sites (for RGL2) or BglII site (for RGL3) in the correct reading frame (Supplemental Table III). The PCR DNA fragments were digested with *Nco*I and *Bam*HI (for RGL2) or BglII (for RGL3) and subcloned into the *Nco*I/*Bam*HI sites or *Bam*HI site of pACTII.

Distribution of Materials

Upon requests, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. The only exception is that we will not be able to distribute the anti-RGA antibodies from rat (DUR18) because we only have a very limited amount.

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