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The Arabidopsis *RGA* Gene Encodes a Transcriptional Regulator Repressing the Gibberellin Signal Transduction Pathway

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The recessive *rga* mutation is able to partially suppress phenotypic defects of the Arabidopsis gibberellin (GA) biosynthetic mutant *ga1-3*. Defects in stem elongation, flowering time, and leaf abaxial trichome initiation are suppressed by *rga*. This indicates that *RGA* is a negative regulator of the GA signal transduction pathway. We have identified 10 additional alleles of *rga* from a fast-neutron mutagenized *ga1-3* population and used them to isolate the *RGA* gene by genomic subtraction. Our data suggest that RGA may be functioning as a transcriptional regulator. *RGA* was found to be a member of the VHIID regulatory family, which includes the radial root organizing gene *SCARECROW* and another GA signal transduction repressor, *GAI*. RGA and GAI proteins share a high degree of homology, but their N termini are more divergent. The presence of several structural features, including homopolymeric serine and threonine residues, a putative nuclear localization signal, leucine heptad repeats, and an LXXLL motif, indicates that the RGA protein may be a transcriptional regulator that represses the GA response. In support of the putative nuclear localization signal, we demonstrated that a transiently expressed green fluorescent protein–RGA fusion protein is localized to the nucleus in onion epidermal cells. Because the *rga* mutation abolished the high level of expression of the GA biosynthetic gene *GA4* in the *ga1-3* mutant background, we conclude that *RGA* may also play a role in controlling GA biosynthesis.

INTRODUCTION

Gibberellins (GAs) comprise a large family of diterpenoid compounds. Some of these are bioactive plant hormones controlling diverse growth and developmental processes, including seed germination, stem elongation, and flower development (Davies, 1995). Despite its complexity, the GA biosynthetic pathway has been well characterized by using biochemical techniques as well as studying mutants defective in biosynthesis. (GA biosynthesis is reviewed in Hedden and Kamiya [1997].) In contrast, much less is known about how plants perceive GA and how the signal is transduced to control GA-regulated gene expression during plant growth and development. Biochemical studies using barley aleurone cells have demonstrated that GA is perceived on the external face of the plasma membrane (Hooley et al., 1991; Gilroy and Jones, 1994). However, the GA receptor has not yet been identified.

Genetic approaches have been successful in identifying GA signal transduction mutants from a variety of species (reviewed in Hooley, 1994; Ross, 1994; Swain and Olszewski, 1996; Ross et al., 1997). GA response mutants fall into two phenotypic categories: elongated slender mutants and GA-unresponsive dwarf mutants. The recessive slender mutants behave as though their GA response pathway is constitutively activated; they can be further subdivided into GA-responsive and GA-unresponsive mutants. In contrast, the GA-unresponsive dwarfs are semidominant mutants whose phenotype resembles GA-deficient biosynthetic mutants. However, their dwarf phenotype cannot be rescued by exogenous GA treatment. Therefore, these mutants appear to be impaired in GA perception or signal transduction. Unfortunately, most of these mutants are of species not amenable to facile map-based cloning and genetic manipulation. In Arabidopsis, the GA-responsive recessive slender mutant *spindly* (*spy*; Jacobsen and Olszewski, 1993; Jacobsen et al., 1996; Silverstone et al., 1997b) and semidominant semidwarf mutant *gai*, whose stem growth is unresponsive to exogenous GA treatment (Koornneef et al., 1985; Peng and Harberd, 1993; Wilson and Somerville, 1995), have been characterized in detail. Because *spy* alleles are recessive, the *SPY* locus has been postulated to encode a negative regulator of GA response (Jacobsen and Olszewski, 1993). On the other hand, because *gai* is semidominant and the loss-of-function intragenic *gai* suppressors confer a wild-type phenotype, *GAI* was originally thought to be a redundant activator of the GA response pathway (Peng and Harberd, 1993). However, further characterization of a null *gai* mutant (*gai-t6*), using paclobutrazol, an inhibitor of GA biosynthetic

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enzymes, demonstrates that this mutant is more resistant to paclobutrazol than is the wild type (Peng et al., 1997). This result indicates that *GAI* may in fact also be a negative regulator of the GA response. Because *spy* is epistatic to *gai*, it was proposed that *spy* is downstream of *gai* on the GA signal transduction pathway (Jacobsen et al., 1996).

Recently, we identified a new Arabidopsis locus, *RGA* (for repressor of the *ga1-3* mutant), involved in GA response (Silverstone et al., 1997b). Mutant alleles at this locus were isolated as recessive suppressors of the GA biosynthetic mutant *ga1-3*, which is a nongerminating, male-sterile, extreme dwarf blocked in the first committed step of GA biosynthesis (Koornneef and Van der Veen, 1980; Sun and Kamiya, 1994). Mutations at the *RGA* locus partially suppress certain aspects of the GA-deficient phenotype of the *ga1-3* mutant, including the defects in stem elongation, flowering time, and leaf abaxial trichome initiation (Silverstone et al., 1997b). These results suggest that the wild-type RGA protein may function as a negative regulator of the response to GA. *spy*, on the other hand, is able to partially suppress all aspects of the *ga1* mutant (Jacobsen and Olszewski, 1993; Silverstone et al., 1997b). We recently proposed that the *RGA* and *SPY* loci may control separate branches on the GA signal transduction pathway based on epistatic analyses showing that the *rga* and *spy* mutations have an additive effect in the *ga1-3* background (Silverstone et al., 1997b). Subsequently, a fourth locus, *PICKLE* (*PKL*), that may be involved in a more specific set of GA responses, was identified based on characterization of the *pkl* mutation that affects GA-induced differentiation of the seedling primary root (Ogas et al., 1997).

Although *SPY* and *GAI* have been cloned, their exact functions are not well understood (Jacobsen et al., 1996; Peng et al., 1997). SPY shows sequence similarity to Ser (Thr)–*O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferases, which play an important role in regulating the activities (via glycosylation) of various nuclear and cytosolic proteins (Kreppel et al., 1997; Lubas et al., 1997). The *GAI* gene encodes a member of the VHIID regulatory protein family and has structural features indicative of a transcriptional regulator (Peng et al., 1997).

To gain more insight into the function of the RGA protein in the GA response, we cloned the *RGA* locus by genomic subtraction. An additional 10 *rga* alleles, *rga-18* through *rga-27*, were isolated from the M_2 generation of a population of *ga1-3* plants mutagenized by using fast-neutron (FN) bombardment. Four arbitrarily chosen FN alleles were analyzed by genomic subtraction, and a DNA fragment deleted in *rga-20* was identified. DNA sequence analyses of the *RGA* gene indicated that RGA is also a member of the newly identified VHIID family of plant regulatory proteins (Di Laurenzio et al., 1996). RNA expression studies showed that the *RGA* gene is ubiquitously expressed in different tissues and may also play a role in regulating GA biosynthesis. Nuclear localization of RGA was illustrated by the location of a green fluorescent protein (GFP)–RGA fusion protein in a transient expression system. The *rga* mutant was identified in the wild-type *GA1* background, and it does not have a dramatic phenotype.

RESULTS

Cloning of the *RGA* Locus by Genomic Subtraction

Previously, we had isolated 17 independent *rga*/*ga1-3* mutants from ethyl methanesulfonate–mutagenized *ga1-3* seeds (Silverstone et al., 1997b). Our initial mapping of the *RGA* gene indicated that it was far from any known marker (Silverstone et al., 1997b), precluding the use of map-based cloning. To use the genomic subtraction technique (Sun et al., 1992a) in cloning the *RGA* gene, we isolated an additional 10 mutant alleles of *rga* (*rga-18* through *rga-27*) from an FN-mutagenized population of *ga1-3* mutants. FN bombardment of seeds generates DNA rearrangements and large deletions (Koornneef et al., 1982; Shirley et al., 1992; Sun et al., 1992a; Bruggemann et al., 1996; Cutler et al., 1996). Because there had been no quantitative measure of the frequency with which FN causes large deletions in Arabidopsis, we chose four of our FN-induced *rga* alleles (*rga-18* through *rga-21*) randomly and analyzed them by using genomic subtraction. The four alleles were examined by subjecting *ga1-3* DNA to five rounds of subtraction with biotinylated genomic DNA from the respective *rga*/*ga1-3* mutant. Afterward, the remaining DNA was amplified and cloned into the pBluescript SK+ plasmid. Individual clones were analyzed for a deletion in the *rga*/*ga1-3* mutant by DNA gel blot analyses.

We identified a 450-bp DNA fragment (in pRG1) that was deleted in *rga-20* but present in the other three alleles analyzed by genomic subtraction. This fragment is also present in *ga1-3* and Landsberg *erecta* (L*er;* Figure 1A). The insert in pRG1 was used as a hybridization probe to isolate overlapping genomic clones pRG2 and pRG3 from a pOCA18 genomic library (Olszewski et al., 1988) (Figure 2). The genomic DNA corresponding to the inserts in pRG2 and pRG3 was completely deleted in *rga-20* (Figures 1 and 2). A DNA gel blot containing HindIII-digested genomic DNA isolated from nine FN *rga*/*ga1-3* alleles was hybridized with a DNA probe containing the 2.5-kb left distal end of the insert DNA in pRG3. Figure 1B shows that two additional alleles, *rga-24* and *rga-26*, also had at least 3-kb deletions (1- and 2-kb HindIII fragments) in this region. We did not obtain any genomic clones from the pOCA18 library that extended beyond the left distal end of the insert in pRG3, probably because the library used was amplified from a fraction of the original library.

We then screened for additional genomic clones from a λGEM-11 ecotype Columbia (Col-0) Arabidopsis genomic DNA library. A 2-kb HindIII fragment that was cloned from the left end of pRG3 was used to identify three additional

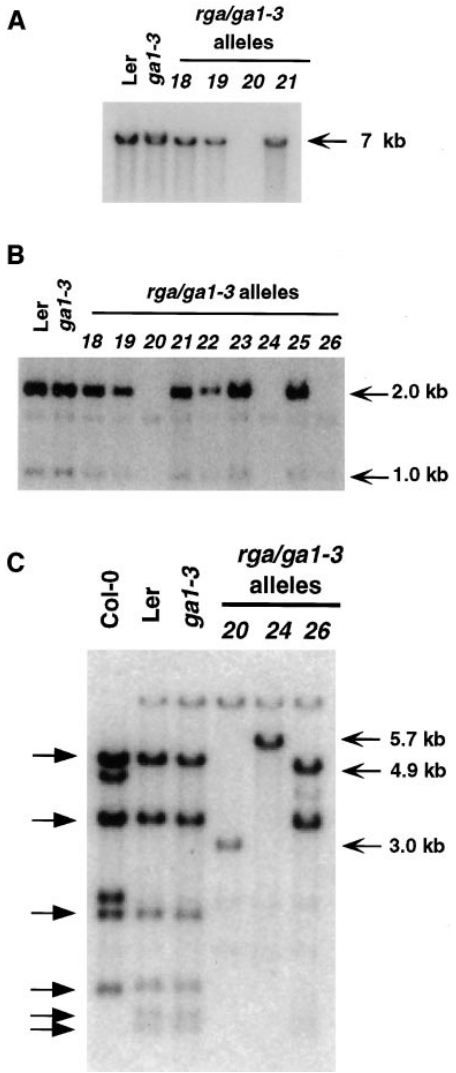

Figure 1. Detection of Deletions in FN-Generated *rga* Alleles.

Shown is autoradiography of DNA gel blots containing HindIII-digested genomic DNA isolated from Col-0, L*er*, *ga1-3*, and FN-generated *rga/ga1-3* mutants. The radiolabeled probes are as indicated.
(A) The 450-bp Sau3A fragment from pRG1.
(B) The 6-kb BamHI-KpnI DNA fragment from pRG3, which includes the 2.5-kb left distal end of the insert DNA.
(C) The AvrII DNA fragment (15 kb) that contains the entire insert DNA of the λRG2 clone.
The arrows at right in **(A)** and **(B)** indicate HindIII fragments near the *RGA* locus. The arrows at left in **(C)** indicate HindIII fragments (5, 3.5, 2, 1.4, 1.2, and 1.0 kb, top to bottom) that are absent in the *rga/ga1-3* deletion mutants. The new HindIII fragments present in the deletion mutants are indicated by the arrows at right.

genomic clones: λRG1, λRG2, and λRG3 (Figure 2). These clones were used to map the deleted regions in *rga-24* and *rga-26* as well as the left junction of the deletion in *rga-20* by using DNA gel blot analyses (Figures 1C and 2). *rga-20* has at least a 33-kb deletion, *rga-24* an 8.4-kb deletion, and *rga-26* a 5.9-kb deletion. The 2-kb HindIII DNA fragment, which is completely missing in all three *rga* deletion alleles, was then used as a hybridization probe to identify a putative *RGA* transcript by RNA gel blot analysis. This putative *RGA* mRNA is 2.4 kb and is present in *ga1-3* but absent in *rga-20*, *rga-24*, and *rga-26* (data not shown). Subsequently, we isolated three cDNA clones by screening the λPRL2 Arabidopsis cDNA library with the 2-kb HindIII fragment. The largest clone (pRG20) carries a 2.3-kb DNA insert containing an open reading frame of 1921 bp that encodes a 587–amino acid protein with a 64-kD predicted molecular mass and is likely to be a full-length cDNA because there is a stop codon three nucleotides upstream of the ATG start site.

DNA sequence analysis of the genomic DNA revealed that the *RGA* locus has an uninterrupted 1921-bp open reading frame with no introns. To prove that the cloned pRG20 corresponds to the *RGA* locus, we characterized the molecular

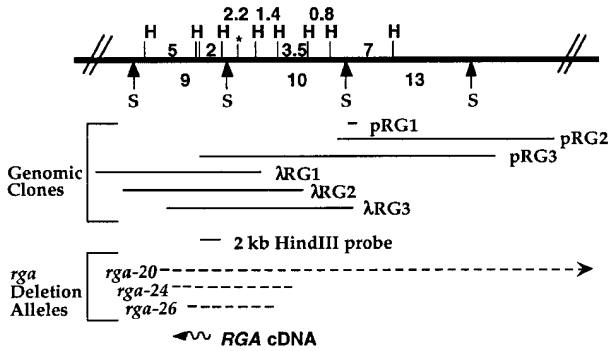

Figure 2. Physical Map around the *RGA* Locus.

The heavy horizontal line shows a SalI (S) and HindIII (H) restriction map around the *RGA* locus. The asterisk indicates a HindIII site that is only present in L*er* but not in Col-0. Distances between restriction sites are indicated in kilobases. The thin horizontal lines labeled Genomic Clones indicate where the original deleted fragment, pRG1, isolated by genomic subtraction, maps in relation to the five overlapping genomic clones. The dashed lines indicate the deleted regions in the three *rga* deletion alleles. The right distal end of the deletion in *rga-20* has not been identified because it is beyond the right distal end of this map. The locations of the left junction of the deletion in *rga-20* and both ends of deletions in *rga-24* are within the HindIII fragment indicated on the map. However, the exact end points have not been determined. The wavy line depicts the coding region of the *RGA* locus. The 2-kb HindIII fragment is located within the deleted regions in all three *rga* deletion alleles and has been used as a hybridization probe in DNA and RNA blot analyses, as described in the text.

lesions in five of the *rga* alleles. DNA gel blot analysis using the radiolabeled 2.3-kb RGA cDNA as a probe indicated that the entire coding region for the *RGA* gene is deleted in *rga-20* and *rga-24* (data not shown). By using DNA sequence analysis, we found that 4.2 kb upstream of the ATG start site and 1.7 kb of the coding region of the *RGA* gene had been deleted in *rga-26* (data not shown). Besides the three FN alleles with large deletions, we also identified single nucleotide changes in two ethyl methanesulfonate alleles. In *rga-1*, the third base in the codon for Trp-521 (TGG) is mutated from G to A, creating a stop codon (TGA) mutation that resulted in a C-terminal truncation; in *rga-2*, there is a missense mutation formed when the first base in the codon for Asp-478 (GAT) is mutated from G to A, which resulted in Asn-478 (AAT) (Figure 3). These results confirm that we have cloned the *RGA* gene.

RGA Is a Member of the VHIID Protein Family

There are several interesting regions in the predicted RGA protein sequence. RGA contains homopolymeric regions of serine and threonine at the N terminus and leucine heptad repeats (Figure 3). RGA also has, beginning at Leu-423, an LHKLL motif, which is identical to the consensus sequence LXXLL (where X stands for amino acid) that was recently demonstrated to mediate the binding of steroid receptor coactivator complexes to nuclear receptors (Heery et al., 1997; Torchia et al., 1997). PSORT analysis (Nakai and Kanehisa, 1992; http://psort.nibb.ac.jp/) indicated a high likelihood of nuclear localization of the RGA protein, and it identified a putative bipartite nuclear localization signal (NLS) beginning at Arg-258. The sequence RKVATYFAELARRIYR fits well with the consensus of bipartite NLSs (Raikhel, 1992). Amino acid sequence comparison between the RGA sequence and those in the database indicated that RGA is a member of the VHIID family of regulatory proteins. *RGA* has some homology to *SCR*, which regulates cellular differentiation in Arabidopsis roots (Di Laurenzio et al., 1996). In their conserved regions, amino acids 176 to 580 in RGA and 245 to 649 in SCR are 38% identical and 44% similar.

While preparing this article, we found that *RGA* was also cloned recently by two other groups. In their search for proteins regulating nitrogen metabolism, Truong et al. (1997) identified two homologous Arabidopsis cDNAs that would complement the yeast *gln3 gdh1* strain, which is affected in the regulation of nitrogen metabolism. They named the cDNAs *RGA1* (GenBank accession number Y11336) and *RGA2* (GenBank accession number Y11337), for restoration of growth on ammonia, and characterized them as VHIID protein family members. By a particularly ironic twist of fate, *RGA1* is identical to *RGA*. Also, Peng et al. (1997) recently cloned the GA signal transduction mutant gene *GAI*. In the course of their study, they also cloned a homologous gene, which they termed *GRS* (for *GAI*-related sequence). However, they only present the sequence data of this gene. After receiving the preprint of the paper by Peng et al. (1997) from N. Harberd, we found that *GAI* is identical to *RGA2* and *GRS* is identical to *RGA* (*RGA1*). Although Truong et al. (1997) used a heterologous system to identify *RGA1* and Peng et al. (1997) only report a *GAI* homologous sequence, these reports did not illustrate the function of RGA in plants. However, we have cloned the *RGA* locus based on its mutant phenotype and have demonstrated clearly *RGA*'s important role in mediating GA signal transduction.

Two other full-length members of the VHIID family in Arabidopsis have recently been identified in contigs at the top of chromosome 4 sequenced by the European Union Arabidopsis Genome Project. The first in contig ATFCA8 (GenBank accession number Z97343) is located at nucleotides 26,164 to 28,937. The second is located in contig ATAP22 (GenBank accession number Z99708) at nucleotides 62,096 to 63,475. Because the deduced amino acid sequences of these two proteins have similar degrees of homology to the first three VHIID proteins, we named the former VHS4 and the latter VHS5 (for VHIID homologous sequence). RGA shows 41% identity and 52% similarity with VHS4 versus 24% identity and 33% similarity with VHS5.

The alignment between RGA, GAI, SCR, VHS4, and VHS5 sequences shown in Figure 3 demonstrates that they all contain the central VHIID conserved region. By comparing RGA with the rest of the VHIID family members, we found two additional conserved motifs besides the VHIID domain (Figures 3 and 4). We have named the one located at the C terminus the RVER domain for the presence of this conserved set of amino acids. At the N terminus, there is the acidic DELLA domain, which is present only in RGA and GAI. Besides these fully sequenced genes, there are a number of partially sequenced expressed sequence tags (ESTs) from various plant species as well as a sequence-tagged site from maize that show homology to RGA and appear to be in the VHIID family. Alignments of their DELLA, VHIID, and RVER domains with those of the completely sequenced proteins are shown in Figures 4A to 4C.

Nuclear Localization of the RGA Protein

Because the predicted RGA amino acid sequence has features that are found in transcription regulators, including a putative NLS, we constructed a cauliflower mosaic virus (CaMV) 35S promoter::*GFP–RGA* gene fusion that could be used in transient assays (Varagona et al., 1992; Haseloff et al., 1997). After biolistic bombardment of an onion epidermal layer with a CaMV 35S::GFP control construct or this reporter construct, the GFP signal from the control was observed in 110 cells to be always in both the cytoplasm and nucleus (Figures 5A and 5B), where it has been shown to accumulate (Haseloff et al., 1997). In contrast, the GFP–RGA fusion protein is located exclusively in the nucleus in 89 cells examined (Figures 5C and 5D), indicating that the RGA sequence targets the fusion protein to the nucleus.

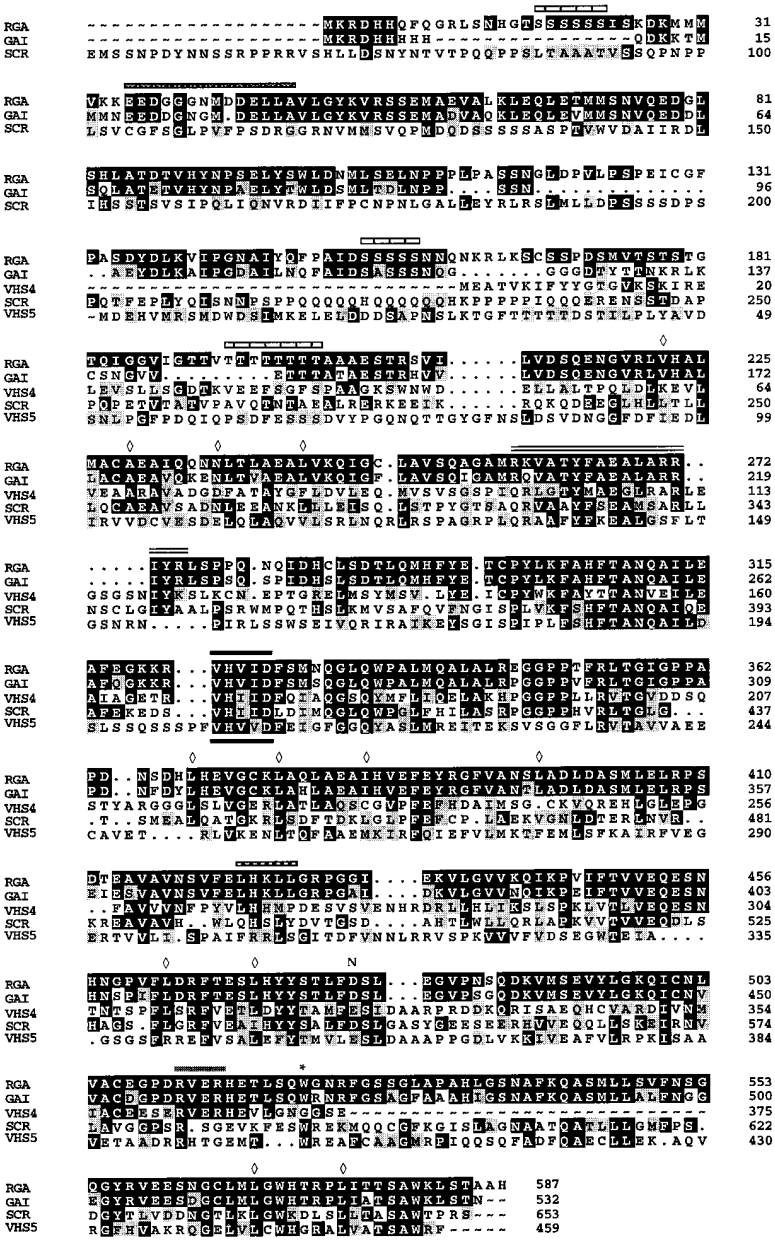

Figure 3. Amino Acid Sequence Alignment of the RGA Protein Compared with Other Members of the VHIID Protein Family.

RGA sequence (L*er* allele) is compared with GAI (Peng et al., 1997; Truong et al., 1997), SCR (Di Laurenzio et al., 1996), VHS4 (GenBank accession number Z97343), and VHS5 (GenBank accession number Z99708). Identical residues conserved between RGA and at least one other family member are displayed in reverse type, and similar residues are in gray boxes. Gaps introduced to improve the alignment are indicated by dots and sequence truncations by wavy dashes. The mutation in *rga-1*, marked above the RGA sequence with an asterisk, changes Trp-520 to a stop codon, and the mutation in *rga-2*, indicated with an N, converts Asp-478 to Asn-478. The acidic DELLA motif is marked by an overhead stippled bar. The conserved VHIID sequence is indicated by thick solid lines above and below the sequences. The RVER motif is marked by an overhead gray bar. The homopolymeric Ser/Thr stretches are indicated by overhead bars with thin stripes. The Leu heptad repeat residues are marked with an open diamond. The putative NLS is indicated by a double line, and the LXXLL motif by a striped bar. The sequence alignment was done using the Pileup program. The boxes were drawn using the BOXSHADE web site (http://ulrec3.unil.ch/software/BOX_form.html).

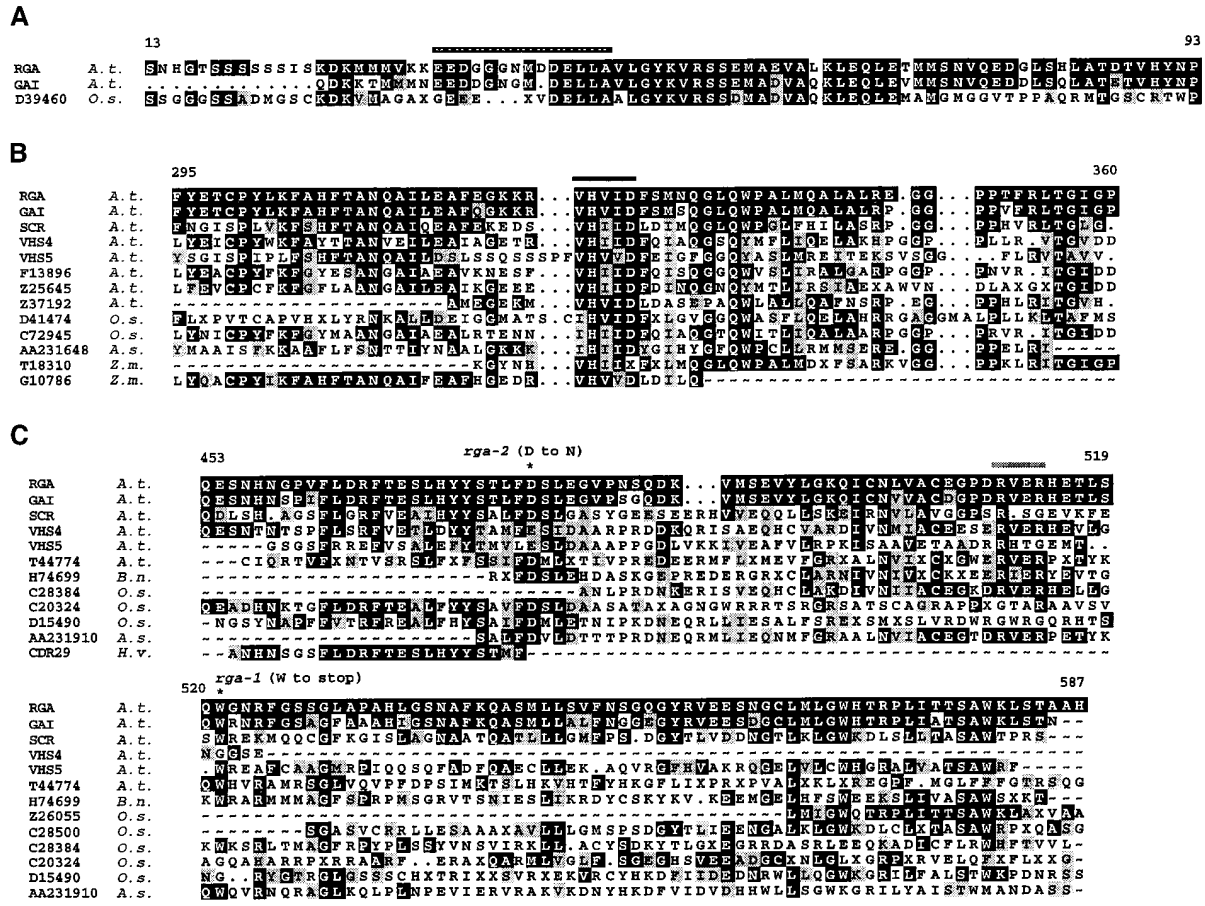

Figure 4. Three Conserved Domains Revealed by Sequence Alignment between RGA, Other Cloned Genes, and ESTs.

Residues conserved between RGA and at least one other family member are displayed in reverse type for identical residues and in gray boxes for similar residues. Gaps introduced to improve the alignment are indicated by dots, and sequence truncations are depicted by wavy dashes. All short sequences are ESTs except for one maize sequence, which is from a sequence-tagged site (G10786). They are labeled according to their GenBank accession numbers. The sequence alignment was done using the Pileup program. The boxes were drawn using the BOXSHADE web site (http://ulrec3.unil.ch/software/BOX_form.html).

(A) The N-terminal DELLA domain.

(B) The central VHIID domain.

(C) The C-terminal RVER domain.

The three motifs are indicated as given in Figure 3. The point mutations in the *rga-1* and *rga-2* mutant alleles are marked above the sequence. *A. t.*, *Arabidopsis thaliana*; *A. s.*, *Avena sativa*; *B. n.*, *Brassica napus*; *H. v.*, *Hordeum vulgare*; *O. s.*, *Oryza sativa*; *Z. m.*, *Zea mays.*

Map Position of the *RGA* Locus

The *RGA* cDNA and genomic DNA clones came from Col-0 libraries. Because our *rga*/*ga1-3* mutants were all in the L*er* background, we also determined the DNA sequence of the L*er* wild-type allele of *RGA* when we searched for the point mutations in *rga-1* and *rga-2.* Four single-nucleotide polymorphisms were found between the wild-type L*er* and Col-0 alleles of *RGA.* Three are silent changes, but one causes an alteration of the final amino acid residue of the RGA protein (His-587 in L*er* and Tyr-587 in Col-0). This last polymorphism also resulted in the presence of an RsaI site in Col-0 that is absent in L*er.* Although we have previously published a weak linkage for *RGA* at the bottom of chromosome 3 (Silverstone et al., 1997b), we were unable to find any markers that were closely linked to confirm the observation. The RsaI polymorphism between the L*er* and Col-0 alleles allowed us to design a cleaved amplified polymorphic sequence marker that would distinguish between the two ecotypes for mapping by means of the recombinant inbred lines (Lister and Dean, 1993). Using this approach, we found that the *RGA* locus maps very close to the top of chromo-

some 2. This agrees well with the results from Peng et al. (1997), who indicated (as unpublished data) that they had mapped *GRS* to the top of chromosome 2, and Truong et al. (1997), who mapped *RGA1* to three yeast artificial chromosomes that map to the top of chromosome 2.

Identification of the *rga*/*GA1* Mutant

Previously, we had surmised that *rga*/*GA1* plants must have a subtle phenotype because we could not identify them in the F_2 generation of a cross between *rga-2*/*ga1-3* and L*er* (Silverstone et al., 1997b). We subsequently identified *rga-2*/*GA1*, as described in Methods. These plants were a little paler than wild-type L*er* plants, but they did not have any dramatic phenotype, and they were similar to L*er* with respect to final height, flowering time, and fertility under long-day conditions (data not shown).

Ubiquitous Expression Pattern of *RGA*

To determine whether the regulation of *RGA* gene expression was involved in controlling GA-mediated growth, we measured the levels of the *RGA* mRNA in a number of tissues, including seedlings, roots, rosette leaves, whole rosette plants, bolting stems, mature stems, flower buds, young siliques, and mature siliques (Figures 6 and 7A). We found that *RGA* was expressed ubiquitously in all tissues examined. Quantitative analyses using cyclophilin as a loading control (Lippuner et al., 1994) indicated that the levels of *RGA* mRNA between tissues did not differ greatly (Figure 6).

Previously, we proposed a model of GA signal transduction that consisted of two branches that converge to regulate several common developmental processes, including stem elongation, flowering time, and leaf abaxial trichome initiation (Silverstone et al., 1997b). Our hypothesis was that the plant could achieve finer control over these events by manipulating the signal flowing through the two pathways.

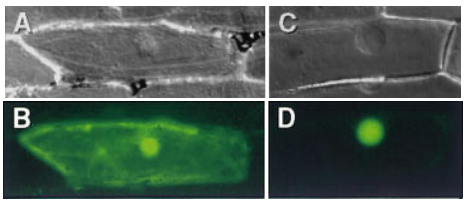

Figure 5. Nuclear Localization of the GFP–RGA protein.

(A) and **(B)** The control GFP protein.
(C) and **(D)** The GFP–RGA fusion protein.
The proteins are transiently expressed in onion epidermal cells. Individual cells are seen in a differential interference contrast image (**[A]** and **[C]**) and a corresponding epifluorescence image (**[B]** and **[D]**), respectively.

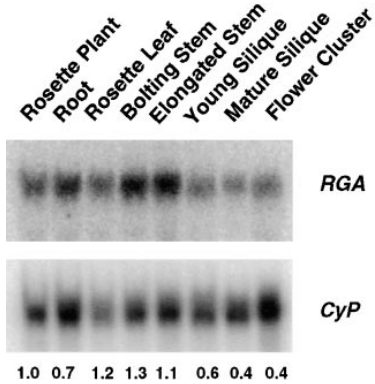

Figure 6. Expression Pattern of the *RGA* Gene.

Shown is autoradiography of an RNA blot containing 10 μg of total RNA isolated from different tissues, as labeled. Rosette plants are the 2-week-old aerial portion of the plant, roots are from tissue culture, rosette leaves are from 3.5- to 4-week-old plants, bolting stems are from 2-cm-tall plants (~3 weeks old), elongated stems are from the bottom internode of 3.5- to 4-week-old plants, young siliques were 5 to 7 mm long, mature siliques had fully developed seeds before desiccation, and flower clusters had the terminal inflorescence with developing buds and open flowers. The blot was hybridized with radiolabeled *RGA* cDNA and then reprobed with radiolabeled cyclophilin (*CyP*) as a loading control. The numbers below the blot indicate the relative amount of *RGA* mRNA after standardization, using cyclophilin as a loading control. The level of *RGA* mRNA in the rosette plant was arbitrarily set as 1.0.

Therefore, if one of the branches were constitutively activated, as in the *spy* or *rga* mutant, then the other branch could be inhibited to compensate. This inhibition could occur by altering gene expression of GA response components and/or by modifying their protein activities.

We compared *RGA* expression in wild-type L*er* seedlings to seedlings in a variety of mutant backgrounds, including the GA biosynthetic mutants *ga1-6* (leaky) and *ga1-3* (null) and the signal transduction mutants *rga*, *spy*, and *gai*, both in the wild-type *GA1* background and mutant *ga1-3* background (Figure 7A). Seedlings of these different plant lines grown for 10 days in Murashige and Skoog (MS; Murashige and Skoog, 1962) medium alone were compared with those grown in MS medium containing 1 μM GA_3. Changes in *RGA* expression were quantified by using cyclophilin as a loading control (Figure 7A). Expression of *RGA* was slightly lower in the GA biosynthetic mutants *ga1-3* and *ga1-6* than in wild-type L*er* in the absence of exogenous GA. Except in the *gai* mutant background, there was a slight increase (less than twofold) in *RGA* mRNA levels in all other genetic backgrounds in response to GA application.

Because RGA2 shares 82% identity and 85% similarity with RGA, we thought that *RGA2* may also be involved in GA response. We also examined the *RGA2* gene expression pattern in different GA biosynthetic or signal transduction

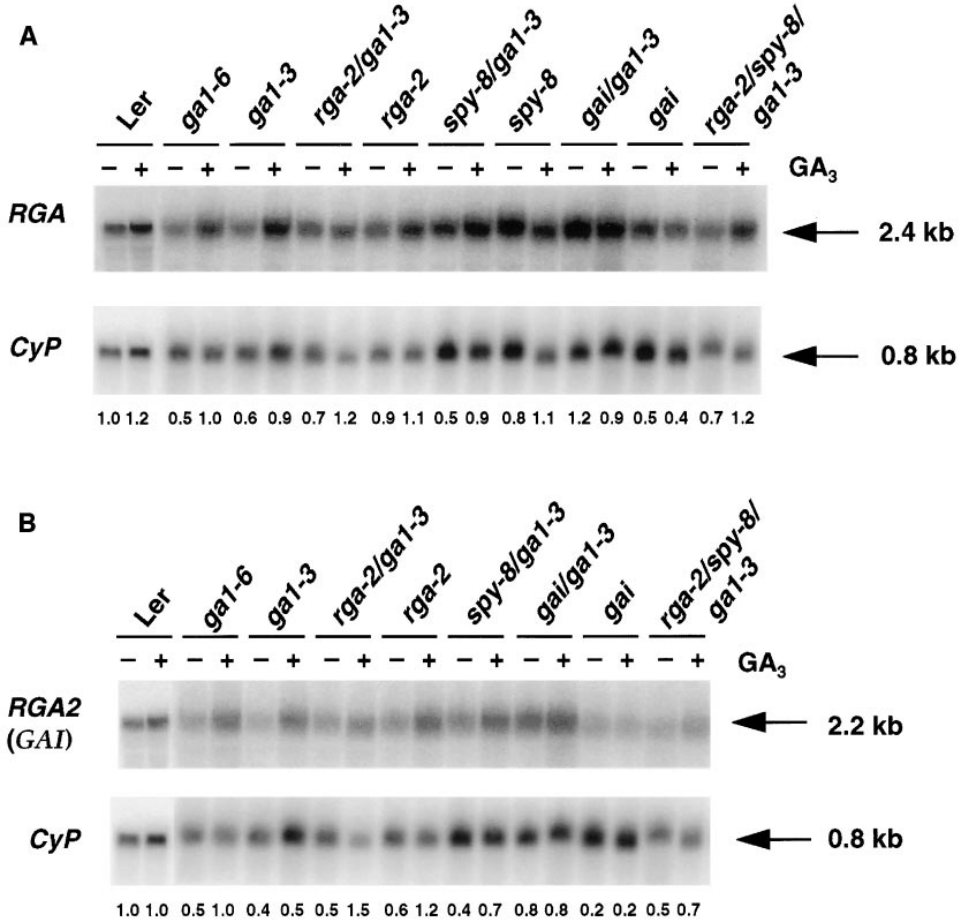

Figure 7. Expression of *RGA* and *RGA2* (*GAI*) in the Wild Type, GA Biosynthetic Mutants, and GA Response Mutants.

Shown is autoradiography of RNA blots containing 10 μg of total RNA isolated from wild-type L*er* and various GA biosynthetic and signal transduction mutants, as labeled. RNA samples were isolated from seedlings grown in media with (+) or without (−) 1 μM GA_3. The arrows at right in **(A)** and **(B)** indicate the sizes of the transcripts.

(A) Blot hybridized with the radiolabeled 2.3-kb *RGA* cDNA and reprobed with cyclophilin (*CyP*). The numbers below each lane indicate the relative amounts of *RGA* mRNA after standardization, using *CyP* as a loading control. The value of L*er* (−GA) was arbitrarily set as 1.0.

(B) Blot probed with a radiolabeled 0.65-kb *RGA2*/*GAI* DNA fragment and reprobed with *CyP.* The relative amount of *RGA2* mRNA is given below each lane, and the value of L*er* (−GA) was set as 1.0.

mutant backgrounds. The pattern of expression we observed for *RGA2* was similar to that of *RGA* (Figure 7B). The difference between expression of the two genes was in the L*er* background, where no increase is seen in *RGA2* expression in response to GA treatment. Now that *RGA2* is known to be *GAI*, the similar expression patterns of these two GA response genes is particularly interesting.

Regulation of GA Biosynthesis

Several of the GA biosynthetic genes have been shown to be under feedback control by GA action, including the GA 20-oxidase genes (Phillips et al., 1995; Xu et al., 1995) and *GA4* that encodes the 3β-hydroxylase, which catalyzes the production of bioactive GAs (Chiang et al., 1995). In the *ga1-3* mutant, which has very low levels of GAs, expression of these genes is elevated, whereas expression in both the *ga1-3* mutant and wild-type plants can be inhibited by GA application. The *gai* mutant is a semidwarf plant blocked in GA signaling, yet it accumulates high levels of GAs (Koornneef et al., 1985). Although GA biosynthesis is upregulated, the *gai* mutant is not able to respond to the increased GA levels. Thus, GA activity has been proposed to modulate GA biosynthesis through feedback inhibition. To determine whether *RGA* is involved in the regulation of GA biosynthesis, we examined

GA4 expression in the different GA biosynthetic and signal transduction mutant backgrounds (Figure 8). *GA4* mRNA level was elevated in the *ga1-3* and *spy*/*ga1-3* mutants only, but not in the *rga*/*ga1-3* mutant. In both *ga1-3* and *spy*/*ga1-3*, the induction of *GA4* expression was inhibited by the application of GA.

DISCUSSION

We have cloned the *RGA* locus by using genomic subtraction. Although the *RGA* sequence is identical to two recently reported genes (Peng et al., 1997; Truong et al., 1997), this study goes beyond these two reports by demonstrating its biological role as a repressor of GA signal transduction. The RGA protein belongs to the VHIID family of regulatory proteins, whose members include SCR and GAI. All three proteins have features indicating that they are transcriptional regulators, and we further showed that the GFP–RGA fusion protein is localized in the nucleus of onion cells in a transient assay (Figure 5). RGA and GAI share a high degree of homology, and both proteins have been suggested to function in GA signal transduction. Based on our analysis of *rga* and *gai* mutant phenotypes (see below) and comparison of the RGA and GAI sequences, we postulate that the two proteins may have overlapping, but not completely redundant, functions in controlling the GA response pathway.

Genomic Subtraction Technique

We have previously shown the utility of the genomic subtraction technique to clone an Arabidopsis gene (Sun et al., 1992a) by using an FN-induced mutant that was likely to contain a large deletion based on genetic fine-structure mapping (Koornneef et al., 1983). In this study, we have demonstrated that the genomic subtraction technique can be used effectively to isolate genes using FN-induced mutant alleles without prior genetic evidence that one or more alleles carry deletions. We found that 33% (three of nine) of our FN *rga* alleles had large deletions affecting the *RGA* gene. In a recent study using FN mutagenesis to identify *hy4* mutants, Bruggemann et al. (1996) found that 15 of 20 mutants contained large (at least 5 kb) deletions. Although the deletion frequency is locus dependent, with a sufficient number of FN alleles (at least four or five), time-consuming fine-structure genetic mapping is not necessary to identify alleles with large deletions before genomic subtraction is performed.

Coding regions in the Arabidopsis genome are very densely organized, with one gene on average every 5 kb (Goodman et al., 1995). It is interesting that the *rga-20*/*ga1-3* mutant, which has a deletion of at least 33 kb, differs from the other *rga*/*ga1-3* mutants only in its reduced germination rate, even in the presence of GA. This suggests that no other major genes are likely to be present in this region.

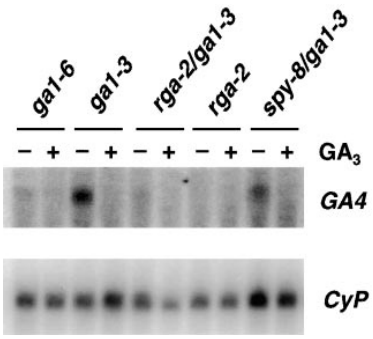

Figure 8. Expression of the *GA4* Gene in GA Biosynthetic and Response Mutant Backgrounds.

Shown is autoradiography of RNA blots containing 10 μg of total RNA isolated from various GA biosynthetic and signal transduction mutants, as labeled. RNA samples were isolated from seedlings grown in media with (+) or without (−) 1 μM GA_3. The blot was probed with the radiolabeled 1.4-kb *GA4* cDNA and then reprobed with cyclophilin (*CyP*) as a loading control.

Characterization and Function of the VHIID Family of Regulatory Proteins

Three proteins identified by studies of mutants (RGA, GAI, and SCR) are members of the VHIID family defined by Di Laurenzio et al. (1996). Using the RGA amino acid sequence to search the database with the BLAST program (Altschul et al., 1990), we have identified two additional completely sequenced Arabidopsis genes from the genome project (VHS4 and VHS5), a number of ESTs from Arabidopsis, and ESTs from rice, oat, oilseed rape, and maize with sequence similarity. Three regions of conserved sequence, including an acidic N-terminal DELLA domain, a middle VHIID domain, and a C-terminal RVER domain, have been identified (Figures 4A to 4C). The DELLA domain may be particular to GA response regulatory proteins because it is found only in RGA, GAI, and one rice EST (GenBank accession number D39460). The full-length sequence of this putative rice *RGA* and *GAI* homolog may prove to be interesting. The eponymous VHIID box is more accurately labeled as a (V/I)H(V/I)-(V/I)D box because positions 1, 3, and 4 can be either valine or isoleucine (Figure 4B). Because the RVER domain appears in many VHIID sequences (Figure 4C), this domain may be important for the function of the proteins. So far, the VHIID proteins are found in diverse plant species but not in yeast, prokaryotes, or animals. They are probably ubiquitous in but unique to plants.

A stretch of 23 amino acids at the C-terminal end of RGA shows 78% identity to the N terminus of a barley protein CDR29 (Figure 4C) that is homologous to acyl–CoA oxidases from a variety of species (Grossi et al., 1995). However, the

domain of CDR29 that shares homology to RGA is not in the conserved acyl–CoA oxidase region. *cdr29* expression is induced in barley in response to both dehydration and cold stress (Stanca et al., 1996). Because GA is important in modulating a plant's response to environmental stimuli, this homologous domain may interact with other factors during periods of environmental stress.

The VHIID proteins may be transcriptional regulators. *SCR* has a number of characteristic features, including a putative NLS, homopolymeric Gln, Pro, and Ser, basic leucine zipper, and acidic regions (Di Laurenzio et al., 1996). RGA and GAI have a putative NLS, Leu heptad repeat regions, and the LXXLL motif, and RGA also has homopolymeric Ser and Thr stretches. SCR is proposed to be a transcriptional activator. In contrast, RGA and GAI may be either transcriptional repressors that block transcription of genes involved in GA-regulated growth and development or they may be transcriptional activators that promote expression of such a repressor. In a transient assay using onion epidermal cells, we detected the GFP–RGA fusion protein exclusively in the nucleus (Figure 5). This provides direct evidence that RGA can be properly targeted to the plant cell nucleus. Truong et al. (1997) found that RGA (RGA1) and GAI (RGA2) behaved as transcriptional activators in a heterologous system to allow a yeast *gln3 gdh1* strain to live on ammonia as a nitrogen source. Whether the roles of RGA and GAI in yeast are similar to their roles in plants is not clear. There does not appear to be a yeast homolog of either *RGA* or *GAI.* In addition, the *rga* and *gai* mutant phenotypes do not display any defects in nitrogen metabolism.

Interaction between RGA and GAI

The *gai* mutant was found to have a 17–amino acid in-frame deletion, which may keep the gai protein constitutively active (Peng et al., 1997). This deletion is located within the DELLA domain, which is unique to RGA, GAI, and one rice EST. Consequently, the DELLA domain may be important for GA signal perception or protein deactivation.

Similarity in chemical structure between GA and mammalian steroid hormones has led to the long-standing hypothesis that the two systems shared a similar method of perception and gene regulation. However, there have not been any proteins from plants identified that are homologous to the steroid hormone receptors. The LXXLL motif, recently identified in a number of steroid receptor coactivators (SRCs) and responsible for SRC binding to steroid receptors in the nucleus (Heery et al., 1997; Torchia et al., 1997), is also found in two GA signal transduction components, GAI (Peng et al., 1997) and RGA.

Although *RGA* and *GAI* are very homologous and may share some role in regulating GA signal transduction, they are not completely functionally redundant. Otherwise, the *rga* mutation would not manifest a phenotype in the *ga1-3* background. The N termini of RGA and GAI comprise the most divergent region, suggesting that this region is important for functional differences between the two proteins. Both proteins have leucine heptad repeats that may be involved in protein–protein interactions. Thus, they may form either homodimers or even heterodimers.

In the wild-type background, the *rga* phenotype is subtle, as is the phenotype of the *gai* null mutant *gai-t6* (Peng et al., 1997). The *rga*/*GA1* plants are a paler green than are L*er* plants, but otherwise flower at the same time, grow to the same height, and have the same fertility. The lack of a dramatic phenotype is discussed further in our model for GA signal transduction, but there is no obvious compensation resulting in increased transcription of one "homolog" in the other mutant background; for example, *GAI* transcription is not affected in the *rga* mutant (Figure 7B). Therefore, if there is any compensation for the loss of one repressor, it would probably occur at the level of translational or post-translational control. Moreover, we did not isolate any *gai* null mutants in our *ga1-3* suppressor mutant screens, even though we did isolate 27 alleles of *rga* and 10 alleles of *spy* (Silverstone et al., 1997b). If *RGA* and *GAI* have similar functions, we would expect *gai* null alleles to suppress partially some aspects of the *ga1-3* phenotype, as *rga* does. Examination of the *gai-t6* mutant in the *ga1-3* background and the *gai-t6*/*rga* double mutant in both the *ga1-3* and wild-type backgrounds is necessary to determine whether *GAI* has a similar function as *RGA.* If there is any functional redundancy, then we would expect to see some additive effects in the double mutants. Because both proteins seem to be ubiquitous in plants, their activities may be modulated to achieve a fine-tuned response to GA in specific tissues.

The point mutation in *rga-2* (Asp-478 to Asn-478) is in the RVER domain at a highly conserved amino acid in all of the VHIID proteins. This amino acid is an Asp in all proteins except in VHS4 and VHS5 (both have a Glu residue at this position), and this Asp residue is next to a highly conserved Phe residue (Figure 4C). Because *rga-2* is as strong an allele as *rga-1*, which is a nonsense mutation resulting in the C-terminal 67 amino acids being deleted, this Asp residue is likely to play a vital role in VHIID protein function. The three deletion mutants *rga-20*/*ga1-3*, *rga-24*/*ga1-3*, and *rga-26*/*ga1-3* are all phenotypically similar to the other *rga*/*ga1-3* mutant alleles. Analysis of other point mutations in *rga* alleles may provide additional insights into important functional domains in the RGA protein and possibly in other VHIID proteins.

Because *RGA* and *GAI* are closely related genes and neither has any introns, they may have evolved by a duplication event. Because GAs are found in all seed plants and GA-like compounds are found in ferns and mosses, *RGA* and *GAI* are likely to be part of a conserved signal transduction pathway in plants. Because *RGA* (*RGA1* and *GRS*) and *GAI* (*RGA2*) have been given different names by several groups, for clarity we propose that the names *RGA* and *GAI* be retained for these two genes because the mutant loci had been identified and registered (http://mutant.lse.okstate.edu/genepage/genepage.html) before the cloning of these genes.

Regulation of GA Biosynthesis by RGA

Expression of a 3β-hydroxylase gene (*GA4*; Chiang et al., 1995) is controlled by a feedback mechanism. Although *GA4* expression was increased in the GA-deficient *ga1-3* mutant, it was not detectable in the leaky *ga1-6* missense mutant that is able to germinate, is semidwarf in stature, and is fertile without GA application (Figure 8). This indicates that moderate levels of GAs are able to reduce *GA4* expression. In the *rga*/*ga1-3* mutant, *GA4* expression was repressed without exogenous GA treatment. Therefore, *RGA* seems to be involved in controlling both GA biosynthesis and GA response. Compared with the *rga*/*ga1-3* mutant, the *spy*/*ga1-3* mutant still exhibited a normal *GA4* feedback inhibition response.

Model of GA Signal Transduction

With the cloning of *RGA*, *SPY* (Jacobsen et al., 1996), and *GAI* (Peng et al., 1997), we can present a revised model for GA signal transduction that combines the genetic and biochemical evidence. Our previous model of a branched GA signal transduction pathway was based solely on the genetic data (Silverstone et al., 1997b). We had proposed that one branch is defined by *SPY* and *GAI* and the second branch by *RGA.* These two branches would converge to regulate a common set of developmental processes. The initial cloning of *SPY* did not provide much information about its function, aside from the presence of tetratricopeptide repeats, which mediate protein–protein interactions and occur in a diverse range of proteins (Jacobsen et al., 1996).

However, several Ser (Thr)–*O*-GlcNAc transferases have been cloned recently, and they are homologous to *SPY* (Kreppel et al., 1997; Lubas et al., 1997). These glycosyltransferases can modify proteins by glycosylation alone or by competing for phosphorylation sites. The sites that are modified typically are rich in Ser/Thr, and both RGA and GAI have such a region at their N termini. A second enzyme is required for removing the GlcNAc residue. This raises the possibility that SPY modifies RGA and/or GAI (Peng et al., 1997). SPY could activate these two proteins by transferring a GlcNAc group onto them, and RGA and GAI would then repress genes involved in GA-mediated growth and development. In response to the GA signal, RGA and GAI would no longer have the GlcNAc group, either through competing phosphorylation or simply removal of the GlcNAc residue, and they would not be able to function as repressors. This would explain both the epistasis of the *spy* mutant to the *gai* mutant as well as the additive effects between the *spy* and *rga* mutants. Because they are not functionally redundant, there may be other interacting or modifying proteins that are specific to either RGA or GAI. Activity of these other regulators could explain why *spy* is not epistatic to *rga.*

Peng et al. (1997) provide an elegant model for how GAI functions as a repressor that is turned off directly or indirectly by GAs, thereby allowing growth to occur. In the semidominant *gai* mutant, GAI would be constitutively active and unable to be inactivated, accounting for the dwarf, GA-deficient phenotype. However, because the *gai*/*ga1* double mutant is an extreme dwarf and can be restored to *gai* semidwarf phenotype by GA application, it is not totally insensitive to GA (Koornneef et al., 1985). At present, the *RGA* and *GAI* homology does not differentiate between whether there are two branches of the signal transduction pathway, with each protein serving a similar role on its respective branch, or whether RGA and GAI actually interact to form a complex that regulates gene expression. In either case, GA would be required to relieve the repression on the pathway, and SPY may be modifying both proteins.

In the *ga1-3* mutant, only a very low level of GA is present, and this is a much more sensitive background in which to observe GA-independent growth. By mutating *rga*, GA signaling is partially de-repressed, and GA-independent stem growth occurs. In the wild-type L*er* plant, there is a higher amount of GA being produced to regulate stem growth. Under these conditions, RGA and GAI may be inactivated by GA directly or indirectly and would only partially repress GA signaling. This may be the reason that a null mutation in either *RGA* or *GAI* does not drastically change the phenotype in the wild-type *GA1* background.

Biochemical studies need to be performed to determine if SPY modifies GAI and/or RGA and to identify other proteins that interact with RGA and GAI. In addition, examination of the genes regulated by RGA and GAI will shed light on the process of GA-mediated growth and development.

METHODS

Plant Materials

Arabidopsis thaliana seeds were stratified for 3 days in the cold before planting. Because *ga1-3* and *rga*/*ga1-3* mutants require gibberellin (GA) treatment for germination, they were incubated with 100 μM GA_3 during stratification, and the seeds were rinsed thoroughly with water before planting. The plants were grown at 22°C under 16-hr-light/8-hr-dark cycles. For wild-type and mutant seedlings, sterilized and stratified seeds were plated on medium with Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with or without 1 μM GA_3. After 10 days, whole seedlings were harvested for RNA extraction.

Isolation of Putative *rga* Deletion Mutants

ga1-3 mutant seeds (56,000) were subjected to fast-neutron (FN) bombardment (at the dose 60 Gy) by H. Brunner (Food and Agriculture Organization/International Atomic Energy Agency Agriculture and Biotechnology Laboratory, Vienna, Austria). M_1 plants were grown in flats and allowed to self-pollinate; their seeds were collected in 30 separate pools. We screened 20,000 M_2 plants from each pool for mutants with the *rga*/*ga1-3* phenotype, as previously described (Silverstone et al., 1997b). Because in our previous screen

all of the plants with the *rga*/*ga1-3* phenotype were allelic, we assumed all our FN mutants with the same phenotype were also alleles of *rga.* Allelism tests were performed at the same time as the genomic subtraction experiments. Allelism was determined for *rga-20* through *rga-27* by crossing the FN mutants with *rga-2*/*ga1-3.* The F_1 plants all had the *rga*/*ga1-3* phenotype.

Identification of *rga* in the Wild-Type *GA1* Background

We backcrossed *rga-2*/*ga1-3* to wild-type Landsberg *erecta* (L*er*) plants. In the F_2 generation, there were no plants with a phenotype differing from L*er*, *ga1-3*, or *rga*/*ga1-3.* Therefore, we had surmised that if the *rga*/*GA1* mutant had any phenotype, it would be subtle (Silverstone et al., 1997b). Among the F_2 progeny, we identified wild-type-looking plants that were heterozygous at the *GA1* locus (*GA1*/*ga1-3*) by using the polymerase chain reaction (PCR) markers described previously (Silverstone et al., 1997b). We then let these individuals self and collected F_3 seeds from each plant individually. The F_3 seeds from each individual were then treated with 100 μM GA_3 for 3 days at 4°C and rinsed thoroughly with water before planting. We could determine the genotype of the original F_2 plant at the *RGA* locus by the following reasoning. If an F_2 plant was homozygous for *RGA*, all of the plants homozygous for *ga1-3* in the F_3 generation would look like *ga1-3.* If an F_2 individual was heterozygous for *RGA*/*rga-2*, then one-quarter of the F_3 plants homozygous for *ga1-3* would look like *rga*/*ga1-3* and the rest would look like *ga1-3.* If an F_2 plant was homozygous for *rga-2*/*rga-2*, then all of the plants homozygous for *ga1-3* would be *rga*/*ga1-3.* Among the F_3 progeny of an F_2 plant homozygous for *rga-2*, we identified plants by PCR analysis that were also homozygous for *GA1.*

Mapping the *RGA* Locus

From our sequencing data, we found that there was an RsaI restriction endonuclease site polymorphic between L*er* and Columbia (Col-0) in the *RGA* locus (Col-0 at 1759 bp [GTAC, RsaI site], L*er* [GCAC]). Genomic DNA from 30 independent recombinant inbred lines (Lister and Dean, 1993) was amplified using two flanking primers 204 (5′-GTTTAAGCAAGCGAGTATGC-3′) and 211 (5′-TTCGATTCAGTTCGGTTTAG-3′), digested with RsaI, and then fractionated by electrophoresis using a 2.5% agarose gel. Each line was then scored for whether the *RGA* allele was L*er* (a 263-bp fragment) or Col-0 (143- and 120-bp fragments). The data were submitted to the NASC web site (http://nasc.nott.ac.uk/), and *RGA* was mapped to the very top of chromosome 2 close to the telomere (LOD 2.9; log-likelihood = −180.28).

Genomic Subtraction

Genomic subtraction was performed in parallel for *rga-18*/*ga1-3* through *rga-21*/*ga1-3* mutants, according to the protocol of Sun et al. (1992a, 1992b), with modifications as noted. The *ga1-3* mutant seedlings grown in sterile MS plates for 2 weeks were used to isolate genomic DNA for subtraction. Plant genomic DNA was purified using a QIAGEN (Valencia, CA) column instead of a CsCl gradient, using a procedure including hexadecyltrimethylammonium bromide and chloroform extraction, as recommended by QIAGEN, with slight modification. We used 3 and 10 g of Arabidopsis tissues for QIAGEN genomic-tip 100/G and 500/G columns, respectively. The DNA was eluted from the column with QF buffer (QIAGEN) preheated to 70°C. Photoactivatable biotin was purchased from Pierce (29987G; Rockford, IL). Four sets of subtractive hybridization reactions, each of which contained one of the four putative deletion *rga*/*ga1-3* mutant DNAs and the *ga1-3* DNA, were performed. After the fifth cycle of subtraction, the remaining DNA fragments were ligated with Sau3A adapters, amplified by PCR, and cloned into the SmaI site of pBluescript SK+ (Stratagene, La Jolla, CA), as described previously (Sun et al., 1992a). Insert DNA of individual clones was amplified using a primer corresponding to the Sau3A adapters, radiolabeled, and used as hybridization probes for DNA blot analyses. Small genomic DNA gel blots containing HindIII-digested DNA isolated from *ga1-3* and one of the *rga*/*ga1-3* mutants were used for initial screening of putative clones.

Isolation of *RGA* Genomic and cDNA Clones

Initially, a pOCA18 Col-0 genomic library (Olszewski et al., 1988) was screened with the ^{32}P-labeled random-primed PCR fragment from pRG1, and two overlapping genomic clones were identified as pRG2 and pRG3. A 2-kb HindIII fragment from pRG3 was cloned into the HindIII site of pBluescript SK+ to make plasmid pRG13. To generate additional overlapping genomic clones spanning the deletions, the ^{32}P-labeled random-primed 2-kb HindIII fragment from pRG13 was used to probe a λGEM-11 Col-0 genomic library. An additional three overlapping genomic λ clones were identified as λRG1, λRG2, and λRG3.

A cDNA that corresponds to the deleted region was found by screening the λPRL2 cDNA library obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH) with the ^{32}P-labeled random-primed 2-kb HindIII fragment from pRG13. Four clones were isolated. The plasmids were excised from the phage DNA, according to the protocol supplied (Gibco BRL), by plating phage with DH10B cells on an LB plate with 100 μg/mL of ampicillin and 10 mM $MgCl_2$. Restriction digestion analyses indicated that two clones contained a 2.3-kb cDNA insert, and two others contained truncated cDNAs that are part of the 2.3-kb cDNA. The cDNA clone containing the 2.3-kb insert was designated pRG20 (pZL1 with a 2.3-kb insert cloned at the SalI-NotI sites).

DNA Sequence Analysis

DNA sequencing was performed using a Perkin-Elmer dye terminator cycle system with an ABI (Foster City, CA) 377 PRISM DNA sequencer. Subcloned fragments from pRG20 and λRG2 were used as templates to conduct sequence analyses to determine the *RGA* cDNA and genomic sequence for both strands. Fragments of the *RGA* gene were amplified by PCR from genomic DNA isolated from L*er* and the *rga-1*/*ga1-3* and *rga-2*/*ga1-3* mutants to identify point mutations in the *rga-1* and *rga-2* alleles. PCR primers and/or internal primers were used for sequencing reactions. DNA sequence analyses were repeated to confirm the point mutations, using template DNA generated by an independent PCR reaction. Primary sequence analysis was performed with MacVector v3.0 (Oxford Molecular, Campbell, CA). Homology searches were performed in the GenBank database, using the BLAST program (Altschul et al., 1990). Align-

ments were made using the Pileup program in the Genetics Computer Group (Madison, WI) package of programs.

DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from 2-week-old L*er*, *rga*/*ga1-3*, and *ga1-3* seedlings grown on MS plates, and the mutants in the *ga1-3* background had 1 μM GA_3 included in the plates. The DNA was purified on QIAGEN columns, using the protocol described earlier.

One microgram of HindIII-digested genomic DNA was fractionated on 0.8% agarose gels, transferred to GeneScreen membranes (Du Pont–New England Nuclear), and hybridized with gel-purified ^{32}P-labeled DNA fragments (Ausubel et al., 1990).

Total RNA was isolated from different Arabidopsis tissues (Ausubel et al., 1990; Lashbrook et al., 1994; Silverstone et al., 1997a), and 10 μg of total RNA was treated with glyoxal, fractionated on a 1% agarose gel, transferred to GeneScreen membranes (Sambrook et al., 1989), and hybridized with a random-primed ^{32}P-labeled 2.3-kb SalI-BamHI fragment from pRG20 (Church and Gilbert, 1984). To avoid cross-hybridization between *RGA* and *RGA2* (*GAI*), hybridization was performed at 65°C, using the buffer described in Church and Gilbert (1984), and the filters were washed under high-stringency conditions of 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 1% SDS at 65°C followed by 0.2 × SSC and 0.1% SDS at 65°C for 30 min and 0.1 × SSC at room temperature. After autoradiography, filters were stripped and reprobed with the ^{32}P-labeled 0.8-kb EcoRI fragment from the cyclophilin gene as a loading control (Lippuner et al., 1994). The *RGA2* probe for the RNA blot was made by amplifying L*er* genomic DNA with primers 300 (5′-CTAGATCCGACATTGAAGGA-3′) and 201 (5′-CAGCTAAGCATCCGATTTGC-3′), which specifically amplified a 652-bp fragment from *RGA2* (Truong et al., 1997). Primer 300 has an eight-base mismatch with *RGA*, including the three nucleotides at the 3′ end. Primer 201 sequence is identical to *RGA* and has only a single base mismatch with *RGA2.* If these primers had also amplified a fragment from *RGA*, there would have been an additional 801-bp band. The *GA4* probe was made by random-prime labeling the 1.4-kb EcoRI fragment of the *GA4* cDNA cloned into pBluescript SK+. The RNA and DNA blots were exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) and quantitated on a PhosphorImager (model 400E; Molecular Dynamics), using Imagequant v4.1 software.

Transient Expression of the GFP–RGA Fusion Protein in Onion Epidermal Cells

The *RGA* cDNA from pRG20 was amplified using primers 216 (5′-AACCAGATCTATGAAGAGAGATCATCACCA-3′; BGIII site underlined) and 217 (5′-ATTAAGATCTTCAGTACGCCGCCGTCGAGA-3′; BGIII site underlined) and the Expand High Fidelity system (Boehringer Mannheim) to generate a BglII site at both the 5′ and 3′ ends of the *RGA* cDNA. This PCR DNA was digested with BglII and ligated with BglII-digested pRTL2ΔNmGFPS65T to create pRG34F, which contains *GFP–RGA* in-frame fusion under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This *GFP–RGA* fusion encodes a fusion protein with GFP at the N-terminal portion and RGA at the C-terminal portion. The onion epidermal layers were prepared and bombarded, as previously described (Varagona et al., 1992), with tungsten particles (Bio-Rad) coated with the control plasmid DNA, pRTL2ΔNmGFPS65T, or pRG34F. The cells were viewed using a Leica (Heerbrugg, Switzerland) DMRB microscope equipped with a fluorescence module.

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