Synergistic Derepression of Gibberellin Signaling by Removing RGA and GAI Function in *Arabidopsis thaliana*

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ABSTRACT

RGA and *GAI* are negative regulators of the gibberellin (GA) signal transduction pathway in *Arabidopsis thaliana*. These genes may have partially redundant functions because they are highly homologous, and plants containing single null mutations at these loci are phenotypically similar to wild type. Previously, *rga* loss-of-function mutations were shown to partially suppress defects of the GA-deficient *ga1-3* mutant. Phenotypes rescued include abaxial trichome initiation, rosette radius, flowering time, stem elongation, and apical dominance. Here we present work showing that the *rga-24* and *gai-t6* null mutations have a synergistic effect on plant growth. Although *gai-t6* alone has little effect, when combined with *rga-24*, they completely rescued the above defects of *ga1-3* to wild-type or GA-overdose phenotype. However, seed germination and flower development defects were not restored. Additionally, *rga-24* and *rga-24/gai-t6* but not *gai-t6* alone caused increased feedback inhibition of expression of a GA biosynthetic gene in both the *ga1-3* and wild-type backgrounds. These results demonstrate that *RGA* and *GAI* have partially redundant functions in maintaining the repressive state of the GA-signaling pathway, but *RGA* plays a more dominant role than *GAI*. Removing both RGA and GAI function allows for complete derepression of many aspects of GA signaling.

THE plant hormone gibberellin (GA) plays an important role in many aspects of plant growth and development (reviewed in Hooley 1994; Ross *et al.* 1997). For instance, GA promotes seed germination, leaf expansion, and stem elongation. In a number of species, GA also regulates flowering time and is necessary for flower and fruit development. The essential role of GA in plant growth is clearly illustrated by the phenotype of severe GA biosynthetic mutants (Phillips 1998). In *Arabidopsis thaliana*, these mutants, for example, *ga1-3*, fail to germinate without exogenously applied GA, produce small, dark green leaves, and are male-sterile dwarfs (Koornneef and van der Veen 1980). These defects are rescued completely by GA treatment.

Progress is being made to identify components involved in the GA signal transduction pathway using both pharmacological and genetic approaches (reviewed in Bethke and Jones 1998; Thornton *et al.* 1999; Lovegrove and Hooley 2000; Sun 2000). Biochemical studies using cereal aleurone cells provide evidence that the GA receptor is localized on the plasma membrane (Hooley *et al.* 1991; Gilroy and Jones 1994), but the nature of the receptor remains elusive. Second messengers and transcription factors implicated in GA signaling include Ca^{2+}, calmodulin, heterotrimeric G-proteins, protein kinases, and GAMYB (Bethke and Jones 1998; Lovegrove and Hooley 2000).

GA-signaling mutants have been isolated from various species and fall into two phenotypic categories, GA-unresponsive dwarf mutants and slender mutants (Thornton *et al.* 1999; Sun 2000). The GA-unresponsive mutants resemble the dwarf mutants that are defective in GA biosynthesis, but fail to respond to exogenous GA application. Recessive mutants in this class are likely to be impaired in positive regulators of GA signaling, *e.g.*, *dwarf 1* (*d1*) in rice (Mitsunaga *et al.* 1994), *gse* in barley (Chandler and Robertson 1999), and *sleepy1* in Arabidopsis (Steber *et al.* 1998). The *D1* gene has been shown to encode the putative α-subunit of the heterotrimeric G-protein (Ashikari *et al.* 1999; Ueguchi-Tanaka *et al.* 2000). A number of semidominant GA-unresponsive dwarf mutants have also been isolated, including *gai-1* in Arabidopsis (Koornneef *et al.* 1985), *D8* and *D9* in maize (Phinney 1956), and the *Rht* mutants of wheat (Borner *et al.* 1996). Isolation of intragenic revertants and recent cloning of these genes showed that they encode negative regulators of GA response (Peng *et al.* 1999; see below). Another GA-unresponsive dwarf mutant is the semidominant *shi* mutant in Arabidopsis (Fridborg *et al.* 1999). The phenotype in this plant is due to overexpression of its product and *SHI* is therefore also a putative negative regulator of GA response.

The recessive slender mutants resemble wild-type plants that have been treated with excess GA, suggesting that they are defective in negative regulators of GA signaling.

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Mutants in this category include *la cry*^{s} from pea (Potts *et al.* 1985), *sln* in barley (Lanahan and Ho 1988), and *spindly* (*spy*) from Arabidopsis (Jacobsen and Olszewski 1993). *SPY* is predicted to encode an *O*-linked N-acetylglucosamine (GlcNAc) transferase (OGT; Thornton *et al.* 1999), which regulates target protein function by glycosylation of serine or threonine residues (Hart 1997).

We have identified another negative regulator of GA signaling, *RGA*, by screening for Arabidopsis mutants that were able to suppress the GA-deficient phenotype of *ga1-3* (Silverstone *et al.* 1997). The homozygous *rga/ga1-3* double mutants, while still nongerminating and male sterile, have larger leaves and a semidwarf stature. Cloning of *RGA* revealed that RGA and GAI are 82% identical at the amino acid level and have hallmarks of transcriptional regulators, such as a nuclear localization signal, homopolymeric serine and threonine sequences, leucine heptad repeats, and an SH2-like domain (Peng *et al.* 1997, 1999; Silverstone *et al.* 1998). Additionally, a green fluorescent protein-RGA fusion protein has been shown to localize to the nucleus in a transient assay in onion cells (Silverstone *et al.* 1998) and in stably transformed Arabidopsis (Silverstone *et al.* 2001).

RGA and GAI are members of the GRAS (*G*AI, *R*GA and *S*CARECROW) family of regulatory proteins (Pysh *et al.* 1999). In Arabidopsis, at least 38 GRAS family members are present, and all of them contain highly conserved central (VHIID) and C-terminal (RVER) regions. Their N termini, however, are more divergent. Interestingly, RGA and GAI have a conserved sequence near their N termini, termed the DELLA domain, after an amino acid motif contained therein (Silverstone *et al.* 1998). This DELLA region may be required for the inactivation of GAI and RGA by the GA signal, because a 17-amino-acid deletion in this domain in either protein causes a GA-insensitive dwarf phenotype (Peng *et al.* 1997; A. Dill and T.-p. Sun, unpublished results). It has been hypothesized that deleting the DELLA sequences turns the mutant protein into a constitutive repressor of GA signaling (Peng *et al.* 1997).

Recently, the functional orthologs of *RGA* and *GAI* in wheat (*Rht*), maize (*d8*), barley (*SLN*; P. M. Chandler, A. Marion-Poll, F. Gubler, personal communication), and rice (*SLR*; Ogawa *et al.* 2000; Ikeda *et al.* 2001) have been isolated. Mutant studies revealed that these genes also function as repressors of GA signaling, indicating that RGA/GAI function is conserved among dicots and monocots (Peng *et al.* 1999; Ikeda *et al.* 2001). The most notable example is the semidwarf wheat cultivars, a crucial component of the "Green Revolution," which all contain deletions in the DELLA region of an *Rht* gene.

Because a loss-of-function mutation at either the *RGA* or *GAI* locus in wild-type GA background does not cause any obvious phenotype, these two homologous genes may have partially or completely redundant functions in GA signaling (Peng *et al.* 1997; Silverstone *et al.* 1998). To test this hypothesis, we generated homozygous *rga/gai* double mutant lines in the wild-type and *ga1-3* backgrounds and showed that *rga* and *gai* null alleles have synergistic effects on a number of GA-mediated processes. GA signaling can control GA biosynthesis through feedback mechanisms (reviewed in Bethke and Jones 1998; Hedden and Phillips 2000; Yamaguchi and Kamiya 2000). By RNA blot analysis, we demonstrated that loss of RGA and GAI function also affects the feedback regulation of the GA biosynthetic gene *GA4*.

MATERIALS AND METHODS

Isolation of mutant lines: We isolated *rga-24*, *rga-24/gai-t6*, *gai-t6/ga1-3*, and *rga-24/gai-t6/ga1-3* homozygous mutant lines from crosses between *rga-24/ga1-3*, *gai-t6*, *ga1-3*, and wild type in the Landsberg *erecta* (L*er*) ecotype. *gai-t6* and *rga-24/gai-t6* also contained *tt1-1*. *gai-t6* was a gift from Nicholas Harberd. Allele-specific primers were designed to identify homozygous mutants from segregating F_2 populations of different crosses. Primers 219 (5′-GTTGATAGACATTTTCAATGA-3′) and 220 (5′-GGTCATCAGTAGAGACTAA-3′), which flank the 8.4-kb deletion in *rga-24*, amplify a 4-kb region in *rga-24* but fail to amplify *RGA* DNA because the distance between these primers is too great for amplification of the product under standard conditions. *RGA* was identified using primers 219 and 212 (5′-GGTGATTTTCACGGTGGTTG-3′), which amplify a 3.3-kb region in *RGA* but fail to amplify *rga-24* because the sequence in primer 212 is deleted in *rga-24*. To detect *gai-t6* we used primer 304 (5′-TCGGTACGGGATTTTCGCAT-3′), and primer 300 (5′-CTAGATCCGACATTGAAGGA-3′), which are located in the Ds insertion and the *GAI* coding sequence, respectively. Together, these primers amplify a 720-bp fragment in *gai-t6*, but not in the *GAI* allele. *GAI* was identified using primers 300 and 302 (5′-AGCATCAAGATCAGCTAAAG-3′), which flank the Ds insertion in *gai-t6* and therefore only amplify a 1.2-kb product in *GAI* but not in *gai-t6*. The *GA1* and *ga1-3* alleles were verified using PCR primers as previously described (Silverstone *et al.* 1997). All the PCR reactions were done using AmpliTaq (Perkin-Elmer, Norwalk, CT) except for genotyping *RGA* and *rga-24*, for which Accu Taq LA (Sigma, St. Louis) was used.

Plant growth conditions: For all experiments except for the examination of *GA4* mRNA levels and germination, seeds were imbibed for 3 days at 4° and then sown on soil. Seeds in the *ga1-3* background were imbibed in 100 μM GA_3 and then rinsed thoroughly with water before sowing on soil. Sterilization of seed surface in bleach solution weakens the seed coat and could result in up to 5% germination in nongerminating *ga1-3* seeds (Silverstone *et al.* 1997). To avoid this effect, seeds for germination percentage measurements were gently washed in 0.02% Triton X-100 to remove most contamination, rinsed with sterile water, and then spread on three layers of moist Whatman filter paper. Long day (LD) plants were grown at 22° with 16-hr light and 8-hr dark cycles supplied under a light intensity of 140 μE. All experiments except flowering time, abaxial trichome initiation, rosette radius, and *GA4* mRNA levels were carried out under LD conditions. Flowering time, abaxial trichome initiation, and rosette radius were studied under short day (SD) conditions of 8-hr light and 16-hr dark cycles at 22° with a light intensity of 160 μE. SD lighting

was supplied by a 3:1 mixture of cool-white:wide spectrum fluorescent bulbs (General Electric).

Measuring flowering time, rosette radius, and germination: The flowering time was scored when the flower bud was first visible without manipulation or magnification. Rosette radius was obtained by measuring the longest rosette leaf of each plant. Germination percentage was scored after 7 days of incubation in LD conditions. A seed was considered germinated if the radicle protruded from the seed coat.

***GA4* mRNA quantitation:** Seeds were washed with 95% ethanol for 1 min, sterilized in bleach for 2 min, and rinsed thoroughly with sterile water. Seeds were then imbibed for 3 days at 4° in either 50 μM GA_4 (*ga1-3*-containing lines) or water (*GA1*-containing lines) and then washed five times with sterile water before plating on agar media containing Murashige-Skoog media and 2% sucrose in 100 × 15 mm plates. The plates were incubated at 22° under continuous light for 13 days with a light intensity of 100 μE. The seedlings were then either harvested or treated with 3 ml of 100 μM GA_3 per plate for 8 hr and then harvested. Total mRNA was isolated and *GA4* mRNA detected using an antisense *GA4* RNA probe as described by Yamaguchi *et al.* (1998). As a loading control 18S RNA levels on the same blot were examined using 5′-^{32}P-labeled oligonucleotide (5′-TGAAGGGATGCCTCCAC-3′). The blot was prehybridized for 2 hr at 42° in 10× Denhardt's, 5× SSPE, 1% SDS, and 100 μg/ml salmon sperm DNA. ^{32}P-labeled 18S oligonucleotides were then added, with the final concentration of oligonucleotides at 15 nM and 5×10^5 cpm/ml, and hybridized overnight at 42°. The filters were washed four times (10 min each wash) in 6× SSC and 0.1% SDS at 48° and analyzed using a PhosphorImager as previously described (Silverstone *et al.* 1998).

RESULTS

To investigate the role of RGA and GAI in GA signaling, we generated homozygous single and double *rga-24/gai-t6* mutant lines in the wild-type and *ga1-3* backgrounds by genetic crosses. Both *rga-24* and *gai-t6* are null alleles due to a deletion spanning the *RGA* locus (Silverstone *et al.* 1998) and a Ds insertion in the *GAI* coding region (Peng *et al.* 1997), respectively. Allele-specific PCR markers for *RGA* and *GAI* loci were designed to identify homozygous mutant and wild-type alleles. A number of GA-controlled developmental phenotypes in the mutants were examined, including seed germination, stem elongation, juvenile-to-adult transition in leaf development, apical dominance, flowering time, and flower development. Our previous mutant analysis suggested that *RGA* plays an important role in most of these processes, except seed germination and flower development (Silverstone *et al.* 1997). Because *RGA* and *GAI* are likely to have overlapping functions in GA signaling, we tested whether *gai-t6* has a similar effect as *rga-24* and whether *rga-24* and *gai-t6* showed additive interactions in these GA-mediated processes.

Phenotypes of mutant lines: Figure 1 shows the phenotypes of the digenic (double homozygous) and trigenic (triple homozygous) mutants, along with wild-type L*er* and *ga1-3*. The *rga-24* and *gai-t6* single mutants (not shown) had a similar phenotype as L*er*. The *gai-t6/ga1-3* mutant did not bolt at all. This is in contrast to *rga-24/ga1-3*, which had partially restored stem height (59% of L*er*, Figure 2). However, the combination of *rga-24* and *gai-t6* had a dramatic effect on stem growth in both wild-type and GA-deficient *ga1-3* backgrounds. Compared to the nonbolting *ga1-3*, the final stem height of the trigenic *rga-24/gai-t6/ga1-3* mutant was remarkable (even taller than L*er* by 32%). The digenic *rga-24/gai-t6* mutant was 35% taller than L*er* (Figures 1 and 2). This synergistic effect of *rga* and *gai-t6* indicates that *RGA* and *GAI* are the major repressors regulating GA-induced stem growth in Arabidopsis.

Phase change and flowering time: The appearance of trichomes on the lower surface of the leaf indicates the developmental transition from juvenile-to-adult stages. GA is essential for this transition because the *ga1-3* mutant does not produce abaxial trichomes at all (Chien and Sussex 1996). Previously, we showed that the *rga/ga1-3* mutants initiate abaxial trichomes, although later than wild type (Silverstone *et al.* 1997). This indicated that *RGA* inhibits the GA-induced transition from juvenile-to-adult stages during leaf development. Here, we examined the timing of the phase change under SD conditions because the plants would have a longer juvenile phase. We found that *rga-24* alone caused the abaxial trichomes to initiate much earlier than the control in both the *ga1-3* and wild-type GA backgrounds (Figure 3A). The *gai-t6* mutant initiated abaxial trichomes only very slightly earlier than L*er*, whereas *gai-t6/ga1-3* did not produce any abaxial trichomes (Figure 3A). However, *GAI* does play an important role in suppressing the juvenile-to-adult phase transition because the trigenic mutant *rga-24/gai-t6/ga1-3* initiated abaxial trichomes even earlier than L*er* (Figure 3A). These results indicate that both *RGA* and *GAI* inhibit the juvenile-to-adult developmental stage transition, although *RGA* plays a more dominant role than *GAI*.

GAs are important for promoting flowering in Arabidopsis and are required for flower initiation in SD (Wilson *et al.* 1992). We showed that *rga* partially rescues the flowering time defect of *ga1-3*, suggesting that *RGA* suppresses flowering (Silverstone *et al.* 1997). In this study we scored flowering time in both days to flower and leaves to flower. The *rga-24* and *gai-t6* single mutants flowered slightly earlier than L*er* (8.8 days and 6.5 leaves for *rga-24* and 3.2 days and 3 leaves for *gai-t6*; Figure 3, A and B). In the GA-deficient background, both *rga-24* and *gai-t6* rescued the nonflowering defect of *ga1-3* although *rga-24/ga1-3* flowered 63.7 days (42%) and 23.2 leaves (30%) earlier than *gai-t6/ga1-3*. The flowering time of the trigenic *rga-24/gai-t6/ga1-3* mutant was even earlier than L*er*. This plant flowered 10 days (16%) earlier with 10 fewer leaves (30%) than L*er*, which was comparable to *rga-24/gai-t6* and *rga-24*.

The earlier flowering time of plants containing *rga-24* or *rga-24/gai-t6* alleles appears to correlate with a shorter juvenile stage of the plants (Figure 3A). In con-

FIGURE 1.—Phenotypes of plants. All plants are 52 days old except *rga-24/gai-t6*, which is 37 days old.

trast, the length of the adult stage was similar in all lines that underwent the phase transition. Therefore, the main role of *RGA* and *GAI* in repressing flowering is by delaying the juvenile-to-adult transition.

Leaf expansion and apical dominance: GAs are known to promote leaf expansion. We examined the effect of *rga-24* and *gai-t6* on rosette size by measuring the maximum radius of each line. Figure 4A shows that *rga-24* partially rescued the leaf expansion defect of *ga1-3* whereas *gai-t6* alone had no effect. The rosette radius of the trigenic mutant is similar to L*er*.

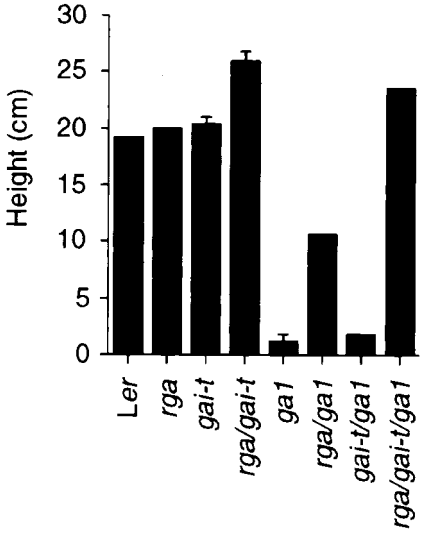

FIGURE 2.—Final heights of plants. The final height of 10 plants per line was measured and the means ±SE are shown. Some error bars are too small to be seen.

The greatly reduced apical dominance in *ga1-3* is partially rescued by *rga* (SILVERSTONE *et al.* 1997). The axillary branch numbers of different mutant lines are shown in Figure 4B. The *gai-t6* mutation alone in either a wild-type or GA-deficient background did not affect this phenotype at all. However, in combination with *rga-24*, apical dominance was restored to wild type (Figure 4B). These results show that *RGA* and *GAI* both function to repress GA-induced leaf expansion and apical dominance and that *RGA* plays a more dominant role than *GAI* in these processes.

Germination, fertility, and flower morphology: GAs are vital for both germination and flower development as reflected in the nongerminating, male-sterile phenotype of *ga1-3*. We showed previously that mutations at the *RGA* locus were unable to rescue these phenotypes. Therefore, we tested whether *gai-t6* or *rga/gai-t6* have any effect on these processes. Neither *gai-t6* nor *rga-24/gai-t6* increases the germination percentage or restores the fertility of *ga1-3* (Table 1). The digenic *rga-24/ga1-3*, *gai-t6/ga1-3* (not shown) and trigenic *rga-24/gai-t6/ga1-3* mutants all had male-sterile flowers with rudimentary petals, which are similar to those of *ga1-3* (Figure 5B).

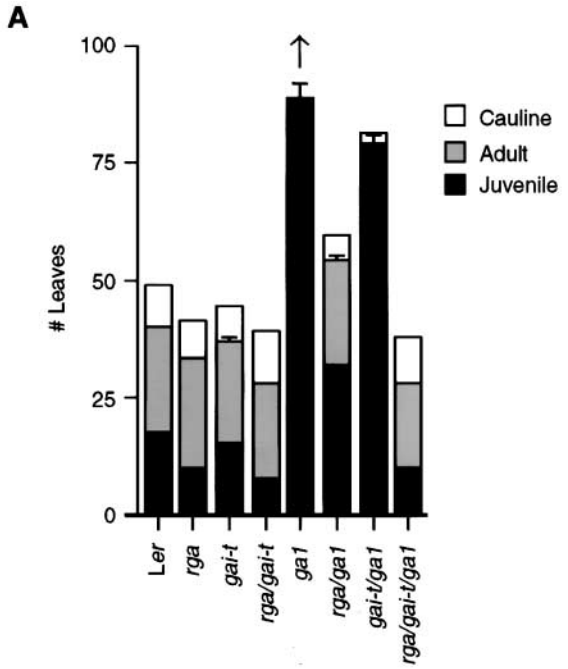

FIGURE 3.—The effect of *rga-24* and *gai-t6* null alleles on flowering time in SD. (A) Number of juvenile, adult, and cauline leaves produced by the primary inflorescence stem of each genotype after bolting. Juvenile leaves do not have abaxial trichomes whereas adult leaves do. (B) Number of days from sowing until floral buds are clearly visible. The values plotted are the means ±SE of 10–20 plants. Some error bars are too small to be seen. ↑ indicates that *ga1-3* produced only juvenile leaves and did not flower after 170 days. SE of *ga1-3* in A represents the variation in total leaf number of plants on day 170.

These results suggest that *RGA* and *GAI* may not control seed germination or flower development. However, we noticed that the carpels of the *rga-24/gai-t6/ga1-3* mutant flower are longer than those in *ga1-3* flowers (Figure 5B). We also found that the digenic *rga-24/gai-t6* mutant had much reduced fertility compared to that of wild-type L*er* (Table 1). In fact, *rga-24/gai-t6* had reduced pollen levels compared to L*er* (not shown) and the stamen filaments were shorter than the carpels (Figure 5B). This developmental defect is probably due to an elevated level of GA signaling, which mimics the effect of GA overdose on flower development. Although GA is required for stamen development, an overdose of GA on wild-type Arabidopsis plants also results in reduced fertility (JACOBSEN and OLSZEWSKI 1993). These data suggest that *RGA* and *GAI* may play only a minor role in flower development.

Feedback control of GA biosynthesis: In addition to the above phenotypes, we also investigated the effect of the *rga* and/or *gai* mutations on the feedback regulation of GA biosynthesis, which is affected by the activity of the GA response pathway (BETHKE and JONES 1998). The *GA4* gene in Arabidopsis encodes GA 3β-hydroxylase, which catalyzes the conversion of GA precursors to bioactive GAs (CHIANG *et al.* 1995). The transcript level of this gene is upregulated in the GA-deficient background, and the elevated *GA4* mRNA level can be reduced by application of GA (CHIANG *et al.* 1995; COWLING *et al.* 1998; SILVERSTONE *et al.* 1998; YAMAGUCHI *et al.* 1998). In addition, the gain-of-function *gai-1*

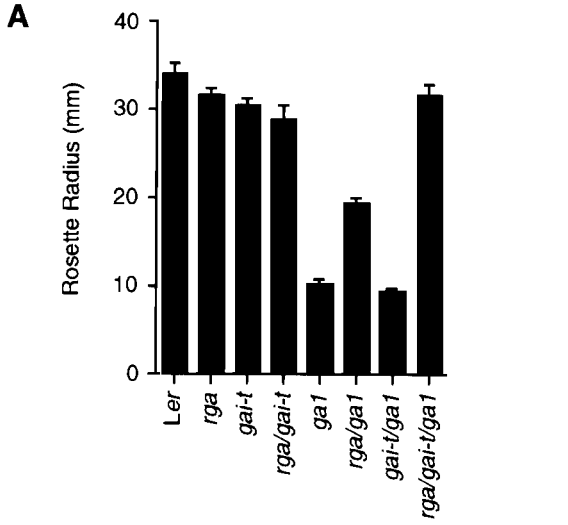

FIGURE 4.—The effect of *rga-24* and *gai-t6* null alleles on rosette radius and apical dominance. (A) Rosette radius of SD-grown plants as labeled. (B) Number of axillary inflorescence stems. Means ±SE were measured for 10–20 plants per line. Some error bars are too small to be seen.

TABLE 1

Germination percentage and fertility in wild type and homozygous GA biosynthesis or response mutants

Plant	Germination[a] (%)	Seeds/silique[b]
L*er*	99.7	42.1 ± 1.2
rga-24	99.3	40.4 ± 2.2
gai-t6	100.0	39.9 ± 1.6
rga-24/gai-t6	99.6	7.6 ± 1.0
ga1-3	3.5 ± 1.3	Sterile
rga-24/ga1-3	5.3 ± 2.4	Sterile
gai-t6/ga1-3	2.1 ± 0.9	Sterile
rga-24/gai-t6/ga1-3	3.1 ± 1.5	Sterile

Values are means ±SE.

[a] A total of 220–310 seeds per line were tested. *ga1-3* background seeds were tested three times, except for *rga-24/ga1-3*, which was tested twice.

[b] Fertility was measured by the number of seeds per silique on the primary inflorescence stem for 10 plants/line. For L*er*, *rga-24*, and *gai-t6*, seed number in 14 siliques per plant was counted. Because the first 4 siliques of *rga-24/gai-t6* were completely sterile, the mean was determined for siliques 5 to 36.

mutant, which shows constitutively repressed GA signaling, has an elevated *GA4* mRNA level compared to that in wild type (Cowling *et al.* 1998). These results indicate that decreased GA-signaling results in increased levels of *GA4* mRNA.

Previously, we found that the digenic *rga/ga1-3* mutant has an intermediate level of *GA4* mRNA compared to L*er* and *ga1-3* (Silverstone *et al.* 2001), probably because loss of RGA function resulted in partially derepressed GA signaling. On the basis of this hypothesis, we predicted that removing both RGA and GAI function in the *ga1-3* background may lead to a completely derepressed GA response, and this, in turn, may lower *GA4* gene expression even further. RNA gel blot analysis showed that the trigenic *rga-24/gai-t6/ga1-3* mutant, in the absence of exogenously applied GA, accumulated a lower level of *GA4* mRNA than that in L*er* (Figure 6). This experiment was repeated four times and the average level of *GA4* mRNA in each line is shown in Table 2. As with the phenotypes we studied, removing RGA function caused a partial reduction (by 25%) in the level of *GA4* mRNA but removing GAI function alone did not. In fact, *gai-t6* and *gai-t6/ga1-3* had higher levels of *GA4* mRNA than L*er* and *ga1-3*, respectively. GA_3 treatment lowered *GA4* mRNA levels in all lines to approximately the same level (Figure 6 and Table 2).

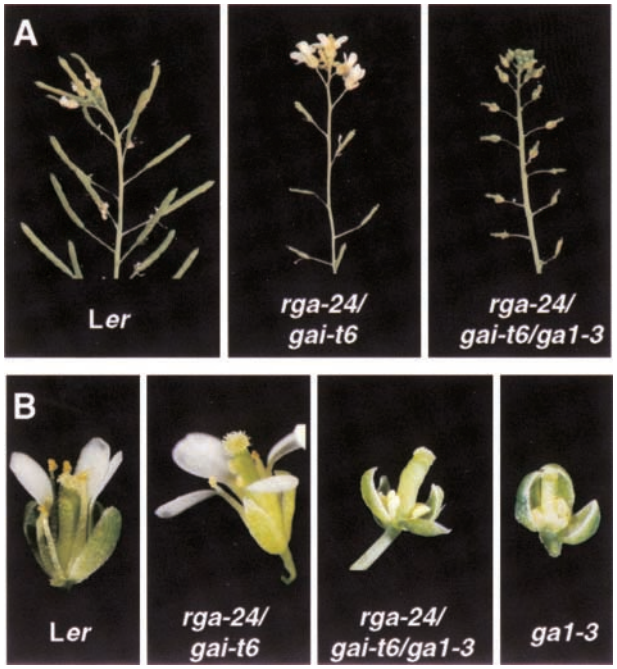

Figure 5.—Inflorescence and flowers of wild-type and mutant plants. Inflorescences (A) and individual flowers (B) of plants as labeled.

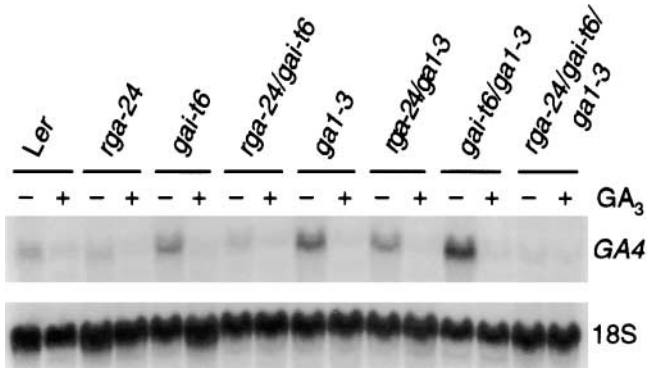

Figure 6.—The levels of *GA4* mRNA in *rga* and *gai* mutants in wild-type and *ga1-3* backgrounds. Shown is an autoradiogram of RNA blots containing 9 μg of total RNA isolated from L*er* and various mutants with (+GA_3) or without (−GA_3) treatment. The blot was hybridized with a labeled *GA4* antisense RNA probe and then reprobed with a labeled 18S rDNA probe.

TABLE 2

Relative *GA4* mRNA levels in L*er* and homozygous mutant lines

	Relative *GA4* mRNA level[a]	
Genotype	−GA_3	+GA_3
L*er*	1.0 ± 0.0	0.4 ± 0.0
rga-24	0.7 ± 0.1	0.3 ± 0.0
gai-t6	1.8 ± 0.1	0.5 ± 0.1
rga-24/gai-t6	0.8 ± 0.1	0.5 ± 0.2
ga1-3	3.5 ± 0.6	0.3 ± 0.1
rga-24/ga1-3	2.1 ± 0.2	0.4 ± 0.0
gai-t6/ga1-3	4.0 ± 0.8	0.4 ± 0.0
rga-24/gai-t6/ga1-3	0.5 ± 0.1	0.3 ± 0.1

[a] The amounts of *GA4* mRNA in each sample were standardized using 18S rRNA as a loading control, and the value of untreated L*er* was arbitrarily set to 1.0. The means ±SE of four experiments are shown.

DISCUSSION

RGA and GAI are not completely redundant: Our data illustrate that RGA and GAI interact synergistically to repress a set of GA-induced growth processes. Phenotypes affected by *rga* and *gai* null mutations include leaf expansion, stem elongation, juvenile-to-adult phase change in leaf development, vegetative-to-reproductive transition, and apical dominance. Removing both RGA and GAI function leads to the complete derepression of the above phenotypes because the trigenic *rga-24/gai-t6/ga1-3* mutant shows a wild-type or even GA-overdose phenotype. Although RGA and GAI interact synergistically in repressing GA signaling, RGA alone is a more active repressor than GAI by itself. This is evident from the observation that *rga-24* alone partially rescues the above defects in *ga1*-3 whereas *gai-t6* individually has little or no effect.

The *rga-24* and *gai-t6* single mutants did have subtle phenotypes when compared to wild-type L*er*, further supporting that RGA and GAI do not have completely redundant functions. Both *rga-24* and *gai-t6* flowered slightly earlier than wild type, and *rga-24* underwent the transition from juvenile-to-adult phase 46% earlier than L*er*.

The effects of single and double *rga-24* and *gai-t6* mutations are more evident in the GA-deficient *ga1-3* background than in L*er*. This result supports the hypothesis that *RGA* and *GAI* are more active repressors of GA signaling in GA-deficient conditions than in wild-type background and that GA derepresses the GA-signaling pathway by inactivating RGA and GAI. In the wild-type GA background, however, RGA and GAI are still functional, although they have a lower activity in modulating GA signaling. This is evident by the subtle phenotypes associated with loss of RGA and/or GAI in wild-type GA background.

Are *RGL* genes involved in controlling seed germination or flower development? Neither *rga-24* nor *gai-t6* singly or in combination rescued the germination and flower development defects of *ga1-3*, suggesting that additional gene(s) must modulate these GA-regulated phenotypes. Candidates that may control these processes are *RGL* (for *RGA-LIKE*; Sanchez-Fernandez *et al.* 1998), *RGA1-LIKE* (GenBank accession no. AC009895), and *RGA-LIKE PROTEIN* (GenBank accession no. AL39-1150), all of which are DELLA domain-containing GRAS family members with 56–60% amino acid sequence identity to RGA and GAI. The current names of these *RGA/GAI* homologs are confusing, and we propose renaming these genes as *RGL1*, *RGL2*, and *RGL3*, respectively. Isolation of knockout mutants that are defective in these genes using a reverse genetic approach will help to test their role in GA-regulated germination and/or flower development. The function of the *RGL* genes may be better revealed in multiple mutant backgrounds because of functional redundancy. Also, their phenotype might be more visible in the *ga1-3* background if RGLs, like RGA and GAI, are more active in the GA-deficient condition. If these genes do play a role in GA signaling, differential expression of their products in various tissues and/or varying the sensitivity to the GA signal for each gene would allow fine-tuning of the GA response.

Gene duplications and the *RGA*, *GAI*, and *RGL* genes: Recent sequence analysis has revealed a large number of gene duplications in the Arabidopsis genome (Vision *et al.* 2000). *RGA* and *GAI*, on chromosomes 1 and 2, are within duplicated block 10 that was estimated to have taken place ~100 million years ago (mya; age class C). *RGL2* and *RGL3* are located in duplicated block 71 in age class E (170 mya) on chromosomes 3 and 5, respectively. *RGL1* (chromosome 1) is in duplicated block 37 in age class F (200 mya). But there is no paralogous gene in the other block 37 on chromosome 5. Our data illustrate that *RGA* and *GAI* have similar, but not completely redundant functions. After the duplication event, they clearly evolved to have slightly different roles in controlling GA signaling. This demonstrates that gene duplications could provide ways for the organisms to evolve more sophisticated regulatory mechanisms in controlling cellular processes. Future studies on the *RGL* genes will reveal whether RGL2 and RGL3 have more similar biological function to each other than to RGA, GAI, and RGL1.

The *RGA/GAI* orthologs in rice (*SLR*) and in barley (*SLN*) appear to function as the single major repressor for GA-mediated stem growth because recessive *slr* and *sln* mutations result in constitutive GA response in rice and barley, respectively (Lanahan and Ho 1988; Ikeda *et al.* 2001; P. M. Chandler, A. Marion-Poll, and F. Gubler, personal communication). These results suggest that the *RGA/GAI* orthologs are not redundant in rice and barley. The oldest gene duplication in Arabidopsis (age class F) identified by Vision *et al.* (2000) occurred at approximately the same time as the divergence of monocots and dicots. If future studies indicate that most dicots with diploid genomes contain multiple *RGA/GAI* orthologs, whereas monocots have only a single *RGA/GAI* ortholog, then it is possible that the first duplication event for *RGA/GAI* occurred after the divergence of monocots and dicots. However, subsequent loss of duplicated genes in monocots after divergence from dicots could also account for such results.

Feedback regulation of GA biosynthesis by GA response: Because RGA and GAI have overlapping functions, removing RGA alone only partially derepresses GA signaling. We showed that *rga-24* partially reduced expression of the GA biosynthetic gene *GA4*. When both RGA and GAI were inactivated, the *GA4* mRNA level in *rga-24/gai-t6/ga1-3* was further decreased to be even lower than L*er*. These results support the current model that increased activity in GA response can downregulate GA biosynthesis by a feedback mechanism. GA treatment only slightly decreased *GA4* mRNA level in the

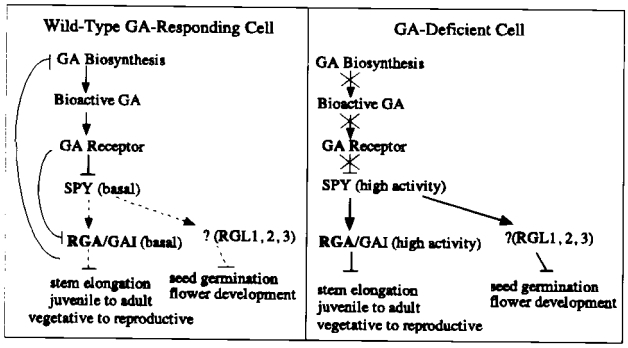

FIGURE 7.—Model of GA-signaling pathway in Arabidopsis. SPY inhibits all GA responses by activating RGA, GAI, and perhaps RGL1, RGL2, and RGL3, through GlcNAc modification. GA signal derepresses its signal transduction pathway by inactivating all repressor proteins. The activity of GA response regulates GA biosynthesis via a feedback mechanism. The extent of GA-mediated growth is determined by the balance between the amount of the GA signal and the levels and/or activities of the repressors. Arrows and T-bars indicate positive and inhibitory effects, respectively. Weaker *vs.* stronger effects are represented by dotted and solid lines, respectively. RGA is in boldface, indicating that RGA alone is a more active repressor than GAI by itself.

trigenic *rga-24/gai-t6/ga1-3* mutant, indicating that feedback inhibition was almost at its maximum. On the basis of the above model, we would expect that *gai-t6* by itself may not affect *GA4* gene expression because it has little effect in all GA-regulated events. However, we were surprised to find that *gai-t6* alone caused increased *GA4* mRNA levels in both wild-type and *ga1-3* backgrounds. One possible explanation is that RGA and/or RGLs may be activated further and overly compensate for the loss of GAI function in repressing GA signaling, and consequently may affect the feedback control of GA biosynthesis.

A working model of GA signal transduction pathway in Arabidopsis: In this article, we demonstrate that RGA and GAI synergistically repress a number of GA-controlled processes. Our previous studies, using the trigenic mutant *rga/spy/ga1-3*, showed that SPY additively interacts with RGA (SILVERSTONE *et al.* 1997). It was postulated that SPY may activate RGA and GAI by GlcNAc modification, because SPY is probably an OGT (reviewed in HARBERD *et al.* 1998; THORNTON *et al.* 1999; SUN 2000). The ground state of the GA-signaling pathway is repressive, and growth and development occur through derepression of the pathway by the GA signal, which inactivates SPY, RGA, and GAI (Figure 7). To achieve differential growth, quantitative differences in the GA signal can determine the degree of derepression of the pathway in different cells. Therefore, a proper balance between the bioactive GA level and activity in GA signaling is important, and this is consistent with our finding that *rga* and *gai* affect not only GA response, but also regulation of GA biosynthesis.

Unlike the *rga* and *gai* null alleles, the *spy* mutation also completely restores seed germination and partially rescues the defect in flower development of *ga1-3* (JACOBSEN and OLSZEWSKI 1993; Silverstone *et al.* 1997). Therefore, we propose that SPY may also activate RGL1, RGL2, or RGL3 to inhibit GA-induced seed germination and flower development (Figure 7). Future studies will be needed to verify this model and to place additional putative activators (*e.g.*, SLEEPY, G-proteins, and GA-MYB) and a repressor (SHI) in the pathway.

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