

Gibberellin regulates *Arabidopsis* seed germination via *RGL2*, a *GAI/RGA*-like gene whose expression is up-regulated following imbibition

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The germination of *Arabidopsis* seeds is promoted by gibberellin (GA). *Arabidopsis* *GAI*, and *RGA* are genes encoding key GA signal-transduction components (*GAI* and *RGA*) that mediate GA regulation of stem elongation. The *Arabidopsis* genome contains two further genes, *RGL1* and *RGL2*, that encode proteins (*RGL1* and *RGL2*) that are closely related to *GAI* and *RGA*. Here, we show that *RGL2* regulates seed germination in response to GA, and that *RGL1*, *GAI*, and *RGA* do not. In addition, we show that *RGL2* transcript levels rise rapidly following seed imbibition, and then decline rapidly as germination proceeds. In situ GUS staining revealed that *RGL2* expression in imbibed seeds is restricted to elongating regions of pre-emergent and recently emerged radicles. These observations indicate that *RGL2* is a negative regulator of GA responses that acts specifically to control seed germination rather than stem elongation. Furthermore, as *RGL2* expression is imbibition inducible, *RGL2* may function as an integrator of environmental and endogenous cues to control seed germination.

[Key Words: *RGL2/RGL1/GAI/RGA*; gibberellin; imbibition; seed germination; *Arabidopsis thaliana*]

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Seeds contain embryonic plants that are arrested in their development, and that await the appropriate environmental conditions to continue with their life cycles (Bewley 1997). The transition of a seed from dormancy to germination is controlled by external environmental cues (including light quality, moisture, and transient exposure to cold) and by the internal growth regulators GA and ABA (abscisic acid). ABA establishes and maintains dormancy, whereas GA breaks dormancy and induces germination (Koornneef and Karssen 1994; McCarty 1995; Bewley 1997; Steber et al. 1998). Under favorable environmental conditions, GA biosynthesis is induced and, in turn, the de novo-synthesized GA induces the expression of genes encoding enzymes such as endo- β -1,3 glucanase (Leubner-Metzger et al. 1995), β -1,4 mannan endohydrolase (Bewley 1997; Sánchez and Miguel 1997), and the extensin-like protein AtEPR1 (Dubreucq et al. 2000). These enzymes hydrolyze the endosperm and release the inhibitory effects of ABA on embryo growth potential (Koornneef and Karssen 1994; McCarty

1995; Bewley 1997). The importance of the balance between ABA and GA signaling for seed germination is perhaps best shown by studies of ABA response mutants and GA-deficient mutants. In *Arabidopsis*, ABA-insensitive mutations (e.g., *abi1*, *abi2*, and *abi3*) reduce seed dormancy and allow germination at ABA concentrations that are normally inhibitory to wild-type germination (Koornneef et al. 1984). On the other hand, GA-deficient mutants (e.g., *ga1-3* and *ga2*) fail to germinate in the absence of exogenous GA (Koornneef and van der Veen 1980). Although it has long been known that GA promotes seed germination, the molecular mechanisms by which GA signaling regulates germination, and the relationship between GA signaling and the environmental cues to germination are poorly understood.

In recent years, genes encoding several GA-signaling components have been identified and cloned (Harberd et al. 1998; Thornton et al. 1999; Sun 2000; Richards et al. 2001). Loss-of-function mutations of *SPINDLY* (*spy* mutant alleles) confer a phenotype that mimics that of GA-treated wild-type plants. *spy* mutants are slender and early flowering and have pale green foliage, indicating that *SPINDLY* is a negative regulator of GA responses (Jacobsen and Olszewski 1993; Jacobsen et al. 1996). In addition, *spy* alleles suppress the nongerminating phenotype conferred by *ga1-2* (an allele of *ga1-3*) as follows:

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spy gai-2 double-mutant seeds germinate in the absence of exogenous GA (Jacobsen and Olszewski 1993), suggesting that SPY plays an important role in the GA-mediated control of both stem elongation and seed germination. GAI and RGA are important negative regulators of the GA-signaling pathway for stem elongation, because the stem elongation of plants homozygous for null alleles of *GAI* or *RGA* (e.g., *gai-t6* or *rga-24*) requires less GA than that of wild-type plants (Peng and Harberd 1993; Peng et al. 1997; Silverstone et al. 1997, 1998). Furthermore, the combination of *gai-t6* and *rga-24* (in a *gai-t6 rga-24* double mutant) completely suppresses the dwarfing phenotype conferred by the *gai-3* mutation (Dill and Sun 2001; King et al. 2001). The *GAI/RGA* genes were originally defined by the cloning of the mutant *gai* allele (Peng et al. 1997). *gai* encodes a mutant protein that lacks a region of 17 amino acids from close to the N terminus and confers a dominant dwarf, reduced GA-response phenotype. Functional *GAI/RGA* orthologs have been identified in wheat (Rht), maize (d8), and rice (SLR1), and mutations that cause alterations in the N termini of these proteins also confer dominant dwarfism combined with reduced GA responses (Peng et al. 1999a; Ikeda et al. 2001). Recently, *PHOR1* (*photoperiod-response 1*), initially identified as a photoperiod response gene in potato (*Solanum tuberosum* ssp. *Andigena*), was also found to be a GA-signaling factor, suggesting that *PHOR1* links photoperiod and GA signaling (Amador et al. 2001).

The plant height regulating factors *GAI/RGA/RHT/d8/SLR1* (Peng et al. 1997, 1999a; Silverstone et al. 1998; Ikeda et al. 2001) belong to a family of putative transcription factors known as the GRAS family. GRAS proteins appear to be unique to plants (Peng et al. 1999a; Pysh et al. 1999; Richards et al. 2000), and there are ~30 candidate GRAS ORFs in the *Arabidopsis* genome. Alignment of the GRAS proteins reveals that they share close homology in approximately two-thirds of their C-terminal regions, contain features with characteristics of transcription factors, and contain an SH2-like domain (Peng et al. 1999a; Pysh et al. 1999; Richards et al. 2000). In contrast, the N-terminal approximately one-third of the GRAS proteins are highly diversified (Peng et al. 1999a; Pysh et al. 1999; Richards et al. 2000). Mutant analysis has revealed that GRAS proteins regulate several different aspects of plant growth and development in addition to GA signaling, including root development (Di Laurenzio et al. 1996; Helariutta et al. 2000; Nakajima et al. 2001), lateral branch initiation (Schumacher et al. 1999), and phytochrome A signal transduction (Bolle et al. 2000). The specificity of the GRAS proteins is thought to reside in the diverse approximately one-third N-terminal regions, because they differ from each other mainly in this region. In fact, two highly conserved N-terminal regions (I and II; Peng et al. 1999a) are found in *GAI* and *RGA*, in *GAI/RGA* functional orthologs in other species, and in the ORFs of three related *Arabidopsis* genes of hitherto unknown function: *RGA-like 1* (*RGL1*, chromosome 1), *RGA-like 2* (*RGL2*, chromosome 3), and *RGA-like 3* (*RGL3*, chromosome 5) (Peng et al. 1999a; Richards

et al. 2000; Dill and Sun 2001). Regions I and II are not found in other *Arabidopsis* GRAS proteins. Analysis of the *Arabidopsis* mutant *gai* gene, of mutant forms of *GAI/RGA* orthologs in wheat and maize, and of the effects of in vitro-synthesized *RGA* and *SLR1* mutant alleles that encode proteins lacking region I, have shown that regions I and II are critical for GA signaling (Peng et al. 1999a; Dill et al. 2001; Ikeda et al. 2001).

Because *RGL1* and *RGL2* contain regions I and II, it seemed likely that they also function as GA-signaling factors. As described above, the absence of *GAI* and *RGA* suppresses the dwarf stem phenotype of *gai-3*, indicating that *GAI* and *RGA* act as negative regulators of GA-mediated stem elongation. However, absence of *GAI* and *RGA* function is not sufficient to restore normal germination or normal floral development to *gai-3* in the absence of exogenous GA, indicating that other signaling components or pathways are involved in controlling these responses (Dill and Sun 2001; King et al. 2001). Here, we present genetic and molecular data showing that *RGL2* is a GA-signaling component, and that it functions as a negative regulator of seed germination. In addition, we show that *RGL2* expression in both wild-type and *gai-3* seeds is induced to high levels following the onset of imbibition. The levels of *RGL2* transcripts in germinating wild-type seeds decrease rapidly once the radicle begins to protrude, but remain at a high level in imbibed, nongerminating *gai-3* mutant seeds. This suggests that the role of *RGL2* is to prevent germination after imbibition and that GA promotes germination by down-regulating *RGL2* expression. Thus, GA regulates stem elongation via *GAI* and *RGA*, and seed germination via *RGL2*. *RGL2* acts as an integrating factor that links GA signaling and environmental cues in the regulation of seed germination.

Results

The N termini of RGL1 and RGL2 contain regions I and II, the conserved GA-signaling regions in GAI/RGA

The *Arabidopsis* genome contains three additional genes, *RGL1*, *RGL2*, and *RGL3* that encode proteins (*RGL1*, *RGL2*, and *RGL3*) whose amino acid sequences are closely related to those of *GAI* and *RGA* (Dill and Sun 2001). Overall, these sequences share 59% (*RGL1* vs. *RGL2*), 58% (*RGL1* vs. *RGL3*), and 68% (*RGL2* vs. *RGL3*) homology. *RGL1*, *RGL2*, and *RGL3* show, respectively, 58%, 55%, and 54% homology to *GAI*, and 55%, 57%, and 53% homology to *RGA*. *RGL1*, *RGL2*, and *RGL3* differ from other GRAS proteins by showing close similarity, not only with the C-terminal regions of *GAI* and *RGA* but also to the N-terminal regions I and II (GA-response regions; Fig. 1). Previous reports have shown that mutant alleles with alterations in regions I and II confer greatly reduced responses to either exogenous or endogenous GA (Peng et al. 1997, 1999a; Dill et al. 2001; Ikeda et al. 2001). The fact that *RGL1*, *RGL2*, and *RGL3* display strong amino acid sequence conservation of regions I and II implies that these three proteins are also GA-response regulators.

publ.). In RNA gel-blot experiments (Fig. 2A), an *RGL2* probe identified a 2.0-kb *RGL2* transcript in wild type. In SGT625, SGT11937, and SGT13975, this 2.0-kb *RGL2* transcript was no longer present and was replaced by larger transcripts that were thought likely to be fusion transcript products containing both *RGL2* and *Ds-GUS* (Fig. 2A) similar to the pattern observed previously for *GAI* transcripts in the *Ds*-insertion *gai-t6* mutant (Peng et al. 2001). As expected, the larger transcripts in SGT625, SGT11937, and SGT13975 also hybridized to a probe derived from the *GUS* gene (Fig. 2A). The *GUS* gene probe also identified additional, smaller, hybridization bands in SGT625, SGT11937, and SGT13975, which possibly represent alternative splicing products of *RGL2-Ds-GUS* fusion transcripts (Fig. 2A).

By use of further information from the SGT database (V. Sundaresan, pers. comm.), *RGL2*-specific primers were designed and paired with primers derived from *Ds-GUS*, thus permitting the amplification via PCR of DNA fragments containing *Arabidopsis* genomic DNA-*Ds* junction regions (for both ends of the *Ds*) from each of SGT625, SGT11937, and SGT13975. The obtained PCR products were sequenced and compared with wild-type *RGL2* DNA sequence to locate the precise *Ds* insertion sites. *Ds*-insertions in *RGL2* were confirmed for all three mutant lines, and the precise position of insertion of *Ds* in each line is shown in Figure 2B. These three lines were

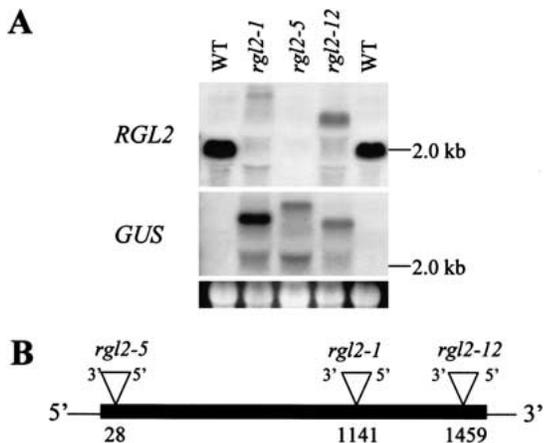


Figure 2. Molecular characterization of three independent *RGL2* *Ds*-insertion mutant lines. (A) RNA gel-blot hybridizations using DIG-labeled *RGL2* and *GUS* probes (see Materials and Methods) and total RNA from inflorescences (28 days old). The *RGL2* probe identified a ~2-kb hybridization band in RNA from the wild-type control. This band was absent in RNA from all three mutant lines. The *GUS* probe identified hybridization bands in RNA from all three mutant lines, but failed to hybridize to RNA from wild-type controls. Below the *GUS*-hybridization panel, the gel fluorescence serves as a control for equal RNA sample loading on the gel. (B) Schematic diagram showing the *Ds*-insertion sites in the *rgl2-1*, *rgl2-5*, and *rgl2-12* mutant alleles (thin line, noncoding region; filled thick line, *RGL2*-coding region, inverted triangle, *Ds* insertion). The *Ds* element insertion in all three alleles orientated 3' to 5' as shown. Numbers represent the site of insertion in the *RGL2* ORF (in nucleotides, the A of the start ATG = 1).

thus designated as *rgl2-1* (SGT625), *rgl2-5* (SGT11937), and *rgl2-12* (SGT13975), respectively.

While searching the SGT database, another line (SGT4763), with a *Ds-GUS* insertion 68 bp upstream of the ATG translation start codon of *RGL1* was identified (V. Sundaresan, pers. comm.; S.C. Lee and J. Peng, unpubl.). RNA gel-blot analysis confirmed that this *Ds-GUS* insertion abolishes *RGL1* gene expression (S.C. Lee and J. Peng, unpubl.). This mutant line was therefore designated *rgl1-1*.

The germination of rgl2 mutants is strongly resistant to the effects of paclobutrazol

When grown in the greenhouse and in controlled-environment conditions (23°C, 16 h light/8 h dark), *rgl2-1* plants were indistinguishable from wild type (data not shown). To determine whether disruption of *RGL2* would affect seed germination, wild-type, *rgl2-1*, *rgl2-5*, and *rgl2-12* seeds were placed on medium containing paclobutrazol (PAC) (Fig. 3A). PAC is a triazole derivative that inhibits GA biosynthesis at the kaurene oxidase reaction (Hedden and Graebe 1985). Seeds imbibed in the presence of PAC contain reduced or depleted endogenous GA levels, as a result of which their germination is inhibited. Previous experiments have shown that seeds of the *gai spy-7 gar2-1* mutant line are strongly resistant to the inhibitory effects of PAC on seed germination (Peng et al. 1999b), and this line was used here as a positive control (Fig. 3A). The germination of wild-type seeds was inhibited completely by PAC at concentrations higher than 10^{-5} M. In contrast, seeds of the *gai spy-7 gar2-1*-positive control, and *rgl2-1*, *rgl2-5*, and *rgl2-12* seeds, exhibited strong resistance to PAC and achieved high germination percentages (>90%) on 10^{-5} M and 10^{-4} M PAC medium (Fig. 3A).

To determine whether the PAC resistance of the *rgl2-1*, *rgl2-5*, and *rgl2-12* mutants is truly conferred by insertional inactivation of *RGL2*, complementation experiments with an *NdeI* genomic DNA fragment containing the entire *RGL2*-coding region plus ~3.3 kb 5' noncoding and ~2.0 kb 3' noncoding sequences were performed. This DNA fragment was cloned into the binary vector pCambia1300 and transformed into *rgl2-1* mutant plants. A number of independent transformants were obtained and seeds homozygous for the *RGL2* transgene (from five independent transformant lines) were tested for their ability to germinate on medium containing PAC. As shown in Figure 3B, PAC sensitivity was restored in the seeds of all five transgenic lines (all of which display <5% germination at 10^{-5} M PAC). This result unequivocally shows that loss of *RGL2* function confers PAC-resistant germination, demonstrating that *RGL2* is a GA-response negative regulator of seed germination.

Experiments comparing the extent of the PAC resistance conferred by the *rgl2* alleles with that conferred by loss-of-function mutations in other gene members of the *GAI/RGA-like* family were performed (Fig. 3C). In these experiments, seeds of the *gai spy-7 gar2*-positive control

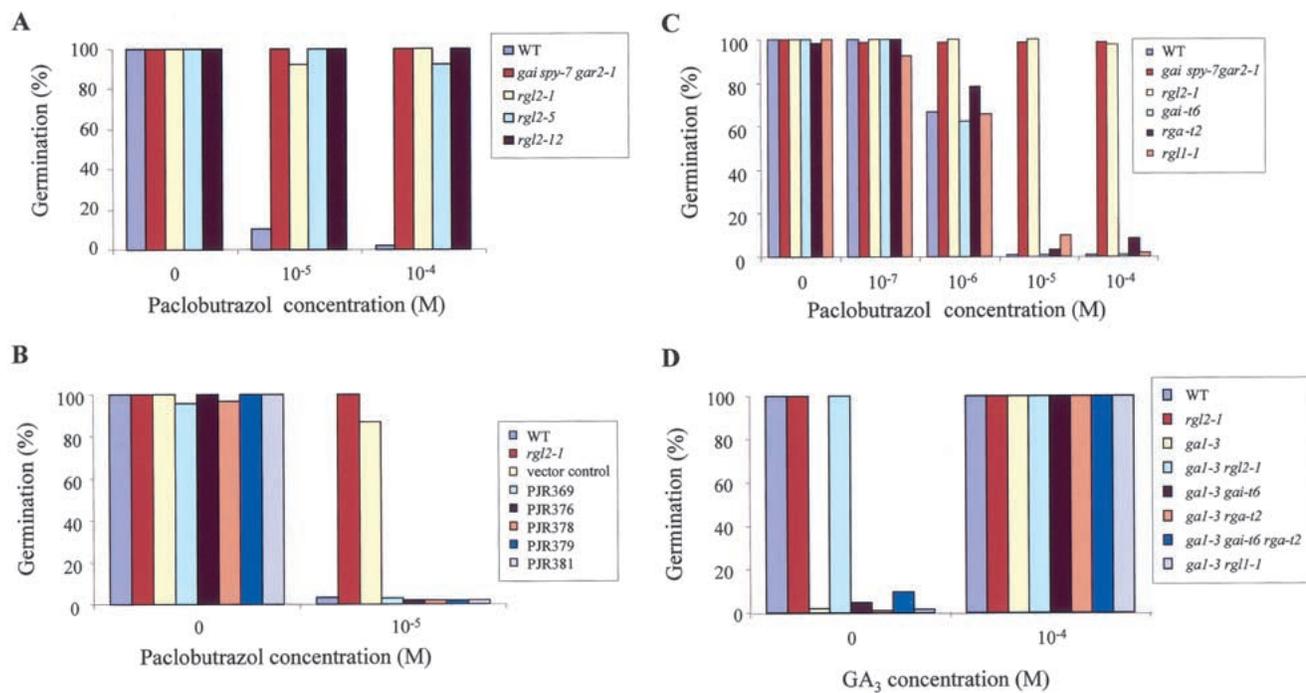


Figure 3. RGL2 is a GA-response negative regulator of seed germination. Germination begins when quiescent dry seeds are imbibed. Following this, the radicle begins to elongate. Germination becomes visible when the radicle protrudes outside of the seed coat. In these experiments, the frequency of radicle protrusion was used as a measure of germination. (A) Seed germination of *RGL2* alleles on medium containing PAC. All three alleles (*rgl2-1*, *rgl2-5*, and *rgl2-12*) confer strong resistance to the inhibitory effects of PAC on seed germination. The *gai spy-7 gar2-1* mutant line also displays PAC-resistant seed germination (Peng et al. 1999b) and was used here as a positive control. (B) A genomic DNA fragment containing *RGL2* complements the *rgl2-1* phenotype in five independent transgenic lines, PJR369, 376, 378, 379, and 381. Vector control, pCambia1300 (Cambia, Australia). (C) Comparison of the effects of increasing concentrations of PAC on the seed germination of wild-type and of the *rgl2-1*, *rgl1-1*, *gai-t6*, and *rga-t2* mutants. At the highest PAC concentration, only the *rgl2-1* mutant, and the *gai spy-7 gar2-1*-positive control seeds can germinate. (D) Suppression of the nongerminating phenotype of *gal-3* by the *rgl2-1* mutation, but not by *rgl1-1*, *gai-t6*, *rga-t2*, or by *gai-t6 rga-t2* in combination. Seeds were germinated on SM medium. All tests yielding germination rates between zero and <2% were given a value of 2% for the convenience of drawing the histogram. The seed germination experiments were repeated multiple times and the results shown are those of a single experiment and are clearly representative of what was seen in the repeat experiment.

mutant achieved >95% germination on 10^{-4} M PAC medium. Although germinating slightly later than the positive control (data not shown), *rgl2-1* seeds also achieved a high germination percentage (>95%) on this medium. However, the *rgl1-1* (loss-of-function *RGL1* allele), *gai-t6* (loss-of-function *GAI* allele), and *rga-t2* (loss-of-function *RGA* allele; see Materials and Methods) mutants all failed to germinate in these conditions, as did the wild-type control (Fig. 3C). These results show that *rgl2* mutants, but not *rgl1-1*, *gai-t6*, or *rga-t2* mutants, can germinate, regardless of the depleted endogenous GA levels conferred by PAC, suggesting that RGL2 and GAI/RGA, although closely related in amino acid sequence, might function in different GA-mediated signaling pathways.

rgl2-1 suppresses the nongerminating phenotype of *gal-3*

GAI encodes *ent*-CDP synthase, an enzyme that catalyzes a relatively early step in the biosynthesis of GA (Sun et al. 1992; Sun and Kamiya 1994). The *gal-3* loss-of-function allele causes GA deficiency and abolishes seed

germination (Koornneef and van der Veen 1980). Because *rgl2-1* confers resistance to the inhibitory effect of PAC on seed germination, it seemed likely that *rgl2-1* would also suppress the nongerminating phenotype conferred by *gal-3*. To test this hypothesis, the seed germination of *gal-3* mutant lines carrying *RGL2* or *rgl2-1* was compared with that of other genotypes (Fig. 3D). As expected, *gal-3* mutants did not germinate, and absence of either or both of GAI and RGA (in *gal-3 gai-t6*, *gal-3 rga-t2*, and *gal-3 gai-t6 rga-t2*) did not substantially suppress the nongermination phenotype of *gal-3* (Dill and Sun 2001; King et al. 2001). The *gal-3 rgl1-1* mutant also did not germinate, indicating that *rgl1-1* germination is not GA independent. However, *gal-3 rgl2-1* achieved >95% germination, showing that the germination of *rgl2-1* is largely independent of GA. As expected, high levels of germination were exhibited by all mutant lines in the presence of exogenous GA_3 (Fig. 3D). These results, together with those obtained from the PAC test, clearly show that RGL2, but not RGL1, acts as a negative regulator of GA responses in the control of seed germination.

rgl2-1 does not confer PAC-resistant stem elongation or leaf expansion

As shown above, wild-type seeds can germinate on medium containing 10^{-7} M PAC (Fig. 3C). However, continued growth on this medium causes dwarfism, with the reduced stem elongation and leaf expansion characteristic of GA deficiency. We tested the effects of long-term growth on PAC-containing medium on the stem elongation and leaf expansion of various genotypes. Growth of the *gai spy-7 gar2-1* mutant line is relatively resistant to PAC, thus serving as a positive control for these experiments (Peng et al. 1999b; Fig. 4A,B). As expected, the absence of RGA had a marked effect on stem growth under these conditions (Silverstone et al. 1997). The *rga-t2* mutant plants displayed strong PAC resistance and grew significantly taller than all of the other genotypes tested. The *gai-t6* mutant plants also grew taller than wild type (Peng et al. 1997), but were shorter than the *rga-t2* plants. However, no obvious differences were observed between the stem lengths of *rgl1-1*, *rgl2-1*, or wild-type plants grown in these conditions (Fig. 4A). The rosette sizes (used here as a measure of leaf expansion) of plants grown on 10^{-6} M PAC medium were also compared (Fig. 4B). The leaf expansion of wild-type and *rgl2-1* plants was severely retarded by PAC. Wild-type and *rgl2-1* rosettes were indistinguishable from one another,

and markedly smaller than those of the *gai spy-7 gar2-1* control. These results indicate that RGL2 is not involved in controlling stem elongation or leaf expansion growth or plays only a minor role in these processes.

rgl2-1 does not suppress the dwarf phenotype of *gai-3*

To further investigate whether RGL2 is involved in controlling stem elongation growth, the growth of *gai-3* mutants carrying *RGL2* or *rgl2-1* was compared with the growth of additional mutant lines. When *gai-3* and *gai-3 rgl2-1* plants were compared at the late rosette stage, no significant phenotypic differences between the two lines could be discerned (Fig. 4C). In further experiments, the growth of *gai-3 rgl2-1* plants was compared with that of plants lacking GAI and/or RGA (Fig. 4D). Consistent with previous reports (Silverstone et al. 1998; Dill and Sun 2001; King et al. 2001), the dwarfism conferred by *gai-3* is partially suppressed by the absence of RGA, and completely suppressed by the absence of both GAI and RGA (Fig. 4D). In contrast, the growth of *gai-3 rgl2-1* plants was not significantly different from that of *gai-3* mutant plants (Fig. 4C,D). Thus, as shown above, *rgl2-1* suppresses the effect of GA deficiency on seed germination, but does not suppress the effect of GA deficiency on stem elongation.

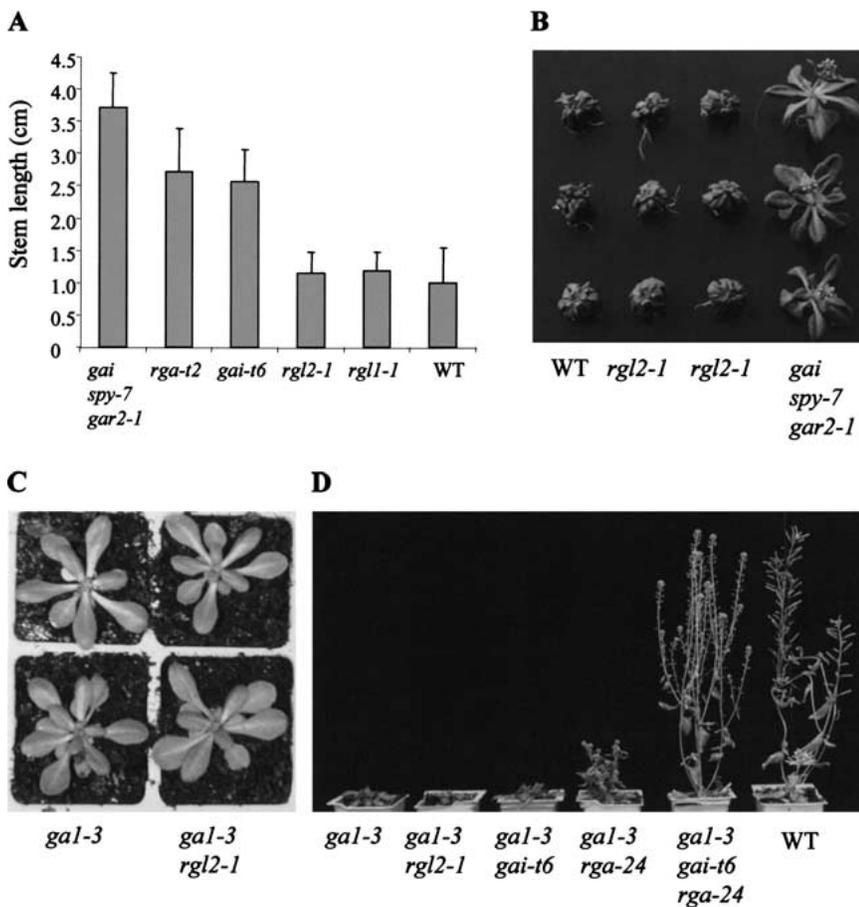


Figure 4. RGL2 plays a relatively minor role in the regulation of stem elongation and leaf expansion. (A) Comparison of stem lengths of wild-type and of *rgl2-1*, *rgl1-1*, *gai-t6*, and *rga-t2* mutants grown on medium containing 10^{-7} M PAC (30 days old; $n = 15-20$). (B) Comparison of rosette size (a measure of leaf expansion) of two *rgl2-1* lines, *gai spy-7 gar2-1* (positive control) and wild-type (negative) control grown on medium containing 10^{-6} M PAC (30 days old). Each genotype is represented by three typical plants arranged in a column. (C) Rosette size of the *rgl2-1 gai-3* double mutant is indistinguishable from that of the *gai-3* single mutant (30 days old). Each genotype is represented by two typical plants arranged in a column. (D) *rgl2-1* does not suppress the dwarf phenotype of *gai-3*. All plants shown (except for the wild-type control) are homozygous for *gai-3*. *rgl2-1* and *gai-t6* do not appreciably suppress *gai-3* phenotype, whereas *rga-t2* partially, and the *gai-t6 rga-24* combination fully, suppresses the dwarfing phenotype conferred by *gai-3* (adult plants, 45 days old, are shown).

RGL2 expression is up-regulated during imbibition of seeds

Imbibition is the process of water uptake by dry seeds, in the course of which the seed becomes hydrated. To further study how *RGL2* is involved in seed germination, RNA gel-blot experiments were used to compare *RGL2* transcript levels in dry seeds and in seeds imbibed at either 23 or 4°C. In dry wild-type seeds, *RGL2* transcripts are present at a relatively low level. *RGL2* transcript levels rise following onset of imbibition, are clearly visible after 12 h (at 23°C) and 48 h (at 4°C), and reach a high level at around 24 h (at 23°C) and 72 h (at 4°C) (Fig. 5A,B). The high levels of *RGL2* transcripts are maintained if seeds are kept at 4°C (Fig. 5A, 168 h). When seeds imbibed at 4°C for 168 h are moved to 23°C, they germinate rapidly, with seedling emergence completed, and with hypocotyl and radicle elongation and cotyledon expansion clearly in progress within 48 h (data not shown). During this time, *RGL2* transcript levels fall rapidly, and *RGL2* transcripts are barely detectable 48 h following the move to 23°C (Fig. 5A).

The *Ds* insertion in the *rgl2* alleles contains an intact but promoterless *GUS* reporter gene, with intron sequences and three alternative splice donor and acceptor sequences fused upstream of the *GUS* ATG codon (Sundaresan et al. 1995). If *Ds* is inserted in a transcribed region (5'-noncoding, intron, or exon), and providing that the *GUS* gene is in the correct orientation, a *GUS* fusion under control of the chromosomal gene promoter will result. Thus, *GUS*-staining patterns conferred by alleles containing such insertions can serve as markers for studying the tissue specificity of gene expression. The *Ds* insertion in *rgl2-5* is between bases encoding G²⁸ and A²⁹ of *RGL2* (Fig. 2B), and the *GUS* reporter gene is in the correct orientation. In addition, a 3.3-kb DNA fragment

upstream of the start ATG in *rgl2-5* was cloned via PCR. The DNA sequence of this fragment is identical to that obtained from a wild-type control (data not shown). Because we had already shown that the *RGL2*-coding region together with this same 3.3 kb upstream DNA sequence is sufficient to restore a *RGL2* germination phenotype to *rgl2-1* (Fig. 3B), we assumed that the *RGL2* promoter in *rgl2-5* is still fully functional. This is further supported by the detection of *GUS* fusion transcripts in *rgl2-5* when a probe derived from *GUS* was used in RNA gel-blot experiments (Fig. 2B). In situ *GUS* staining of germinating seedlings heterozygous for *rgl2-5* revealed staining in the pre-emergent radicle (embryonic axis; Bewley 1997) of seeds that had been imbibed for 168 h at 4°C (Fig. 5E, 0 h). This staining disappeared rapidly and was faintly detectable 24 h (Fig. 5E, 24 h), and barely detectable 48 h after the seeds were moved to 23°C (Fig. 5E, 48 h).

Imbibed *gal-3* mutant seeds maintain a high level of *RGL2* transcripts

As shown above, *rgl2-1* suppresses the nongerminating phenotype of *gal-3*. We therefore reasoned that *RGL2*, as a repressor of seed germination, might be present at high levels in imbibed *gal-3* seeds, thus preventing their germination. To test this hypothesis, we assayed *RGL2* transcript levels in *gal-3* mutant seeds. As for wild-type seeds, *RGL2* transcript levels are relatively low in dry *gal-3* seeds, accumulate to much higher levels upon imbibition, and stay at high levels while imbibed seeds are maintained at 4°C (Fig. 5C). However, *RGL2* transcript levels do not decline following transfer of *gal-3* seeds from 4 to 23°C. In marked contrast to what is seen in wild-type seeds, *RGL2* transcripts are still at high levels

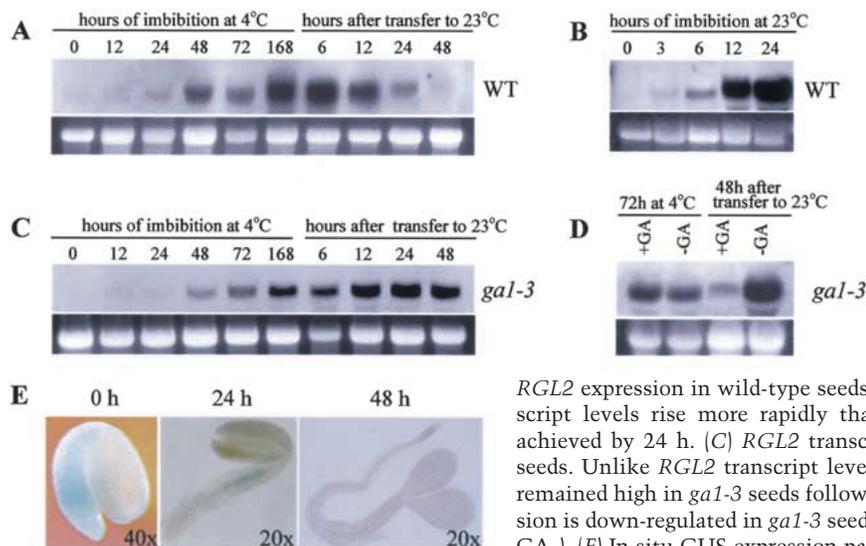


Figure 5. *RGL2* expression in seeds is imbibition inducible. (A) RNA gel-blot analysis of *RGL2* expression in wild-type seeds at different stages of imbibition and germination. *RGL2* transcripts first become detectable at 24 h after the onset of imbibition at 4°C, then rise to a peak that is maintained for as long as the seeds are kept at 4°C. When wild-type seeds are subsequently moved to 23°C, *RGL2* transcript levels begin to fall rapidly, and are barely detectable 48 h after the seeds were moved into the warmth, by which time germination had been completed. (B)

RGL2 expression in wild-type seeds is also induced by imbibition at 23°C. Transcript levels rise more rapidly than they did at 4°C, with high levels being achieved by 24 h. (C) *RGL2* transcripts remain at high levels in *gal-3* mutant seeds. Unlike *RGL2* transcript levels in wild-type seeds, *RGL2* transcript levels remained high in *gal-3* seeds following transfer from 4 to 23°C. (D) *RGL2* expression is down-regulated in *gal-3* seeds grown at 23°C and treated with GA (10^{-4} M GA₃). (E) In situ *GUS* expression patterns in seeds of *rgl2-5* heterozygotes during

seed germination. *GUS* staining is observed in the radicle of seeds that have been imbibed at 4°C (seed coat was removed after staining for observation). Following transfer to 23°C, *GUS* staining rapidly disappears, being still faintly detectable in the radicle at 24 h and barely detectable at 48 h, by which time germination is relatively advanced. In A, C, D, and E, seeds were chilled at 4°C for 7 d and then moved to a 23°C growth chamber with a 24 hr photoperiod.

in *ga1-3* seeds after 48 h at 23°C (Fig. 5C; refer to 5A for wild-type control). In further experiments, the effect of GA₃ on *RGL2* transcript levels in *ga1-3* seeds was studied. No detectable differences were observed between *RGL2* transcript levels in GA-treated and GA-untreated *ga1-3* seeds imbibed for 72 h at 4°C (Fig. 5D). However, *RGL2* transcript levels were dramatically reduced by GA treatment when imbibed *ga1-3* seeds were transferred to 23°C (Fig. 5D). These results are consistent with our genetic analysis, and with the hypothesis that *RGL2* likely functions as a repressor of seed germination. High levels of *RGL2* transcripts are maintained in *ga1-3* seeds, and reduced by GA treatments. Germination of normal seeds presumably requires down-regulation of *RGL2* expression by GA.

RGL2 is highly expressed in young inflorescences

We also investigated the expression of *RGL2* in stages of the plant life cycle following seed germination. RNA gel-blot hybridization was used to measure the steady-state levels of *RGL2*, *GAI*, and *RGA* transcripts in tissues including young flower buds, siliques, bolting stem, cauline leaves, rosette leaves, and roots. With the exception of siliques, in which both *RGA* and *GAI* were expressed at low, but detectable levels, *RGA* and *GAI* were ubiquitously expressed in all tissues tested, with young flower buds showing the highest levels of transcript accumulation (Fig. 6A). In contrast, *RGL2* transcripts are only detectable in the inflorescence, with high levels in young flower buds and significant levels in siliques, but not detectable in leaves, bolting stems, or roots (Fig. 6A).

Following the reasoning outlined above, we also studied in situ GUS staining patterns in *rgl2-5* heterozygotes, beginning at the seedling stage and assaying at 2-day intervals until the plants were 40 days old. GUS expression was not detected in young seedlings (6–12 d after sowing; data not shown). At day 14, GUS staining began to appear in young flower buds (clearly visible by day 20, Fig. 6B), and is maintained in inflorescences, but is not detectable in stem, leaves, or roots of bolting plants (Fig. 6B, 24 and 28 d). Clearly, the GUS expression pattern in *rgl2-5* is consistent with the results obtained via RNA gel-blot analysis of *RGL2* expression (Fig. 6A).

In a more detailed analysis of the GUS staining patterns in floral organs of *rgl2-5* heterozygotes, young flower buds were examined microscopically. GUS staining was observed in almost all floral organs with particularly strong staining in stamen filaments, the top of the style (just below the stigma), and sepals (Fig. 6C). In young siliques, GUS staining was observed at the base of the young developing seed (Fig. 6D).

Discussion

The *GAI/RGA/RHT/d8/SLR1* proteins mediate GA signals in a wide range of vascular plants (Peng et al. 1997, 1999a; Silverstone et al. 1998; Ikeda et al. 2001). Studies of loss-of-function alleles of *GAI* and *RGA* in *Arabidop-*

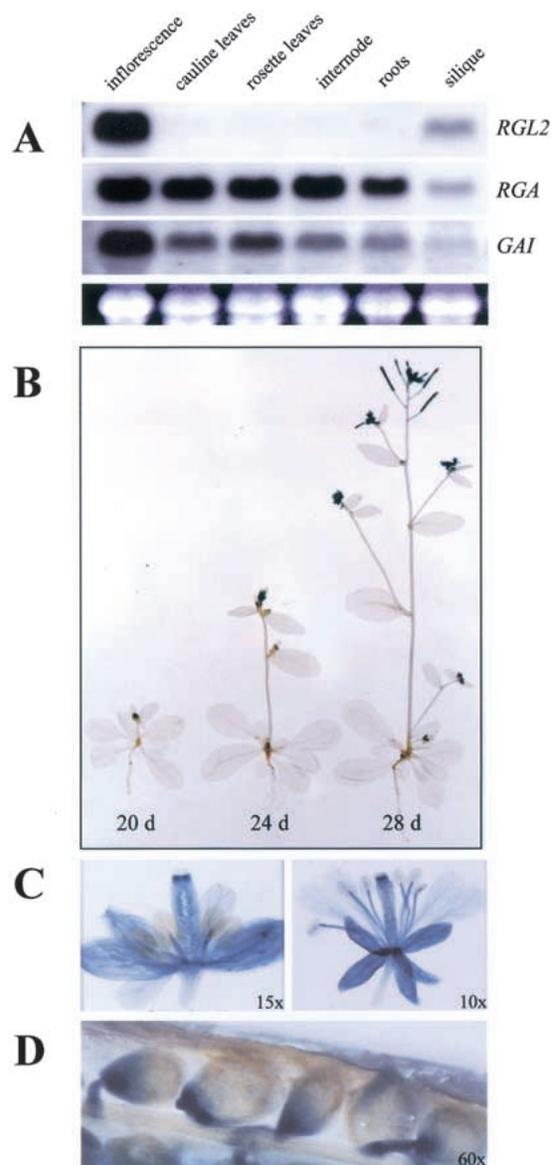


Figure 6. *RGL2* is highly expressed in the inflorescences of bolting plants. (A) RNA gel-blot analysis of *RGL2*, *GAI*, and *RGA* transcripts in different plant tissues (28 days old). (B) GUS staining was detected mainly in the inflorescences and siliques of bolting plants heterozygous for *rgl2-5*. No GUS activity was detected at the seedling stage from day 4 to 12 after sowing (data not shown). (C) GUS staining was displayed in almost all flower organs. (D) Close up of a silique showing GUS staining at the base of the young developing seeds.

sis have shown that *GAI* and *RGA* function additively as negative regulators of GA responses controlling stem elongation growth (Peng et al. 1997; Silverstone et al. 1997, 1998; Dill and Sun 2001; King et al. 2001). The conserved N-terminal regions I and II of *GAI/RGA/RHT/d8/SLR1* have been shown to be crucial for mediating GA signals. Alterations in regions I and II confer greatly reduced responses to both exogenous and endogenous GA (Peng et al. 1997, 1999a; Ikeda et al. 2001; Dill et al.

2001). RGL1 and RGL2 are related in amino acid sequence to GAI/RGA/RHT/d8/SLR1 throughout their entire lengths. The high homology shared by RGL1, RGL2, and GAI/RGA in N-terminal regions I and II (Fig. 1) implies that RGL1 and RGL2 are also regulators of GA responses.

In this study, we report our analyses of loss-of-function alleles of *RGL1* (*rgl1-1*) and *RGL2* (*rgl2-1*, *rgl2-5*, and *rgl2-12*). Under normal growth conditions, *rgl1* and *rgl2* mutant plants are indistinguishable from wild-type plants. However, *rgl2* alleles confer resistance to the inhibitory effect of PAC on germination, whereas *rgl1-1*, *gai-t6*, and *rga-t2* (loss-of-function alleles of *RGL1*, *GAI*, and *RGA*, respectively) do not. Furthermore, *rgl2-1* suppresses the nongerminating phenotype of the GA-deficient *ga1-3* mutant, whereas *rgl1-1*, *gai-t6*, or *rga-t2* alone do not. Even the combined absence of *GAI* and *RGA* (in *ga1-3 gai-t6 rga-t2*) fails to suppress the nongerminating phenotype of *ga1-3*, as reported previously (Dill and Sun 2001; King et al. 2001). These observations suggest that *RGL2* plays a major role in mediating the GA-regulated control of seed germination, whereas *RGL1*, *GAI*, and *RGA* play relatively minor roles.

On the other hand, *rgl2-1* does not confer obvious resistance to the inhibitory effects of PAC on rosette leaf expansion and stem elongation. *gai-t6* and *rga-t2* mutants have significantly larger rosettes and display longer floral bolt stems than wild-type control plants when grown on medium containing PAC. *rgl2-1* also fails to suppress the leaf expansion and stem elongation phenotypes of *ga1-3*. *rgl2-1 ga1-3* double mutant plants are indistinguishable from the *ga1-3* single mutant. This is in marked contrast to the significant suppression of the *ga1-3* dwarfing phenotype conferred by *rga* alleles and to the complete suppression of the *ga1-3* dwarfing phenotype conferred by the *gai-t6 rga-24* double knockout (Dill and Sun 2001; King et al. 2001).

Taken together, our results show that loss-of-function mutations in *RGL2* suppress the seed germination, but not the leaf expansion and stem elongation components of the GA-deficient phenotype conferred either by treatments with PAC, or by *ga1-3*. Our observations indicate that *RGL2* (but not *RGL1*, *GAI*, or *RGA*) acts as a negative regulator of GA-promoted seed germination, whereas *GAI* and *RGA* act as negative regulators of GA-promoted stem elongation and leaf expansion.

After they have been stored for a while (normally >1 mo post harvesting), wild-type seeds will germinate ~3 d following imbibition at room temperature and ~2 wk following imbibition at 4°C (H. Cheng and J. Peng, unpubl.; Koornneef and Karssen 1994). However, seeds of the severely GA-deficient mutant *ga1-3* do not germinate in either of these conditions, implying that GA is necessary for germination to proceed (Koornneef and van der Veen 1980; this work). Here, we have shown that *RGL2* transcripts accumulate following imbibition of both wild-type and *ga1-3* mutant seeds, either at 23 or 4°C. However, whereas *RGL2* transcript levels decline rapidly in wild-type seeds during germination, high levels of *RGL2* transcripts are maintained in the nongerminating

ga1-3 seeds during the same period of time. These observations are significant and have three important implications. First, the expression of *RGL2* is imbibition inducible. This induction may be a direct effect of imbibition, or it may simply be that the hydration of seeds sets in motion a developmental program, of which *RGL2* up-regulation is a part. Second, down-regulation of *RGL2* transcript levels is regulated via GA. Third, because loss-of-function *RGL2* alleles suppress the nongerminating phenotype of *ga1-3*, it is likely that the elevated levels of *RGL2* transcripts in *ga1-3* prevent seed germination.

Previous analyses of *GAI* and *RGA* function have led to the concept that these proteins operate as repressors of GA responses, and that their repressive activities are opposed by GA (the GA-derepressible repressor hypothesis; Peng et al. 1997, 1999a; Harberd et al. 1998; Dill et al. 2001; Fu et al. 2001; King et al. 2001; Richards et al. 2001). Here, we have shown that *RGL2* represses germination in the absence of GA (in *ga1-3* or in the presence of PAC), and that this repression is released by GA. Thus, *RGL2* operates as a GA-derepressible repressor of seed germination. RNA gel-blot analyses have indicated that GA treatments have little effect on the abundance of *GAI* and *RGA* transcripts (Silverstone et al. 1998). However, recent experiments have shown that GA treatments cause rapid disappearance of the *RGA* protein from the nuclei of plant cells. One interpretation of these observations is that GA counteracts the repressive effects of *RGA* by targeting the protein for destruction, rather than by altering the levels of transcripts that encode it (Dill et al. 2001; Silverstone et al. 2001). Although it is possible that GA also alters the stability of *RGL2*, we have shown here that GA causes a rapid decline in the levels of *RGL2* transcripts during seed germination. Thus, in the case of *RGL2*, GA counteracts *RGL2*-mediated repression of seed germination by down-regulating the abundance of *RGL2*-encoding transcripts. It is possible that there are several different mechanisms by which GA can counteract the action of the *GAI/RGA* family of GA-derepressible repressors.

Seed germination is complete when embryo growth overcomes the mechanical restraint imposed by the testa, and the radicle protrudes from the seed coat (Koornneef and Karssen 1994; Bewley 1997). Bioactive GAs promote seed germination by inducing the expression of genes encoding enzymes that hydrolyze the endosperm and loosen cell walls, thus facilitating the protrusion of the radicle (Bewley 1997). As discussed above, *RGL2* is an imbibition-inducible GA-repressible negative regulator of seed germination. However, the exact mechanism of *RGL2* function is still not clear. The *GAI/RGA/RHT/d8/SLR1* proteins are putative transcription factors (Ikeda et al. 2001; Richards et al. 2001). *RGL2* may therefore act as a negative regulator that represses the expression of genes encoding hydrolyzing enzymes, such that this repression can be released by GA. Alternatively, *RGL2* may act as negative regulator that prevents cell expansion by other means. We have shown, via *in situ* GUS staining, that *RGL2* is expressed early during germination, and especially in the pre-emergent

radicle. It is therefore reasonable to suggest that RGL2 functions as a restraint on radicle cell expansion, a restraint that can be counteracted by GA.

rgl2-1 strongly suppresses the nongerminating phenotype of *ga1-3*. However, seed germination can be rendered even more GA independent, because germination of the *gai spy-7 gar2-1* triple mutant is even more resistant to PAC than is *rgl2-1*. Thus, GA-response mediators additional to RGL2 are also involved in controlling seed germination. On the basis of sequence alignments, RGL3 (68% identity to RGL2) is a likely candidate. Previous reports have shown that, whereas the absence of GAI or RGA alone can partially suppresses GA-deficiency phenotypes (Peng et al. 1997; Silverstone et al. 1998), it is necessary to remove both GAI and RGA to achieve full suppression of the dwarfed stem phenotype of *ga1-3* (Dill and Sun 2001; King et al. 2001). RGL2 and RGL3 might be coupled in a similar way in the control of seed germination. Analysis of the effects of combined loss-of-function alleles of both *RGL2* and *RGL3* on *ga1-3* seed germination would allow us an improved understanding of the relative roles of RGL2 and RGL3.

The absence of an obvious role in controlling stem elongation for RGL2 is consistent with *RGL2* expression patterns in the adult plant. RNA gel-blot analysis and in situ GUS staining showed that *RGL2* is specifically expressed in the young inflorescence and is not detectable in expanding leaves and internodes. *GAI* and *RGA* are ubiquitously expressed in all of these tissues, and are known to play key roles in internode and leaf expansion. *RGL2*, *GAI*, and *RGA* are all highly expressed in inflorescences. Intriguingly, *ga1-3* mutants exhibit clear floral phenotypes, with retarded stamen and petal development, suggesting an important role for GA signaling in the regulation of floral organ growth. However, loss of *GAI* and *RGA*, either singly or in combination, does not suppress the floral organ phenotypes of *ga1-3* (Dill and Sun 2001; King et al. 2001). Loss of *RGL2* or *RGL1*, in a *ga1-3 rgl2-1* or *ga1-3 rgl1-1* mutant, also fails to suppress the *ga1-3* floral phenotype (J. Peng, unpubl.). These observations suggest either that there is substantial redundancy of function between RGL2, RGL1, GAI, and RGA in the regulation of floral organ growth, or that these processes are largely regulated by RGL3. Analysis of *ga1-3 gai-t6 rga-t2 rgl2-1* mutant plants in the future will enable us to understand more fully the relative roles of RGL2, GAI, and RGA in regulating the growth of stamens and petals.

The *Arabidopsis* genome contains five members (*GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3*) of the *GAI/RGA* gene family (Dill and Sun 2001). Genetic and physiological studies of mutant alleles of four of these genes (*RGL1*, *RGL2*, *GAI*, and *RGA*) have shown, perhaps not surprisingly, that they act, sometimes alone, sometimes collectively, to control different plant developmental processes. Figure 7 provides a summary of our current understanding of the relative roles of the proteins encoded by *RGL2*, *GAI*, and *RGA*. Signaling through RGL2 is the main pathway for the GA-mediated control of seed germination. Signaling through GAI/RGA is the main path-

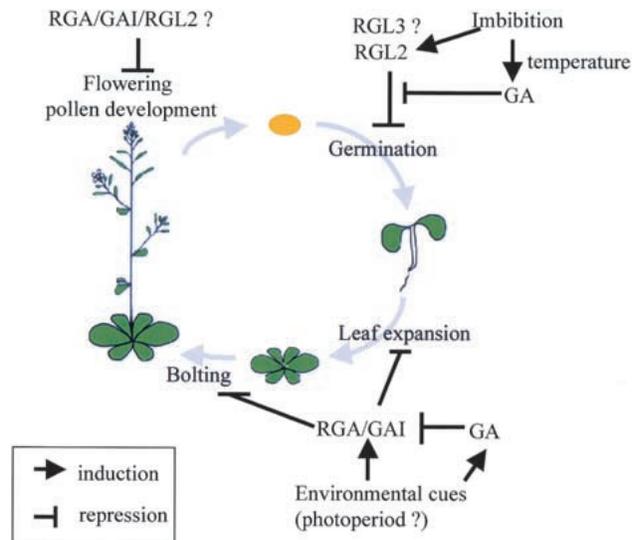


Figure 7. Model outlining the roles of GAI/RGA-like GA-signaling components in the plant life cycle. In this study, we have shown that RGL2 links an environmental cue (moisture) with the GA-signaling pathway during the regulation of seed germination. Sequence similarity suggests that RGL3 may also regulate seed germination in response to GA. Signaling through GAI and RGA mediates GA-promoted stem elongation and leaf expansion (Peng et al 1997, 1999a; Silverstone et al. 1998; Dill and Sun 2001; King et al. 2001; this work). Signaling through GAI, RGA, and RGL2 may mediate GA-promoted flower and pollen development (Dill and Sun 2001; S.C. Lee, H. Cheng, and J. Peng, unpubl.). The activities of GAI, RGA, and RGL1 may well be modulated in response to environmental variables at stages other than seed germination, and possible variables are shown.

way for GA-mediated control of stem elongation and leaf expansion. It is also possible that RGL2, GAI, and RGA are redundantly involved in the regulation of floral organ growth.

Plant growth hormones, such as GA, are often held to be endogenous factors that regulate plant growth and development in response to environmental change. How the GA-signaling system interacts with environmental signals is likely to be an important topic of research in the coming years. Recent observations have identified PHOR1, a possible link between photoperiod sensing and GA signaling in plants (Amador et al. 2001). Here, we have shown that, while absorbing water from the environment in the process of hydrating, *Arabidopsis* seeds accumulate relatively high levels of RGL2-encoding transcripts. RGL2 appears to act as an inhibitor of seed germination, and GA promotes germination by counteracting RGL2.

Materials and methods

Genetic nomenclature and plant materials

In this study, genotypes are written in italics; the wild-type genotype is in capitals (e.g., *RGL2*), and the mutant genotype is in lowercase letters (e.g., *rgl2-1*). The polypeptide products of genes are written in nonitalic capitals (e.g., RGL2).

All mutants described here were derived from Landsberg *erecta* (wild type). Single mutant (*gai-t6* and *gai-3*), double mutant (*gai-t6 gai-3*; *rga-24 gai-3*), and triple mutant (*gai spy-7 gar2-1*; *gai-t6 rga-24 gai-3*) lines were obtained as described previously (Peng et al. 1997; Silverstone et al. 1998; King et al. 2001). Five *Ds*-insertion lines (*rgl1-1*, *rgl2-1*, *rgl2-5*, *rgl2-12*, and *rga-t2*) were obtained from a previously described *Ds*-tagging population (Sundaresan et al. 1995; Parinov et al. 1999). During segregation analysis, a semidwarfing mutation was found to be closely linked (~2.3 cM; data not shown) to *rgl2-1* and *rgl2-5*. This semidwarfing mutation was separated from *rgl2-1* and *rgl2-5* via genetic recombination, and *rgl2-1*, *rgl2-5*, *rgl2-12*, *rgl1-1*, and *rga-t2* were then backcrossed twice with wild type to further purify the genetic background. These backcrossed lines were used for all experiments described in this work. Double mutants (*gai-3 rgl2-1*, *gai-3 rgl1-1*, and *gai-3 rga-t2*) were obtained from crosses between the relevant single mutant and *gai-3*. The triple mutant *gai-t6 rga-t2 gai-3* was obtained from a *gai-t6 gai-3* × *rga-t2 gai-3* cross.

For plants grown in soil, seeds were allowed to imbibe on water-moistened filter paper (in the case of *gai-3*, using GA₃ solution-moistened filter paper) at 4°C for 7 d (to break dormancy), and then planted on *Arabidopsis* mix (three parts Florobella potting compost/1 sand). The plants were then grown in a growth room (16 h light/8 h darkness photoperiod, 20–23°C), or in a greenhouse. For germination tests, seeds (stored more than 1 mo) were surface sterilized and sown on SM or GM medium supplemented (where appropriate) with GA₃ or PAC. The seeds were then chilled in a cold room for 7 d. Germination was recorded 8 d after the plates were moved to the above-mentioned growth room.

To confirm the exact locations of the *Ds*-insertions in *rgl1-1*, *rga-t2*, *rgl2-1*, *rgl2-5*, and *rgl2-12*, DNA was amplified from genomic DNA using primers flanking the *Ds* region, and the products were cloned in the pGEMTeasy (Promega) vector and sequenced. For *rga-t2*, primers 906F (5'-GCCGGAGCTATGA GAAAAGTGG-3') and DS3-2 (5'-CCGGTATATCCCGTT TCG-3') were used to amplify DNA adjacent to the 3' end of the *Ds*, and primers 2076R (5'-AAGAATTTTAAACAAGTGA ACG-3') and DS5-3 (5'-CGGTCGGTACGGGATTTTCC-3') were used to amplify sequences adjacent to the 5' end of *Ds*. Primers 906F and 2076R were derived from *RGA* sequence (Peng et al. 1997; Silverstone et al. 1998) and DS3-2 and DS5-3 were derived from *Ds* sequence (Parinov et al. 1999). The *Ds* insertion in *rga-t2* was located at 1521 nucleotides from the ATG start codon of *RGA*. For *rgl1-1*, primers DS3-2 and 2295R (5'-CCACAGAGCGCGTAGAGGATAAC-3') were used to amplify DNA adjacent to the 3' end of the *Ds*, and primers Ds5-P1 (5'-CATGGGCTGGGCCTCAGTG-3') and 1670F (5'-AAGC TAGCTCGAAACCCAAAT-3') were used to amplify DNA adjacent to the 5' end of *Ds*. Primers 2295R and 1670F were derived from *RGL1* sequence and Ds5-P1 is derived from *Ds* sequence. The *Ds* insertion in *rgl1-1* was located at 68 nucleotides upstream of the ATG start codon of *RGL1*. For *rgl2-1*, primers 856F (5'-GCTGGTGAACCGCTGGGAACA-3') and DS3-2 were used to amplify DNA fragments adjacent to the 3' end of *Ds*, whereas 1883R (5'-ACGCCGAGGTTGTGATGAGTG-3') and DS5-3 were used to amplify DNA fragments adjacent to the 5' end of *Ds*. For *rgl2-5*, primers 78F (5'-GTAACCAATCA CAACAAAGA-3') and DS3-2 were used to obtain the sequence adjacent to the 3' end of *Ds*, whereas 700R (5'-GCTGC TAGCTTCTCGTCAAAA-3') and DS5-3 were used to amplify DNA adjacent to the 5' end of *Ds*. For *rgl2-12*, primer 1355F (5'-TTCGAAACCCGACCC-3') and DS3-2 were used to obtain the sequence adjacent to the 3' end of *Ds*, whereas RGL2(FL)Rv (5'-TCAGGCGAGTTTCCACGCCGAGGTT-3') and DS5-3

were used to amplify DNA adjacent to the 5' end of *Ds*. Primer sequences 856F, 1883R, 78F, 700R, 1355F, and RGL2(FL)Rv were all derived from the *RGL2* sequence. For the *rgl2* mutant alleles, *Ds* was inserted at 1141, 28, and 1459 nucleotides from the ATG start codon of *RGL2* in *rgl2-1*, *rgl2-5*, and *rgl2-12*, respectively. The primers used to identify the *gai-3* mutation were as described (Silverstone et al. 1997).

RNA gel-blot hybridization, DNA sequencing

Total RNA was extracted from different tissues from 28-day-old plants using the Tri Reagent (Molecular Research Center) according to the manufacturer's protocols. RNA from seeds was extracted using the method of Vicent and Delseny (1999) and RNA from siliques was extracted following the method of Bekesiova et al. (1999). DNA-RNA hybridizations were carried out using procedures that are standard for the DIG system, as advised by the manufacturer (Roche). Gene-specific probes were used for the RNA gel-blot hybridizations as follows: *RGL2* (-165–142 nucleotides), *RGA* (-97–402 nucleotides), *GAI* (-150–219 nucleotides) probes were labeled by use of the PCR DIG probe synthesis kit (Roche). DNA sequences were determined by use of the Big Dye terminator cycle sequencing kit (Perkin Elmer).

Plant transformation and histochemical localization of β -glucuronidase (*GUS*) activity

An *NdeI* DNA fragment of 6.95 kb was obtained from the BAC clone T21P5 (*Arabidopsis* Biological Resource Center). This region encompasses the *RGL2* ORF, 3.3 Kb upstream sequence, and 2.0 kb downstream sequence. The DNA fragment was cloned into the binary vector pCAMBIA 1300 (CAMBIA) and transformed into *Agrobacterium* EHA105. The construct was transformed into *rgl2-1* mutant plants following the method of Clough and Bent (1998).

In situ GUS staining was performed using the method of Jefferson (1987). Whole plants were transferred to microfuge tubes or 15-mL falcon tubes and incubated at 37°C overnight in GUS-staining solution containing 100 mM Na Phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 1 mg/mL X-Gluc (Biosynth AG). Imbibed seeds were vacuum infiltrated together with GUS staining buffer for 2 d. The staining solution was then removed and replaced with several changes of 70% ethanol.

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