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

Aron L. Silverstone, Charles N. Ciampaglio, and Tai-ping Sun

Developmental, Cell and Molecular Biology Group, Department of Botany, Box 91000, Duke University, Durham, North Carolina 27708-1000

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 J ones, 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; J acobsen and Olszewski, 1993; J acobsen et al., 1996; Silverstone et al., 1997b) and semidominant semi-dwarf 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 (J acobsen 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
enzymes, demonstrates that this mutant is more resistant to paclobutrazol than is the wild type (Peng et al., 1997). This result indicates that GA1 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 (Jacobson 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 (Koomneef 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 (Jacobson 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 pk1 mutation that affects GA-induced differentiation of the seedling primary root (Ogas et al., 1997).

Although SPY and GA1 have been cloned, their exact functions are not well understood (Jacobson 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 GA1 gene encodes a member of the VHIIID 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 M2 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 VHIIID 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 (Koomneef 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 (Ler; 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., 1998) (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 xGEM-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
RGA, a Transcriptional Regulator of GA Response 157

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 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, Ler, 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, 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.

Figure 2. Physical Map around the RGA Locus.

The heavy horizontal line shows a Sall (S) and HindIII (H) restriction map around the RGA locus. The asterisk indicates a HindIII site that is only present in Ler 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 gel 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 co-activator 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 RKVATYFAELARRYR 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 Y11136) and RGA2 (GenBank accession number Y11137), 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 ATFC8 (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 (Ler 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).
Map Position of the RGA Locus

The RGA cDNA and genomic DNA clones came from Col-0 libraries. Because our rga/gal1-3 mutants were all in the Ler background, we also determined the DNA sequence of the Ler 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 Ler 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 Ler and Tyr-587 in Col-0). This last polymorphism also resulted in the presence of an Rsal site in Col-0 that is absent in Ler. 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 Rsal polymorphism between the Ler 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-

---

**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 VIHD 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.

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>
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 F2 generation of a cross between rga-2/ga1-3 and Ler (Silverstone et al., 1997b). We subsequently identified rga-2/GA1, as described in Methods. These plants were a little paler than wild-type Ler plants, but they did not have any dramatic phenotype, and they were similar to Ler 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.

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 Ler 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 3 μM GA3. 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 Ler 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
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 Ler 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.

**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)V(V/I)-V 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
The Plant Cell

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 steroidal 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/GAI plants are a paler green than are Ler 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-termini of 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 (RG2) 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 resistance. Compared with the rga/ga1-3 mutant, the spyl/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 Ler 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 GA₃ 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 GA₃. After 10 days, whole seedlings were harvested for RNA extraction.

Isolation of Putative rga Deletion Mutants

gai-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). M₂ plants were grown in flats and allowed to self-pollinate; their seeds were collected in 30 separate pools. We screened 20,000 M₂ 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 F1 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 (Ler) plants. In the F2 generation, there were no plants with a phenotype differing from Ler, 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 F2 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 F3 seeds from each plant individually. The F3 seeds from each individual were then treated with 100 μM GA3 for 3 days at 4°C and rinsed thoroughly with water before planting. We could determine the genotype of the original F2 plant at the GA locus by the following reasoning. If an F2 plant was homozygous for GA1, all of the plants homozygous for ga1-3 in the F2 generation would look like ga1-3. If an F2 individual was heterozygous for RGA/ga1-2, then one-quarter of the F3 plants homozygous for ga1-3 would look like rga/ga1-3 and the rest would look like ga1-3. If an F2 plant was homozygous for rga-2/ga1-2, then all of the plants homozygous for ga1-3 would be rga/ga1-3. Among the F3 progeny of an F2 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 Rsal restriction endonuclease site polymorphic between Ler and Columbia (Col-0) in the RGA locus (Col-0 at 1759 bp [GTAC, Rsal site], Ler [GCAC]). Genomic DNA from 30 independent recombinant inbred lines (Lister and Dean, 1993) was amplified using two flanking primers 204 (5′-GTTTAAAGCAAGCCGATGC-3′) and 211 (5′-TTCCATGTACGTTCCGTTAG-3′), digested with Rsal, and then fractionated by electrophoresis using a 2.5% agarose gel. Each line was then scored for whether the RGA allele was Ler (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 Smal site of pBlue-script 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 32P-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 pBlue-script SK+ to make plasmid pRG13. To generate additional overlapping genomic clones spanning the deletions, the 32P-labeled random-primed 2-kb HindIII fragment from pRG13 was used to probe a xGEM-11 Col-0 genomic library. An additional three overlapping genomic clones were identified as xRG1, xRG2, and xRG3.

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 32P-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 MgCl2. 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 Sall-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 xRG2 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 Ler 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-
DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from 2-week-old Ler, rga/ga1-3, and ga1-3 seedlings grown on MS plates, and the mutants in the ga1-3 background had 1 μM GA3 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 (Sambrook et al., 1989), and hybridized with a random-primed 32P-labeled 2.3-kb SalI fragment from the cyclophilin gene as a loading control (Lippuner et al., 1994). The genomic DNA with primers 300 (5'-CTAGATCCGACATTG-3') and 201 (5'-CAGCTAAGCATCCGATTTGC-3'), which specifically amplified a 652-bp fragment from the cyclophilin gene as a loading control (Lippuner et al., 1994). The RGA2 probe for the RGA blot was made by amplifying Ler genomic DNA with primers 300 (5'-CTAGATCCGACATTG-AAGGA-3') and 201 (5'-CAGCTAAGCATCCGATTTGC-3'), which specifically amplified a 652-bp fragment from the cyclophilin gene as a loading control (Lippuner et al., 1994). Primer 300 has an eight-base mismatch with the RGA2 probe.

Acknowledgments

We thank Bryan Baranowski, Alyssa Dill, Ava Krol, Annie Mak, Wendy Watford, and Keni Willa for technical assistance; Zheng-hui He for the genomic DNA from the recombinant inbred lines; Mark Kinkema for assistance with GFP analysis; Shinjiro Yamaguchi for the GA4 clone and beneficial discussions; Nick Harberd for sharing his GA1 data before publication; and John Boynton and Jim Siedow for critical review of this manuscript. We also thank Dr. H. Brunner for FN bombardment of the ga1-3 seeds, Neil Oliszewski for the pOCA18 library and helpful discussions, Ron Davis for the Col-0 genomic library, Charles Gasser for the cyclophilin clone, and Albrecht von Armin for the GFP clone. This work was supported by National Science Foundation Grant No. IBN-9723171.

Received November 7, 1997; accepted December 9, 1997.

References


The Arabidopsis \textit{RGA} Gene Encodes a Transcriptional Regulator Repressing the Gibberellin Signal Transduction Pathway

Aron L. Silverstone, Charles N. Ciampaglio and Tai-ping Sun

\textit{Plant Cell} 1998;10;155-169

DOI 10.1105/tpc.10.2.155

This information is current as of August 4, 2017

| References | This article cites 43 articles, 24 of which can be accessed free at: /content/10/2/155.full.html#ref-list-1 |
| eTOCs | Sign up for eTOCs at: http://www.plantcell.org/cgi/alerts/ctmain |
| CiteTrack Alerts | Sign up for CiteTrack Alerts at: http://www.plantcell.org/cgi/alerts/ctmain |
| Subscription Information | Subscription Information for \textit{The Plant Cell} and \textit{Plant Physiology} is available at: http://www.aspb.org/publications/subscriptions.cfm |