Arabidopsis RGL1 Encodes a Negative Regulator of Gibberellin Responses

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In Arabidopsis, the DELLA subfamily of GRAS regulatory genes consists of GAI, RGA, RGA-LIKE1 (RGL1), RGL2, and RGL3. GAI and RGA are known to be negative regulators of gibberellin (GA) responses. We found that RGL1 is a similar repressor of GA responses, as revealed by RGL1 gain-of-function and loss-of-function phenotypes. Repression of GA responses in Arabidopsis was conferred by a dominant 35S-rgl1 transgene carrying a DELLA domain deletion analogous to the GA-insensitive gai-1 mutation. As in GA-deficient Arabidopsis, the transgenic plants were dark green dwarfs with underdeveloped trichomes and flowers. Expression levels of GA4, a feedback-regulated GA biosynthetic gene, were increased correspondingly. Conversely, a loss-of-function rgl1 line had reduced GA4 expression and exhibited GA-independent activation of seed germination, leaf expansion, flowering, stem elongation, and floral development, as detected by resistance to the GA biosynthesis inhibitor paclobutrazol. RGL1 plays a greater role in seed germination than do GAI and RGA. The expression profile of RGL1 differed from those of the four other DELLA homologs. RGL1 message levels were predominant in flowers, with transcripts detected in developing ovules and anthers. As with RGA, green fluorescent protein (GFP)-tagged RGL1 protein was localized to the nucleus, but unlike GFP-RGA, there was no degradation after GA treatment. These findings indicate that RGL1 is a partially redundant, but distinct, negative regulator of GA responses and suggest that all DELLA subfamily members might possess separate as well as overlapping roles in GA signaling.

INTRODUCTION

Gibberellins (GAs) are a large family of tetracyclic diterpenoid compounds, many of which play important roles in plant growth and development (Hooley, 1994; Swain and Olszewski, 1996). Bioactive GAs are known to promote diverse processes, including seed germination, leaf expansion, shoot/stem elongation, floral initiation, floral organ development, and fruit development (Hooley, 1994; Swain and Olszewski, 1996). Mutants in GA biosynthesis have helped to determine the GA biosynthesis pathway (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000), but less is known at present about the GA signal transduction pathway. A receptor for GA has been localized to the plasma membrane, but the identity of the receptor remains unknown (Gilroy and Jones, 1994). Biochemical and pharmacological data have indicated that Ca$^{2+}$, calmodulin, cyclic GMP, heterotrimeric G proteins, GAMYB, and protein kinases all are involved in GA signaling (Bethke and Jones, 1998; Lovegrove and Hooley, 2000; Sun, 2000). Genes involved in GA signal transduction have been uncovered through GA response mutants. These mutants are either GA-insensitive dwarfs or constitutive GA response mutants (Sun, 2000). The GA-insensitive dwarfs resemble GA-deficient mutants, but they are not rescued by GA treatment. They typically exhibit compact, dark green leaves, inhibition of seed germination, delay of flowering, and abnormal flower development. Recessive GA-insensitive mutants such as dwarf1 (d1) in rice (Mitsunaga et al., 1994), pickle (pk) in Arabidopsis (Ogas et al., 1999), GA sensitivity (gse) in barley (Chandler and Robertson, 1999), and sleepy1 (sl1) in Arabidopsis (Steber et al., 1998) likely represent the loss of positive regulators of GA response. D1 encodes a putative heterotrimeric G protein α-subunit (Ashikari et al., 1999; Ueguchi-Tanaka et al., 2000), and PKL encodes a chromatin remodeling factor (Ogas et al., 1999). Dominant and semidominant GA-insensitive mutants include the gain-of-function gibberellin insensitive (gai-1) allele in Arabidopsis (Koornneef et al., 1985), D8 and D9 in maize (Phinney, 1956), reduced height (Rht) mutants in wheat (Bomer et al., 1996), and short internodes (shi) in Arabidopsis (Fridborg et al., 1999). shi encodes a putative zinc finger protein (Fridborg et al., 1999). The GAI, D8, and Rht genes encode negative regulators of GA responses, and all are DELLA subfamily

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010325.
members of the GRAS (or VHIID) family of regulatory proteins described below.

GA constitutive response mutants appear as though they are exposed to high doses of GAs, yet they show resistance to inhibitors of GA biosynthesis (thus displaying GA-independent activation of GA responses). They generally have longer stems, paler leaves, longer petioles, and reduced fertility. All of these mutants are recessive, indicating that the corresponding genes are negative regulators of GA responses. gai (Peng and Harberd, 1993), repressor-of-gai-3 (rga) in Arabidopsis (Silverstone et al., 1997), and slender (slr1) in rice (Ikeda et al., 2001) are all mutants in the DELLA subfamily. gai loss-of-function alleles were isolated as intragenic revertants of the semidominant GA-insensitive gai-1 mutant (Peng et al., 1997). rga alleles were isolated based on partial suppression of defects in the GA biosynthetic mutant gai-1-3 (Silverstone et al., 1997). Another constitutive GA response mutant in Arabidopsis is spindly (spy) (Jacobsen et al., 1996). Epistasis analysis suggests that SPY and GAI act in the same pathway to suppress GA signaling (Jacobsen et al., 1996). SPY encodes a Ser(Thr)–O-linked GlcNAc transferase (Jacobsen et al., 1996), which possibly modifies GAI and RGA at the Ser/Thr–rich N termini (Harberd et al., 1998; Thornton et al., 1999; Sun, 2000).

GAI, RGA, D8, RHT, and SLR all belong to the plant-specific GRAS gene superfamily of regulatory proteins (Pysh et al., 1999). Members of the GRAS family have a conserved central region characterized by a highly conserved VHIID motif (of unknown function) flanked by leucine heptad repeats, which may be involved in protein–protein interactions (Peng et al., 1997; Silverstone et al., 1998; Pysh et al., 1999). Their less conserved N termini may be involved in conferring specificity. The Arabidopsis genome contains nearly 40 GRAS family members. SCARECROW (SCR) is involved in radial patterning of the root and shoot (De Laurenzi et al., 1996), and PHOTOCROME A SIGNAL TRANSDUCTION1 (PAT1) functions in phytochrome A light signal transduction (Bole et al., 2000). The GRAS genes that are known to be involved in GA signaling all contain an acidic N-terminal DELLA domain, which has been shown to be required for proper regulation of the protein. The C-terminal portion of the DELLA subfamily contains an SH2-like domain (Peng et al., 1999) and an LXXLL consensus motif, which mediates the binding of steroid receptor coactivator complexes to nuclear receptors in mammals (Peng et al., 1997; Silverstone et al., 1998). The semidominant gai-1 allele encodes a 17–amino acid deletion that removes the DELLA motif, causing GA insensitivity (Peng et al., 1997). The DELLA domain appears to be required for GA-mediated inactivation, and loss of the DELLA domain is believed to result in GA insensitivity because of the lack of inactivation (Harberd et al., 1998; Sun, 2000). Several GA-insensitive alleles of wheat Rht and maize D8 were found to contain N-terminal truncations of the DELLA domain or nearby sequences (Peng et al., 1999). In the rice SLR gene, a dominant allele conferring GA insensitivity was created by introducing a deletion analogous to that in gai-1 (Ikeda et al., 2001).

The Arabidopsis genome has five DELLA subfamily members: GAI, RGA, RGA-LIKE (RGL) (Sánchez-Fernández et al., 1998), locus AT3 g03450, and locus AT5 g17490. As proposed by Dill and Sun (2001), we refer to the latter three genes as RGL1, RGL2, and RGL3, respectively. GAI and RGA each lie on duplicated segments of the genome, as do RGL2 and RGL3 (Dill and Sun, 2001). RGL1, however, has no paralogous gene in its duplicated block of sequence (Dill and Sun, 2001). GAI and RGA are thought to be transcriptional regulators that do not bind DNA directly. The RGA protein is localized to the nucleus, and it was found to be degraded rapidly by GA treatment, indicating a mechanism for GA homeostasis (Silverstone et al., 1998, 2001). GAI and RGA have overlapping functions, with RGA playing a larger role (Silverstone et al., 1998; Dill and Sun, 2001; King et al., 2001). GAI and RGA interact synergistically to repress certain GA-induced growth processes (Dill and Sun, 2001; King et al., 2001). In the gai-1-3 background, the gai-t6 rga-24 double null mutations give a wild-type or GA “overdose” phenotype in leaf expansion, flowering time, stem elongation, and apical dominance. gai and rga mutants have minimal phenotypes, however, with respect to seed germination and floral development (Peng and Harberd, 1993; Peng et al., 1997; Silverstone et al., 1997; Dill and Sun, 2001; King et al., 2001).

In this study, we found that the Arabidopsis RGL1 gene encodes a nucleus-localized negative regulator for a range of GA responses. As shown for other DELLA subfamily proteins, deletion of the DELLA domain in RGL1 conferred dominant GA-insensitive repression of GA responses. Loss of RGL1 function conferred GA-independent activation of GA responses. Phenotypic analyses indicated that RGL1 has overlapping, but distinct, roles in GA signaling compared with GAI and RGL, as suggested by their expression profiles. A green fluorescent protein (GFP)–RGL1 fusion protein was localized to the nucleus, but GFP–RGL1 was stable with GA treatment, in contrast to results reported for GFP-RGA (Silverstone et al., 2001).

RESULTS

RGL1 cDNA Clones

RGL1 was first identified through the sequence of a genomic DNA clone (Sánchez-Fernández et al., 1998). cDNA clones of RGL1 have not been reported previously, and BLAST searches (Altschul et al., 1990) failed to identify RGL1 cDNA sequences in the current expressed sequence tag databases. In the course of screening a two-hybrid cDNA library made from etiolated seedlings (Arabidopsis Biological Resource Center catalog number CD4-22), we serendipitously isolated several cDNA clones of the Arabidopsis RGL1 gene. The longest of these cDNA clones (1664 nucleotides) contains an open reading frame encoding a polypeptide of 511 amino acid residues starting at 11 bp
from the 5’ end and having a 121-nucleotide 3’ untranslated region followed by a poly(A) tail. The entire cDNA is colinear with the genomic DNA (bacterial artificial chromosome clone T27F4, chromosome 1), indicating a lack of introns in this sequence. In the genome sequence, a putative TATA box lies 143 nucleotides upstream of the 5’ end of the cDNA, suggesting that the cDNA may be close to full length.

The deduced RGL1 protein sequence contains all of the characteristic motifs of the GRAS family (Figure 1). RGL1 also has a conserved DELLA domain, but with an alanine-to-valine substitution (DELLV). The predicted RGL1 sequence aligns well with those of the Arabidopsis DELLA subfamily members GAI, RGA, RGL2, and RGL3 (Figure 1). (The RGL1 N-terminal sequence predicted by Sánchez-Fernández et al. [1998] is completely divergent, because apparently it was based on the sequence of a chimeric genomic DNA clone.) Over the entire sequence, RGL1 shares 61 and 62% amino acid identity, respectively, with GAI and RGA, whereas GAI and RGA are 80% identical. RGL1 has 63 and 59% identity with the predicted RGL2 and RGL3 sequences, respectively, which are 71% identical. GAI and RGA share 57 to 59% identity with RGL2 and RGL3. Consistent with these data, phylogenetic analysis using ClustalW (Thompson et al., 1994) indicated three subgroups of Arabidopsis DELLA proteins (GAI and RGA, RGL2 and RGL3, RGL1), but it did not allow us to determine the relationships among these groups or to assign the monocot DELLA proteins to any of these three groups (data not shown).

GA Insensitivity Conferred by a Dominant Mutant rgl1 Transgene

To examine the function of the RGL1 gene, we constructed an RGL1 transgene, called rgl1Δ17, encoding a 17-amino acid deletion identical to the dominant mutation that confers GA insensitivity in the gai-1 mutant (Peng et al., 1997). rgl1Δ17 and wild-type RGL1 were fused to the 35S promoter of Cauliflower mosaic virus and transformed into wild-type Arabidopsis. Thirty-five 35S-rgl1Δ17 transformants were obtained, and all displayed a dominant dwarf phenotype more severe than that of the dominant gai-1 mutant (Figures 2A and 2B), whereas transformants of the wild-type transgene showed no obvious phenotypes (data not shown).

All of the 35S-rgl1Δ17 transgenic plants were dwarfed and exhibited a delay in bolting, consistent with the lack of either GA biosynthesis or GA perception. The rosettes were small and dark green, measuring only several millimeters in diameter, and leaf trichomes were both poorly developed and reduced in number (Figures 2A to 2D). Similar phenotypes are displayed by the Arabidopsis GA biosynthesis mutant ga1-3 (Koornneef et al., 1985) and by wild-type plants treated with the GA biosynthesis inhibitor paclobutrazol (PAC) (Jacobsen and Olszewski, 1993).

When the 35S-rgl1Δ17 lines finally bolted and flowered, morphological defects also were seen in the flowers. The sepals, petals, and stamens were underdeveloped such that the pistils, which were stunted themselves, protruded from the floral buds (Figures 2E to 2K). The flowers were male sterile (producing a few seed), but they were fertile when crossed with wild-type pollen. Comparable defects are displayed by flowers of the GA biosynthesis mutant ga1-3 (Koornneef et al., 1985) and by wild-type plants treated with the GA biosynthesis inhibitor paclobutrazol (PAC) (Jacobsen and Olszewski, 1993).

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The floral phenotype is not seen in any of the known GA-insensitive mutants, including *gai-1*. None of the phenotypes of the transgenic 35S-rgl1Δ17 plants was rescued by GA₃ treatment (data not shown). Therefore, the defects conferred by the dominant 35S-rgl1Δ17 transgene were consistent with the repression of GA responses. As in *gai-1*, these were likely to be gain-of-function defects.

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Expression Pattern of *RGL1* and Comparison with Those of Other DELLA Subfamily Members

The expression pattern of wild-type *RGL1* was examined and compared with those of the other DELLA subfamily members (*RGA*, *GAI*, *RGL2*, and *RGL3*) by RNA gel blot analysis (Figure 3). The level of *RGL1* message was highest in inflorescences and weak or undetectable in rosette

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leaves, etiolated seedlings, siliques, mature stems, and roots (Figure 3 and data not shown). GA treatment did not enhance message levels (Figure 3 and data not shown). RGA and GAI transcripts were detected at slightly varying levels in all tissues examined. RGL2 signal was undetected, and RGL3 signal was very weak in all tissues examined (rosette leaves, seedlings, inflorescences, and siliques) except inflorescences.

The floral expression pattern of RGL1 was examined more closely using in situ hybridization. Hybridization signals were detected in developing ovules as well as in developing anthers throughout microspore development (Figure 4). This expression pattern together with the 35S-rgl1Δ17 floral phenotype suggested that RGL1 might play a role in ovule and anther development. RGL1 expression in ovules might affect carpel or seed development. Although we cannot exclude the possibility that the RGL1 probe might cross-hybridize to other DELLLA family members in this experiment, we did not detect significant cross-hybridization when RGL1 was used as a probe on DNA (data not shown) or RNA gel blots.

**RGL1 Gene Silencing in Phenotypic Revertants of 35S-rgl1Δ17 Lines**

In the T1 and T2 generations, the 35S-rgl1Δ17 transgene phenotypes showed a high degree of instability. Of the 10 35S-rgl1Δ17 lines that were maintained, nine showed phenotypic reversion in the T2 generation. That is, flowers and leaves that developed at later times on the same T2 plants were wild type in appearance, and the self-progeny of these plants inherited the reverted phenotypes. We focused on one line designated rgl1Δ17-R (Figure 2A), for which we confirmed that the reverted phenotype was inherited stably for at least five generations. The presence and homology of the 35S-rgl1Δ17 transgene in this line were verified by polymerase chain reaction (PCR) amplification of the transgene from genomic DNA and by the fact that 100% of the progeny showed kanamycin resistance, which presumably was conferred by the T-DNA insertion (data not shown).

The presence or absence of GA-insensitive phenotypes was correlated with the level of rgl1Δ17 message. RGL1 (and rgl1Δ17 transgene) message levels were examined in a nonrevertant 35S-rgl1Δ17 line, the rgl1Δ17-R revertant line, and the wild type by RNA gel blot analysis and reverse transcriptase–mediated (RT)-PCR. The GA-insensitive 35S-rgl1Δ17 line (inflorescences and rosette leaves) exhibited high message levels. The strong signals in this line were attributed to the expression of the 35S-rgl1Δ17 transgene, because the same tissues in the wild type had very low levels of RGL1 expression (Figures 5A and 5B). In contrast to the 35S-rgl1Δ17 line, the rgl1Δ17-R revertant line showed dramatically less RGL1 (rgl1Δ17) expression (Figures 5A and 5B). Moreover, the level of RGL1 message in rgl1Δ17-R was lower than that in the wild type, as detected by RT-PCR (Figure 5B), indicating that gene silencing or cosuppression had occurred (Vance and Vaucheret, 2001). Message levels of the other DELLLA subfamily members (RGA, GAI, RGL2, and RGL3) remained essentially unchanged among the 35S-rgl1Δ17 line, rgl1Δ17-R, and the wild-type samples (Figures 5A to 5C), suggesting that the reduction of the RGL1 message was specific. On the basis of these findings, the rgl1Δ17-R line was considered to be an rgl1 loss-of-function mutant.

**Feedback Regulation of the GA Biosynthesis Gene GA4 in the rgl1 Gain-of-Function and Loss-of-Function Lines**

The GA4 gene was used as a molecular marker to examine GA response in the dominant 35S-rgl1Δ17 and cosuppressed rgl1Δ17-R lines. GA4 encodes GA 3β-hydroxylase, which catalyzes the conversion of inactive GAs to active GAs (Chiang et al., 1995). The expression of GA4 appears to be regulated by negative feedback through the GA response pathway (Cowling et al., 1998; Silverstone et al., 2001). GA4 expression in seedlings is downregulated by exogenous GA
...and rosette leaves of the 35S-rgl1-R17 lines had enhanced levels of GA4 transcripts (Figures 5A and 5B), as seen in the dominant gai-1 mutant (Cowling et al., 1998). These results are consistent with the idea that RGL1 negatively regulates the GA response pathway.

rgl1 Loss-of-Function Phenotype

Under normal growth conditions, rgl1-R17-R plants lacked any obvious phenotypes and were comparable to the wild type (Figure 2A). Null mutants of GAI and RGA also lack appreciable phenotypes: gai-t6 and rga-24 have a slightly earlier flowering time (Peng and Harberd, 1993; Peng et al., 1997; Silverstone et al., 1998; Dill and Sun, 2001), and rga null mutants also are somewhat paler (Silverstone et al., 1997). Under GA-deficient conditions (e.g., PAC treatment or the gai-1 mutation), gai and rga loss-of-function mutants display several GA-independent phenotypes (Peng and Harberd, 1993; Peng et al., 1997; Dill and Sun, 2001). Prevention of GA biosynthesis in the wild type results in inhibition of seed germination, leaf expansion, and stem elongation as well as defective trichome development, delayed flowering, and defective flower development (Harberd et al., 1998). In rgl1-R17-R, all of these processes were PAC resistant, which was indicative of GA-independent activation of GA responses.

Seed germination in rgl1-R17-R, in particular, was highly resistant to PAC. rgl1-R17-R seed were able to germinate on concentrations of PAC that severely inhibited the germination of wild-type seed (Figures 6A and 6B). For instance, >95% of rgl1-R17-R seed, but <20% of wild-type seed, were capable of germinating in the presence of 60 μM PAC. The germination frequency of rgl1-R17-R was comparable to that of the constitutive GA response mutant spy-3 on PAC (Figure 6A) (although immediately subsequent to germination on high concentrations of PAC, the rgl1-R17-R seedlings became stunted, whereas the spy-3 seedlings produced leaves [data not shown]). Notably, germination of the gai-t6 null mutant was highly sensitive to PAC (Figure 6B). Thus, RGL1 plays a substantial role in seed germination.

rgl1-R17-R plants grown on Murashige and Skoog (MS) medium (1962) supplemented with 1 μM PAC (under a 24-hr daylength) exhibited PAC-resistant leaf expansion, flowering time, stem elongation, and flower development (Figure 7). In the absence of PAC, the wild type and rgl1-R17-R were indistinguishable, and both began to flower at ~18 days. When grown on 1 μM PAC, the wild-type plants were highly dwarfed and did not begin flowering even 10 weeks after germination. In contrast, rgl1-R17-R exhibited expanded leaves and began to flower 5 weeks after germination (Figure 7A). The spy-3 mutant, which was treated in parallel, began to flower 18 days after germination. In addition, the flowers of rgl1-R17-R plants appeared normal and were fer-
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PAC resistance in rgl1Δ17-R also was examined in parallel with that in rga-24 and gai-t6 null mutants (Figure 8). In the absence of PAC, all of the plants began to flower at 17 or 18 days after germination, but when grown on MS medium supplemented with 0.75 μM PAC, rga-24 began flowering at 19 days, rgl1Δ17-R began flowering at 25 days, and gai-t6 began flowering at 33 days after germination. It should be noted that the gai-t6 and rga-24 mutants are in the Landsberg erecta (Ler) background, so direct comparisons cannot be made with rgl1Δ17-R, which is in the Columbia (Col-0) background. Both rgl1Δ17-R and rga-24 were capable of producing fertile flowers, whereas flower development in gai-t6 appeared to be more sensitive to PAC (Figure 8B).

Overall, the PAC resistance of the cosuppressed rgl1Δ17-R line indicated that the loss of RGL1 function results in GA-independent activation of GA responses. The phenotypes were opposite to the repression of GA responses seen in the gain-of-function 35S-rgl1Δ17 lines, which is consistent with a proposed role of RGL1 as a negative regulator of GA responses.

Nuclear Localization of the RGL1 Protein

GAI and RGA are thought to be transcriptional regulators on the basis of sequence features, such as a nuclear localization signal, homopolymeric Ser and Thr, leucine heptad repeats, and SH2-like domains (Peng et al., 1997, 1999; Peng et al., 1999) transcripts are increased in inflorescences and leaves of the 35S-rgl1Δ17 line, whereas expression levels of the other genes are relatively constant among all of the different RNA samples.

(B) DNA gel blot of RT-PCR indicating that RGL1 and GA4 transcripts are increased in inflorescences and leaves of the 35S-rgl1Δ17 line but decreased in inflorescences and leaves of the rgl1Δ17-R line compared with those of the wild type. RT-PCR products for RGL1, GA4, and RGA (from the same RNA samples indicated at the top in [A]) were visualized by DNA gel blot hybridization to their respective probes. GAI and RGA results serve as amplification controls. GA4 message levels also are reduced in GA-treated inflorescences compared with those of the wild type. Inflorescence tissue was collected 2 hr after spraying with 100 μM GA3.

(C) RT-PCR indicating no obvious reduction in RGL2 and RGL3 transcripts in rgl1Δ17-R inflorescences. RT-PCR products were generated using RGL2 and RGL3 gene-specific primers, run on an agarose gel, and visualized by ethidium bromide staining. Total RNA (before reverse transcription) was used as a control template (data not shown).
Silverstone et al., 1998, 2001). Consistent with this role, GFP-RGA has been shown to be localized to the nucleus (Silverstone et al., 1998, 2001). In addition, RGA and a GFP-RGA fusion protein have been shown to be degraded rapidly in response to GA treatment (Silverstone et al., 2001). Because RGL1 shares these sequence features, including a nuclear localization signal, RGL1 may function similarly in the nucleus (Figure 1). We examined the subcellular localization of the RGL1 protein using a GFP-RGL1 fusion protein expressed under the 35S promoter of Cauliflower mosaic virus in stably transformed Arabidopsis. GFP was observed in cell nuclei, indicating that RGL1 is localized to the nucleus (Figure 9). When we examined the effect of GA treatment on the stability or localization of GFP-RGL1, we found that GA treatment did not alter the GFP-RGL1 signal (Figures 9E and 9F). Both the GA and mock GA treatments caused a slight reduction in fluorescence by 3 hr after treatment.

**DISCUSSION**

The Arabidopsis RGL1 gene is a DELLA domain-containing GRAS gene family member with sequence similarity to GAI and RGA. We have found that RGL1 acts as a negative regulator of GA responses, confirming that the sequence conservation extends to functional conservation. rgl1 loss-of-function phenotypes revealed that RGL1 has overlapping functions with GAI and RGA and that RGL1 plays a greater role in seed germination than either GAI or RGA. Unlike GFP-RGA, GFP-RGL1 does not degrade upon GA treatment, suggesting that perhaps there is a different mechanism for regulating RGL1.

A gain-of-function rgl1 transgene was created by deleting the DELLA motif, which is known to be important for the function of this subfamily (Peng et al., 1997, 1999; Ikeda et al., 2001). The DELLA domain is thought to be required for

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**Figure 6.** Cosuppression of the RGL1 Gene Confers PAC-Resistant Seed Germination.  
(A) Germination frequency in the rgl1 cosuppressed line (rgl1Δ17-R) compared with that of the constitutive GA response mutant spy-3 (in the Col-0 ecotype) (Jacobsen et al., 1996) and the wild type (Col-0). Error bars show standard deviations. Germination was scored 6 days after stratification.  
(B) Germination frequency in the rgl1 cosuppressed line (rgl1Δ17-R) compared with that of the gai-t6 null mutant (in the Ler background) and Ler. Error bars show standard deviations. Germination was scored 6 days after stratification.

**Figure 7.** Cosuppression of the RGL1 Gene Confers PAC-Resistant Leaf Expansion, Flowering, Stem Elongation, and Flower Development.  
(A) The wild type (Col-0), rgl1Δ17-R, and spy-3 were grown on MS-agar medium plus 1 μM PAC, except for Col-0 (left), which was grown without PAC. All plants are 2 months old. Bar = 1 cm.  
(B) Flowers and siliques from plants in (A). Bar = 2 mm.
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inactivation of the DELLA domain protein by the GA signal; in the presence of the GA signal, the protein is inactivated through the DELLA domain, and in the absence of the DELLA domain, the protein maintains active repression of GA responses (Harberd et al., 1998; Sun, 2000). Consistent with this, rgl1 transgenic lines with the DELLA deletion exhibited repression of GA responses that were phenotypically similar to GA deficiency, but they were not rescued by GA treatment. The phenotypes included dwarfism, dark green leaves, defective trichome development, delayed flowering, short stems, and an increase in GA4 message levels, none of which was conferred by the wild-type RGL1 construct.

Because the 35S-rgl1 transgene conferred a GA-insensitive floral phenotype similar to that seen in GA-deficient flowers, whereas no previously described GA signaling mutant (including gai-1) has been reported to have this floral phenotype. Transgenic rice plants in which the dominant Arabidopsis gai-1 gene also was overexpressed showed dwarfing, but apparently they were self-fertile (Peng et al., 1999; Fu et al., 2001).

For unknown reasons, the 35S-rgl1 transgene was silenced in 90% of the transgenic lines. Cosuppression of RGL1 resulted in constitutive GA responses, which were opposite those of the dominant 35S-rgl1 lines. Phenotypes of the cosuppressed line included repression of GA4 gene expression and GA-independent (PAC-resistant) seed germination, leaf expansion, flowering time, stem elongation, and flower development. Some of these phenotypes overlap those of gai and rga mutants. Both GAI and RGA mediate stem and hypocotyl elongation in response to GA, but they have a lesser role in seed germination and flower development (Peng and Harberd, 1993; Peng et al., 1997; Silverstone et al., 1997; Dill and Sun, 2001). Our results suggest that RGL1 plays a larger role in seed germination than does GAI or RGA. Seed germination in the rgl1 cosuppressed line was highly resistant to PAC and was comparable to that conferred by the spy-3 allele (which codes for a C-terminal amino acid substitution in SPY) (Jacobsen et al., 1996). In contrast, seed germination in the gai-t6 null mutant showed

Figure 8. PAC Resistance in the Cosuppressed RGL1 Line, the rga-24 Null Mutant, and the gai-t6 Null Mutant.

(A) The wild type (Col-0), rgl1Δ17-R, rga-24, and gai-t6 were grown on MS-agar medium plus 0.75 μM PAC. All plants are 50 days old. rga-24 and gai-t6 both are in the Ler background. Bar = 2.5 cm.

(B) Inflorescences of rgl1Δ17-R, rga-24, and gai-t6 from plants in (A). A wild-type (Col-0) inflorescence in the absence of PAC is shown for comparison (left). Bar = 2 mm.

Figure 9. Nuclear Localization of GFP-RGL1 in Transgenic Arabidopsis.

(A) GFP-RGL1 detected in the nucleus of a root cell (top), with DAPI staining of the nucleus (bottom) for comparison.

(B) GFP alone in a root cell (top), with DAPI staining of the nucleus (bottom) for comparison.

(C) GFP-RGL1 localized in the nucleus of a trichome.

(D) GFP-RGL1 localized in guard cell nuclei.

(E) GFP-RGL1 at 5 and 180 min after GA treatment.

(F) GFP-RGL1 at 5 and 180 min after mock GA treatment.
the same sensitivity to PAC as in the wild type. Seed germination in rga mutants also appears to require GA; rga loss-of-function mutations do not suppress the seed germination defect of ga1-3, nor do they confer the degree of PAC resistance exhibited by spy mutants (Silverstone et al., 1997; Dill and Sun, 2001). The gai-t6 rga-24 double null mutant also does not suppress the seed germination defect of ga1-3 (Dill and Sun, 2001).

RGL1 also plays a role in flower development, as indicated by PAC-resistant flower development in the cosuppressed line. When plants were grown on PAC, flowers produced by the wild type were male sterile with poorly developed sepals, petals, and stamens and a protruding but stunted carpel, whereas flowers of the rgl1 cosuppressed line appeared normal and were fertile. RNA gel blots showed high expression of RGL1 in inflorescences compared with other tissues examined. RGL1 expression was localized to ovules and developing anthers by in situ hybridization, suggesting that RGL1 might be a repressor of ovule and stamen development. The expression of RGL1 in ovules also might be related to later seed germination ability. We cannot exclude the possibility that RGL1 is involved in petal or sepal development; in the particular floral sections used for the in situ hybridizations, it may have been difficult to view the sepals and petals at the correct stage because sepals develop early and petal primordia remain quite small until late floral stages (Smyth et al., 1990).

The absence of obvious phenotypes for gai, rga, and rgl1 loss-of-function mutants under normal growth conditions likely is attributable to the functional redundancy of the DELLA subfamily members. The genes probably play differing roles, as indicated by their different expression patterns and by the distinct and varied phenotypes of gai, rga, and transgenic rgl1 mutants. Despite the high GAI and RGA transcript levels detected in inflorescences, the developmental defects of ga1-3 flowers are not suppressed by the rga or gai null mutations, either alone or in combination (Silverstone et al., 1997; Dill and Sun, 2001; King et al., 2001). Because RGL1 plays a role in flower development, it is likely that a combination of rgl1, gai, and rga would suppress the ga1-3 floral defects, just as rga-24 and gai-t6 act synergistically to suppress certain phenotypes of ga1-3 (Dill and Sun, 2001). Dill and Sun (2001) proposed that both seed germination and flower development might be regulated by RGL1, RGL2, and/or RGL3 acting downstream of SPY. Unfortunately, the instability of the 35S-rgl1.Δ17 dominant mutant phenotype interfered with the construction and analysis of a double mutant between the 35S-rgl1.Δ17 lines and spy, so we were unable to determine whether spy can suppress the GA-insensitive phenotype of the 35S-rgl1.Δ17 transgene. Curiously, rgl1 mutants have not been identified in genetic screens for constitutive GA response mutants, which have been obtained on the basis of either PAC-resistant seed germination and stem elongation (Jacobsen and Olszewski, 1993) or suppression of ga1-3 stem and floral defects (Silverstone et al., 1997). On the basis of our findings, mutations in rgl1 should be recoverable in a screen for PAC-resistant seed germination. When Jacobsen and Olszewski (1993) screened for mutants capable of germinating on 120 μM PAC, however, the putative mutants were rescreened for stem elongation and leaf expansion at high levels of PAC, which most likely inhibited stem elongation and leaf expansion in rgl1 mutants relative to spy mutants. As we observed, spy-3, but not the rgl1.Δ17-R cosuppressed line, developed leaves after germinating on high concentrations of PAC. The latter mutant screen yielded many alleles of rga and spy and is thought to have been saturated (Silverstone et al., 1997). rgl1 mutations may have evaded this screen if stem elongation or flower development remained relatively inhibited, for example, if stem elongation and flower phenotypes of the rgl1.Δ17-R line on PAC are weaker than in spy-3 (Figures 7 and 8). Alternatively, suppression of ga1-3 by rgl1 mutations may have been masked through compensatory actions by DELLA subfamily members.

All DELLA subfamily members may have overlapping as well as distinct roles in GA signaling. The action of multiple genes of the DELLA domain subfamily might allow for the fine-tuning of GA responses in different tissues at different stages in the plant. Future isolation of null mutations in RGL2 and RGL3 and analyses of mutant combinations, both within the subfamily and with other mutants such as ga1-3, will help to further elucidate the redundant and specific roles of these gene family members.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana seed were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus), except for rga-24 gai-t6 ga1-3 triple mutant seed, which were provided by T. Sun (Duke University, Durham, NC), and gai-t6 seed, which were provided by N. Harberd (John Innes Centre, Norwich, UK). Plants were grown at 20°C in controlled environment chambers under either 24- or 16-hr daylengths.

To test for gibberellin (GA) response, plants were sprayed every other day with 100 μM GA3 (Sigma, St. Louis, MO) dissolved in 0.2% DMSO. Controls were sprayed with water containing 0.2% DMSO. For RNA isolation after GA treatment, inflorescences and siliques developed leaves after germinating on high concentrations of PAC. The latter mutant screen yielded many alleles of rga and spy and is thought to have been saturated (Silverstone et al., 1997). rgl1 mutations may have evaded this screen if stem elongation or flower development remained relatively inhibited, for example, if stem elongation and flower phenotypes of the rgl1.Δ17-R line on PAC are weaker than in spy-3 (Figures 7 and 8). Alternatively, suppression of ga1-3 by rgl1 mutations may have been masked through compensatory actions by DELLA subfamily members.

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Analysis of the RGL1 cDNA Sequence

Six RGL1 cDNA clones were obtained from a cDNA library (ABRC catalog no. CD4-22) made from etiolated seedling RNA. Nucleotide sequencing was performed at the Center for Agricultural Biotechnology
Sequencing Facility (University of Maryland Biotechnology Institute, College Park). The longest open reading frames were determined using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Homology searches were performed with BLAST (Altschul et al., 1990). Sequences were aligned and formatted using ClustalW (Thompson et al., 1994) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html), respectively. PSORT (Naik and Kanehisa, 1992) predicted a nuclear localization signal in RGL1. The RGL1 sequence 

\[ RKVATYFAEGLA RRYR \] 

matches the consensus nuclear localization sequence described by Robbins et al. (1991), in which two basic amino acid residues are followed by a 10–amino acid spacer, which is followed by more than three basic amino acid residues out of five. A putative tyrosine phosphorylation site in RGL1 was predicted with ScanProsite (Appel et al., 1994).

Construction of RGL1 Transgenes and Plant Transformation

The wild-type RGL1 cDNA (1.6 kb), containing what we determined to be the complete open reading frame, was released from plasmid pACT (converted from the cDNA library \( \lambda \) vector according to instructions provided by ABRC) using BamHI and BglII and cloned into the BamHI site of binary plant transformation vector pCGN18, which is pCGN1547 (Calgene, Davis, CA) containing the 35S promoter and the 3' nonpalline synshe sequence. To create pGf1.17, oligonucleotide primers 5'-AGTCAGCTAGCTTCGTAGCTTAGTG-TCC-3' and 5'-GATTGCTCAAGCTTTCAGGCGCAGACATCACTCG-GCAAGTCTCTCC-3', corresponding to sequences on pACT and RGL1, respectively, were used in a polymerase chain reaction (PCR) to create a HindIII fragment containing the 51-bp in-frame deletion. The wild-type HindIII fragment (internal to RGL1) was swapped with the mutant HindIII fragment. The resulting HindIII DNA was then released from pACT with BamHI and BglII and cloned into pCGN18 as for the wild-type RGL1 clone. The final constructs carrying the wild-type or deletion mutant gene were transformed into Agrobacterium tumefaciens strain ASE (Monsanto, St. Louis, MO) for Arabidopsis transformation.

For the green fluorescent protein (GFP)-RGL1 fusion protein construct (with GFP at the N terminus of RGL1), the wild-type RGL1 BamHI-BglII fragment was first ligated into plasmid pRTL2\2N-mGFPS65T (Kinkema et al., 2000), which was linearized at the BglII site that lies at the 3' end of the GFP reading frame. pRTL2\2N-mGFPS65T::RGL1 DNA was digested with HindIII to release a fragment containing 35S-mGFP fused to the 5' portion of RGL1. This HindIII fragment was ligated to the 5S-RGL1 wild-type construct in pCGN18 (described in the preceding paragraph), which had been digested with HindIII to remove the pCGN18 35S promoter fragment and the 5' portion of the RGL1 sequence. In the resulting clone, the RGL1 gene was reconstituted, but now it was preceded by 35S-mGFP derived from pRTL2\2N-mGFPS65T. This construct was transformed into strain ASE as described above.

A. tumefaciens-mediated in planta transformation of Arabidopsis was performed according to Bechtold and Pelletier (1998). Transgenic plants were selected on 1 × Murashige and Skoog (MS; 1962) salts (Sigma) and 0.8% agar plus kanamycin (100 mg/L) and then transferred to soil. For 35S-gf1.17 transformsants, dwarfing appeared in the first generation of transformants, indicating that the 35S-gf1.17 transgene was dominant to wild type. All of the mutant phenotypes described here cosegregated with the 35S-gf1.17 transgene on the basis of kanamycin resistance.

RNA Gel Blot Analyses

Total RNA was isolated from inflorescences, rosette leaves, etiolated seedlings, siliques, mature stems, and roots according to Wen et al. (1999). Etiolated seedlings were harvested 2 days after germination and used in total RNA isolation. RNA samples that were isolated from etiolated seedlings and siliques were further purified using phenol-chloroform-isooamyl alcohol (CTAB). RNA pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 0.8 M NaCl, 5 mM EDTA, 0.5% (v/v) β-mercaptoethanol, and 2% CTAB. After incubation at 65°C for 30 min, the solution was extracted with chloroform. RNA was precipitated with precipitation buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% β-mercaptoethanol, and 1% CTAB) by adjusting NaCl to 0.35 M. The resulting RNA pellet was resuspended in 1 M NaCl in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA (TE) and then precipitated with an equal volume of isopropanol.

For RNA gel blotting, ~12 μg of total RNA was loaded in each lane. Hybridizations were performed at 65°C according to Church and Gilbert (1984). cDNA probes were synthesized by either PCR labeling or random priming (Boehringer Mannheim). PCR labeling was performed in the presence of 0.125 pmol of gene-specific primers, 5 μM each of dATP, dTTP, and dGTP, and 50 μCi of [α-32P]dCTP (specific activity, 3000 Ci/mmol). cDNA clones of the GAI gene were isolated by the authors (our unpublished data). GAI and GA4 gene clones were provided by T. Sun, and RGL2 and RGL3 genes were obtained by PCR. To avoid cross-hybridization among the DELLA subfamily members, regions of divergent nucleotide sequences from RGL2 (nucleotides 708 to 1679 of the open reading frame) and RGL3 (nucleotides 630 to 1262 of the open reading frame) were amplified by PCR and then cloned to use as probes. For RGL1, GAI, and GA4, full-length cDNAs were used as probes because weak to no cross-hybridization was detected in blots containing 200 ng of DNA (data not shown). Hybridization results were viewed using a Storm phosphorimager (Molecular Dynamics, Piscataway, NJ).

Reverse Transcriptase-Mediated PCR

For all genes, first-strand cDNA was synthesized using SuperScript (Life Technologies, Rockville, MD) in a 42°C reaction with the primer 5'–CCACCGGCTGCACTAGTACT(7)–3' and 1 μg of total RNA as the template, according to the manufacturer’s instructions. PCR then was performed starting at 94°C for 5 min and followed by 30 cycles at 94°C (25 sec), 55°C (50 sec), and 72°C (1.5 min) using gene-specific forward primers and the common reverse primer 5'–CCACGGCTGCACTAGTAC–3', except for RGL2 and RGL3 cDNAs, which were amplified with gene-specific forward and reverse primers. The gene-specific primers were 5’–GACGAGAATTGAATCGGAG–GT–3’ (for RGL1), 5’–GGCGAGGAGATACGTATACT–3’ (for GA1), 5’–ATCATGTCGATCTCTTGC–3’ (for RGA), 5’–CAGCGGCGGACGGACGG–GT–3’ and 5’–CGCGTTGCTAGCCGATCC–3’ (for RGL2), 5’–CAGCGGCGGACGGACGG–GT–3’ and 5’–CGCGTTGCTAGCCGATCC–3’ (for RGL3), and 5’–CGGATTATCCTTCCCGG–3’ (for GA4). The products were viewed by ethidium bromide staining after agarose gel electrophoresis, and in some cases they were analyzed further by DNA gel blot hybridization with gene-specific probes synthesized by random priming (Boehringer Mannheim). As a negative control for reverse transcriptase-mediated (RT)-PCR, total RNA was used as a template.
In Situ Hybridization

Slides of floral sections (ecotype Landsberg erecta [Ler]), which were prepared as described by Song et al. (2000), were kindly provided by Zhongchi Liu (University of Maryland, College Park). For the probes, an RGL1 cDNA (1.6 kb) subclone in pBluescript SK+ (Stratagene, La Jolla, CA) was first linearized at either end of the cDNA using XbaI and HindIII. RGL1 antisense and sense strand RNA probes were transcribed in vitro using T7 (Epicentre, Madison, WI) and T3 (New England BioLabs, Beverly, MA) RNA polymerases, respectively. The manufacturers’ instructions were followed, except that the T3 RNA polymerase reaction contained ATP, CTP, and GTP (1.4 mM each), UTP (0.7 mM), and digoxigenin-labeled UTP (0.5 mM), which improved the synthesis of the probe. The DNA template was removed using RNase-free DNase I (Epicentre). Hybridizations were performed as described by Vielle-Calzada et al. (1999), and the signals were detected as described by Song et al. (2000).

Assays for PAC Resistance

Paclorbutrazol (PAC) powder (Syngenta Crop Protection, Richmond, CA) was dissolved in absolute ethanol to make 2.5, 50, and 250 mM stocks. For seed germination assays, we followed the procedure described by Jacobsen and Olszewski (1993), keeping the ethanol concentration <0.1% because we found that small amounts of ethanol inhibited germination. Petri dishes containing the seed were placed under constant light at 20°C. Seed germination was scored under a dissecting microscope 6 days after stratification. Cotyledon expansion was scored as germination. The percentage germination and standard deviation for each genotype were determined from six trials with 60 to 100 seed per trial. Germination of gai-t6 was examined in three trials with 20 to 30 seed per trial.

To grow plants on PAC, seed were surface-sterilized with 70% ethanol and then sown on 1 × MS salts, 0.8% agar, plus 0.75 or 1 μM PAC and 0.01% Tween 20 in Magenta boxes. Flowering in the wild type (Figure 2) was obtained by plants grown on the same medium but at high density in Petri dishes. Plants were grown under constant light at 20°C.

GFP Analysis

GFP was visualized in roots, guard cells, and trichomes. Tissue samples were placed on a slide and covered with a cover slip. For roots, light pressure was applied to the cover slip to release single layers of cells. For guard cells and trichomes, the epidermis was peeled from leaves and stems and treated with 4’,6’-diamidino-2-phenylindole (DAPI) as described above. GFP was viewed with a Nikon (Tokyo, Japan) Labophot-2 microscope equipped with a fluorescein isothiocyanate filter. To visualize nuclei, tissues were soaked in 0.2 mg/L DAPI in McIlvaine’s buffer, pH 7.0 (18.2 mM citric acid and 0.16 mM disodium phosphate) for 30 min at room temperature and then rinsed with water. To view DAPI staining, seedlings were excited with 359 nm. DAPI staining in the guard cells and trichomes was very weak.

Roots excised from seedlings expressing the GFP-RGL1 fusion protein were placed in either 100 μM GA3 (dissolved in 0.2% DMSO) or in 0.2% DMSO at room temperature as described by Silverstone et al. (2001). Root cells were observed every 10 to 30 min for up to 180 min. In separate experiments, whole seedlings were treated as described above and observed every 30 min for up to 6 hr (data not shown). Fluorescence was quantitated using NIH Image 1.62 (obtained at http://rsb.info.nih.gov/nih-image/download.html) (data not shown).

Accession Numbers

The GenBank accession number for the RGL1 cDNA is AY048749. The GenBank accession numbers for RGL2 and RGL3 are AC009895 and AL391150, respectively.

ACKNOWLEDGMENTS

We are especially grateful to Tai-Ping Sun and Alyssa Dill for sharing their unpublished results and for providing advice and reagents (rga-24 gai-t6 gai-1-3 seed, 35S-GFP transgenic seed, and RGA and GA4 clones). We also thank Amy Lin for assistance with the GFP localization, Zhongchi Liu for donating floral tissue sections and helping to interpret the in situ hybridizations, Nicholas Harberd for gai-t6 seed, and Xinnian Dong for plasmid pRTL2An-mGFP56ST. We thank our colleague Stephen Mount for valuable discussions and both Tai-ping Sun and Stephen Mount for comments on the manuscript. Zeneca Agrochemicals provided PAC. Seed stocks and the two-hybrid library were obtained from the ABRC. This work was supported by Grant No. 98-35304-67-95 from the United States Department of Agriculture National Research Initiative Competitive Grants Program to C.C. and by a grant from the Howard Hughes Medical Institute through the Undergraduate Biological Sciences Education Program at the University of Maryland, College Park.

Received August 1, 2001; accepted October 11, 2001.

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