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Repressing a Repressor: Gibberellin-Induced Rapid Reduction of the RGA Protein in Arabidopsis

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RGA **(for repressor of *ga1-3*) and *SPINDLY* (*SPY*) are likely repressors of gibberellin (GA) signaling in Arabidopsis because the recessive *rga* and *spy* mutations partially suppressed the phenotype of the GA-deficient mutant *ga1-3*. We found that neither *rga* nor *spy* altered the GA levels in the wild-type or the *ga1-3* background. However, expression of the GA biosynthetic gene *GA4* was reduced 26% by the *rga* mutation, suggesting that partial derepression of the GA response pathway by *rga* resulted in the feedback inhibition of *GA4* expression. The green fluorescent protein (GFP)–RGA fusion protein was localized to nuclei in transgenic Arabidopsis. This result supports the predicted function of RGA as a transcriptional regulator based on sequence analysis. Confocal microscopy and immunoblot analyses demonstrated that the levels of both the GFP-RGA fusion protein and endogenous RGA were reduced rapidly by GA treatment. Therefore, the GA signal appears to derepress the GA signaling pathway by degrading the repressor protein RGA. The effect of *rga* on *GA4* gene expression and the effect of GA on RGA protein level allow us to identify part of the mechanism by which GA homeostasis is achieved.**

INTRODUCTION

Gibberellins (GAs) are members of a large family of diterpenoid compounds, some of which are plant growth regulators that control such diverse processes as seed germination, stem growth, and flower development. Although the GA biosynthetic pathway has been elucidated (reviewed in Lange, 1998; Hedden and Proebsting, 1999; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000), much less is known about its signal transduction pathway in plants. Recent molecular and pharmacological studies in cereal aleurone showed that Ca^{2+}, calmodulin, cyclic GMP, heterotrimeric G proteins, GAMYB, and protein kinases may play a role in GA signaling (reviewed in Bethke and Jones, 1998; Lovegrove and Hooley, 2000). Isolation of GA response mutants and molecular cloning of corresponding genes in Arabidopsis also have identified several novel components of the GA signal transduction pathway (reviewed in Thornton et al., 1999; Sun, 2000). The putative repressors include *SPINDLY* (*SPY*; Jacobsen et al., 1996), *RGA* (for repressor of *ga1-3*; Silverstone et al., 1998), *GAI* (for GA insensitive; Peng et al., 1997), and *SHORT INTERNODES* (*SHI*; Fridborg et al., 1999), and the potential activators are *SLEEPY* (*SLY*; Steber et al., 1998) and *PICKLE* (*PKL*; Ogas et al., 1999).

SPY was identified originally because *spy* mutations allowed the seed to germinate in the presence of the GA biosynthesis inhibitor paclobutrazol (PAC; Jacobsen and Olszewski, 1993). The defect in the SPY function also was able to partially suppress the phenotype of the GA biosynthetic mutant *ga1-3*, which is a nongerminating, male-sterile, extreme dwarf (Silverstone et al., 1997). Sequence analysis of *SPY* and in vitro enzyme assays using the recombinant SPY protein suggest that SPY probably is a Ser/Thr *O*-linked *N*-acetylglucosamine transferase (OGT; Thornton et al., 1999).

We identified *RGA* as a repressor of GA signaling because the recessive *rga* alleles partially rescued the stem growth defect of the *ga1-3* mutant (Silverstone et al., 1997). The role of *GAI* in GA signaling was defined initially by the semidominant allele *gai-1*, which caused the plant to be insensitive to exogenous GA treatment and produced an appearance that was similar to that of GA biosynthetic mutants (Koornneef et al., 1985). Subsequently, recessive (loss-of-function) *gai* alleles were found to have the wild-type phenotype (Peng and Harberd, 1993; Wilson and Somerville, 1995), but they conferred resistance to PAC, indicating that GAI negatively regulates GA signaling (Peng et al., 1997).

Cloning of the *RGA* and *GAI* genes revealed that their encoded proteins share 82% sequence identity and are members of the GRAS family of regulatory proteins (Peng et al., 1997; Silverstone et al., 1998; Pysh et al., 1999). Currently,

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at least 38 GRAS family members have been identified in Arabidopsis, and most were identified from the Arabidopsis sequencing project. In Arabidopsis, three additional GRAS members that were defined by mutant analysis are SCARECROW (SCR; Di Laurenzio et al., 1996) and Short-Root (SHR; Helariutta et al., 2000), which are determinants of radial root organization, and PAT1, a phytochrome A signaling component (Bolle et al., 2000). All GRAS members contain conserved central and C-terminal regions, named VHIID and RVER, respectively, after highly conserved amino acid motifs (Silverstone et al., 1998; Pysh et al., 1999). The specificity of different GRAS members seems to lie within their N-terminal regions, which are more divergent. RGA and GAI contain a unique conserved sequence (named DELLA) near their N termini (Peng et al., 1997; Silverstone et al., 1998). This sequence appears to be important for modulating the activity of these proteins by the GA signal, because the GA-insensitive dwarf phenotype of *gai-1* is caused by an in-frame deletion in the DELLA region of the gai protein (Peng et al., 1997). Recently, the functional orthologs of *RGA* and *GAI* were identified in wheat and maize (Peng et al., 1999). Mutations in the wheat ortholog *Rht* were responsible in part for the increased yields of wheat that occurred during the "green revolution." Interestingly, the semidwarfing mutations in *Rht* are similar to that of *gai-1*.

It has been shown that changes in GA signaling can affect GA biosynthesis and catabolism by feedback mechanisms, which contribute to a homeostasis of GA levels (reviewed in Bethke and Jones, 1998; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). The semidominant *gai-1* mutant, which has reduced GA response, contains a higher level of bioactive GAs than do wild-type plants (Talón et al., 1990b). This mutant also accumulates higher levels of GA 20-oxidase (*GA5*; Xu et al., 1995) and 3β-hydroxylase (*GA4*; Cowling et al., 1998) mRNAs, which encode enzymes that catalyze the final reactions for the synthesis of active GAs. In GA-deficient mutants (e.g., *ga1-3*), the expression of *GA4* and *GA5* is higher than in wild type, and this increased expression can be reduced by GA application (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995). Although GA response acts to decrease GA biosynthesis, expression of the genes that encode the GA catabolic enzyme GA 2-oxidase is increased by GA treatment (Thomas et al., 1999). These results indicate that increased GA response causes a reduction in the levels of bioactive GAs by inhibiting GA biosynthesis and activating GA catabolism.

To elucidate the roles of RGA and SPY in GA signaling, we examined whether the *rga* and *spy* mutations altered the level of bioactive GAs. In this work, we measured GA content and *GA4* mRNA levels in the *rga* and *spy* mutants by gas chromatography–mass spectrometry and RNA blot analysis, respectively. In so doing, we were able to glean some information on how the GA response pathway interacts with GA biosynthesis to cause feedback inhibition. We found that these mutations altered *GA4* gene expression but did not change GA content. These results demonstrated that the *rga* and *spy* mutant phenotypes were caused by changes in GA response.

Both RGA and GAI contain sequence features that are common in transcriptional regulators, including homopolymeric Ser and Thr, nuclear localization signals, Leu heptad repeats, and SH2-like domains (Peng et al., 1997, 1999; Silverstone et al., 1998). We have shown that the transiently expressed green fluorescent protein (GFP)–RGA fusion protein was localized to the nucleus in onion epidermal cells (Silverstone et al., 1998). In this report, we demonstrate that the GFP-RGA fusion protein is localized to the nucleus in transgenic Arabidopsis seedlings and that GA treatment rapidly decreased the levels of both the GFP-RGA fusion protein and the endogenous RGA protein. Our studies indicate that the GA signal activates plant growth and development through the degradation of the repressor RGA.

RESULTS

GA Content of *rga* and *spy* Mutants

To rule out the possibility that the *rga* and *spy* mutant phenotypes were caused by increased GA levels and not by derepressing GA signaling, we analyzed the GA content of these mutants. Because leaves and flowers may have different GA contents, we harvested whole rosette plants before flowering for GA measurements. Gas chromatography–selected ion monitoring (GC-SIM) analysis was performed to determine the concentration of endogenous GAs in *ga1-3*, *rga-2*/*ga1-3*, *spy-9*/*ga1-3*, wild-type Landsberg *erecta* (L*er*), *rga-2*, and *spy-9* plants. Both the early 13-hydoxylation pathway and the non-13-hydroxylation pathway are present in Arabidopsis (Talón et al., 1990a). Therefore, we measured metabolic, bioactive, and catabolic GAs in both pathways to determine if one or both pathways are affected by the *rga* and *spy* mutations. ^{2}H-labeled GAs were used as internal standards to quantify the level of each GA.

Table 1 summarizes the results of our quantitation. The GAs in the top half are in the early 13-hydoxylation pathway, and the GAs in the bottom half are in the non-13-hydroxylation pathway. As has been reported (Talón et al., 1990a), GA_4 is the primary bioactive GA in Arabidopsis, although GA_1 also is present. The *rga* and *spy* mutations did not cause detectable changes in GA content in the *ga1-3* background (Table 1). The *ga1-3* mutant is an extreme dwarf because it contains a very low level of GAs. The *rga-2*/*ga1-3* and *spy-9*/*ga1-3* mutants show partially elongated stem growth (Silverstone et al., 1997), although neither the *spy* nor the *rga* mutation resulted in an increase in GA levels in the *ga1-3* background (Table 1). Thus, the dramatic phenotypic changes caused by these mutations are due to alterations in the GA response pathway.

The *rga* mutations in the wild-type *GA1* background were phenotypically indistinguishable from wild-type plants. In

contrast, mutations in the *SPY* gene displayed the phenotype of wild-type plants that had been treated with an excess amount of GAs. Neither mutation affected GA levels substantially (Table 1). Although our data show 17 and 32% reductions in GA_4 levels in *rga-2/GA1* and *spy-9/GA1*, respectively, these changes are not large enough to be considered significant using the current GC-SIM procedure.

Changes in Expression of the *GA4* Gene by the *rga* and *spy* Mutations

The GA-deficient mutant *ga1-3* accumulates a high level of *GA4* mRNA, which could be downregulated by exogenous GA treatment. In a previous article, we reported that *rga* appeared to alter the feedback regulation of the GA biosynthetic gene *GA4* (Silverstone et al., 1998). We found that the untreated digenic *rga-2/ga1-3* mutant contained a much lower level of *GA4* mRNA, which was as undetectable as that in the GA-treated *ga1-3* mutant. Our hypothesis is that partial derepression of the GA signaling pathway by the *rga* mutation may cause some degree of downregulated expression of the GA biosynthetic genes. However, in the previous RNA blot analysis, we had used a *GA4* cDNA probe, and it was difficult to quantify the low levels of *GA4* transcript in these samples. To examine more accurately the effect of *rga* and *spy* on *GA4* transcript levels, we used a sensitive antisense *GA4* RNA probe in the current study. Another difference here is that we examined the effect of GA on *GA4* expression 8 hr after treatment with GA_3, whereas in our previous experiment the "GA-treated" seedlings had been grown for 10 days on medium containing 1 μM GA_3 (Silverstone et al., 1998).

Figure 1 shows the levels of the *GA4* transcripts in *ga1-3*, *rga-2/ga1-3*, *spy-8/ga1-3*, wild-type L*er*, *rga-2*, and *spy-8* with and without GA treatment. The *rga* mutation caused 10 and 26% reductions of *GA4* expression in the wild-type and *ga1-3* mutant backgrounds, respectively. These degrees of reduction of *GA4* expression were found in four independent experiments and support our hypothesis that an increase in GA signaling decreases *GA4* expression. In contrast, the *spy-8/ga1-3* mutant had an even higher level of *GA4* transcript than did *ga1-3*. Exogenous GA treatment decreased the *GA4* mRNA levels in *rga-2*, *spy-8*, *rga-2/ga1-3*, and *spy-8/ga1-3* as in the wild type. This result is consistent with the finding that *rga* and *spy* remain responsive to exogenous GA treatment.

Nuclear Localization of the GFP-RGA Fusion Protein in Transgenic Arabidopsis

Sequence analysis showed that RGA contains several structural features of a transcription regulator, including a putative nuclear localization signal. In support of this finding, we showed previously that a transiently expressed GFP-RGA fusion protein is localized to the nucleus in onion epidermal cells (Silverstone et al., 1998). To analyze the subcellular localization of RGA in Arabidopsis, we introduced a similar

Table 1. Quantitation of GAs in Different Mutants and Wild-Type Plants

GA	*ga1-3*	*rga-2/ga1-3*	*spy-9/ga1-3*	L*er*	*rga-2/GA1*	*spy-9/GA1*
GA_{53}	Undetectable	Undetectable	Undetectable	4.2	3.9	6.5
GA_{44}	Trace	Trace	Trace	1.6	0.7	2.7
GA_{19}	1.6	3.2	2	6.9	10.4	8.4
GA_{20}	0.1	0.2	0.1	0.3	0.4	0.3
GA_1	0.1	Trace	0.1	0.4	0.4	0.5
GA_{29}	0.1	0.1	0.1	0.3	0.3	0.3
GA_8	2.4	0.4	Trace	Trace	1.9	Trace
GA_{12}	Trace	0.9	0.5	44.4	42.0	56.2
GA_{15}	0.7	0.6	0.6	7.2	8.3	10.4
GA_{24}	Trace	Trace	Trace	40.6	41.3	46.0
GA_9	Trace	Trace	Trace	2.3	2.5	1.7
GA_4	Undetectable	Undetectable	Undetectable	22.5	18.6	15.3
GA_{34}	0.4	0.3	0.4	6.4	5.5	4.3

[a] Values are in ng of GA/g dry weight. Trace indicates <0.1 ng of GA/g dry weight. Undetectable indicates that no corresponding peak was detected by GC-SIM. The GAs in the top half of the table are part of the early 13-hydroxylation pathway: $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1 \rightarrow GA_8$, with $GA_{20} \rightarrow GA_{29}$. Those in the bottom half are part of the non-13-hydroxylation pathway: $GA_{12} \rightarrow GA_{15} \rightarrow GA_{24} \rightarrow GA_9 \rightarrow GA_4 \rightarrow GA_{34}$. GA_1 and GA_4 are bioactive GAs, whereas the other GAs are either precursors or deactivated GAs (GA_{29}, GA_8, and GA_{34}).

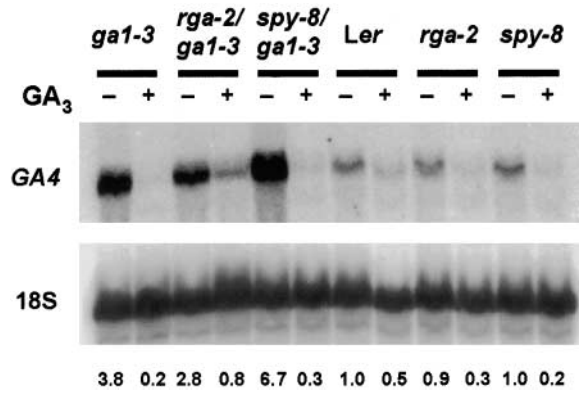

Figure 1. Effect of the *rga* and *spy* Mutations on *GA4* mRNA Levels.

Shown are autoradiographs of RNA blots containing 15 μg of total RNA isolated from different GA biosynthetic and signal transduction mutants, as labeled. (−) or (+) GA_3 indicates that the RNA samples were isolated from untreated seedlings or seedlings treated with GA_3 for 8 hr, respectively. The blots were hybridized with a radiolabeled antisense *GA4* RNA probe and then reprobed with the oligonucleotides corresponding to the 18S rDNA sequence. The numbers under the blots indicate the relative amounts of *GA4* mRNA after normalization using 18S rRNA as a loading control. The value of untreated L*er* was arbitrarily set at 1.0.

construct (pRG36) carrying the *cauliflower mosaic virus 35S*::*GFP-RGA* fusion gene into wild type (L*er* and Columbia [Col-0]) and *rga-24*/*ga1-3* and *rga-26*/*ga1-3* mutants via *Agrobacterium tumefaciens*–mediated transformation. The GFP-RGA fusion protein was functional in Arabidopsis because it was able to rescue the phenotype of null *rga* mutations (Figure 2). In fact, expression of the *35S*::*GFP-RGA* transgene in the *rga-24*/*ga1-3* mutant resulted in a more severe dwarf phenotype than that of *ga1-3*. Therefore, overexpression of the GFP-RGA fusion protein could repress GA signaling more efficiently than the endogenous wild-type RGA protein. Using confocal microscopy, we detected in the nucleus the GFP fluorescence produced by the GFP-RGA fusion protein (Figure 3).

To avoid potential artifacts caused by ectopic expression using the *35S* promoter, we made a new construct, pRG51, in which the *GFP-RGA* fusion gene was flanked by 8-kb 5′ upstream and 5.8-kb 3′ downstream sequences around the *RGA* locus (*RGA* promoter::*GFP-RGA*). pRG51 was used to transform both L*er* and *rga-24*/*ga1-3*. As predicted, the *RGA* promoter::*GFP-RGA* fusion gene rescued the phenotype caused by the *rga-24* null mutation (data not shown), and GFP fluorescence was detected in the nuclei (Figure 4). The confocal images are of root expression, because the low autofluorescence allows clear demonstration of GFP activity. The pattern of *GFP-RGA* expression in the roots of transgenic lines carrying the *RGA* promoter::*GFP-RGA* fusion gene was similar to that in lines expressing *35S*::*GFP-RGA*.

Effects of GA and PAC on the GFP-RGA Fusion Protein

We had found previously that *RGA* mRNA levels remained almost constant among different tissues and were not affected dramatically by the GA status of the plant (Silverstone et al., 1998). Therefore, we hypothesized that the major control of expression of RGA might be on the subcellular localization, concentration, and/or activity of the protein. GFP fluorescence allowed us to monitor the GFP-RGA fusion protein in living cells by epifluorescence and confocal laser microscopy (Sheen et al., 1995; Haseloff et al., 1997). This is informative because the GFP-RGA fusion protein is functionally active in planta.

Using this approach, we observed dynamic alteration of the GFP-RGA protein in response to both exogenously applied GA and an inhibitor of GA biosynthesis, PAC (Figures 4 and 5). Root tips of transgenic plants expressing *35S*::*GFP-RGA* were treated with GA_3 or water and scanned at intervals for 30 min using confocal microscopy (Figure 5). Whereas the water-treated control had only a small loss of GFP fluorescence in the nuclei resulting from bleaching by the laser, there was a dramatic decrease in the GFP signal in response to GA treatment. Similar results were observed after the application of GA to transgenic plants carrying the *RGA* promoter::*GFP-RGA* fusion. Within 2 hr after GA treatment, GFP fluorescence was no longer detectable (Figure 4). Because PAC inhibits GA biosynthesis, we sought to determine whether PAC treatment would have an opposite effect from GA on RGA protein levels. Indeed, we observed a slight increase of GFP fluorescence in the nuclei at 24 hr (data not shown) and a much increased GFP signal in the nuclei at 48 hr (Figure 4). The slower response to PAC probably reflects the time required for PAC to inhibit GA biosyn-

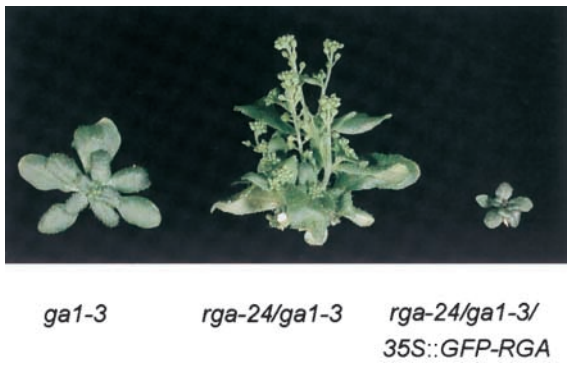

Figure 2. The *GFP-RGA* Fusion Rescues the Phenotype Caused by the *rga* Mutation.

The phenotype of a transgenic plant (*rga-24*/*ga1-3* background) that was homozygous for the *35S*::*GFP-RGA* fusion gene was compared with the phenotypes of *ga1-3* and *rga-24*/*ga1-3*. All plants were 50 days old.

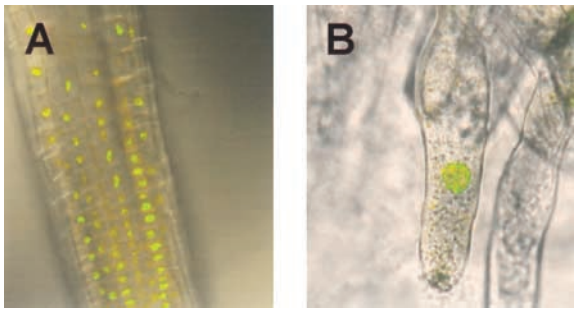

Figure 3. Fluorescence in the Root of Transgenic *rga/ga1-3* Plants Expressing the GFP-RGA Protein.

Shown are overlays of fluorescent and bright-field images generated by confocal laser microscopy. Exclusive nuclear localization of GFP-RGA is seen in a region of a root behind the tip in the elongation zone **(A)** and in a single root hair cell with a fluorescent nucleus **(B)**.

thesis plus the time needed for GA catabolism to reduce the concentration of active GAs.

We then examined GFP-RGA protein levels by immunoblot analysis to determine whether GA and PAC caused this rapid loss or increase of GFP fluorescence by affecting the levels or conformation of the fusion protein. Transgenic lines expressing either the GFP-RGA protein with the constitutive *35S* promoter or the *RGA* promoter were examined. Figure 6 shows that GFP-RGA protein levels in both lines were reduced as early as 30 min after GA treatment and increased 24 to 48 hr after the application of PAC. Thus, GA activity seemed to cause the reduced level of the RGA protein, possibly through targeted degradation of the protein.

Downregulation of the Endogenous RGA Protein by GA

Although the GFP-RGA fusion protein is functional in the plant, it is still a reporter protein. Therefore, it was important to confirm the results with the fusion protein by analyzing the behavior of the native RGA protein. Toward this end, we generated anti-RGA rabbit antibodies using an *Escherichia coli*–expressed 65-kD RGA protein with six additional His residues at its N terminus. The predicted molecular masses of RGA and GAI are 64 and 59 kD, respectively. Indeed, this antiserum detected a 64-kD protein band, which was present in L*er* and *ga1-3* but absent in *rga-24* (Figure 7). Consistent with the data presented for the GFP-RGA fusion protein, we found a much higher level of the RGA protein in the GA-deficient *ga1-3* plant than in L*er*. Moreover, there was a dramatic reduction in RGA protein level after GA treatment for 2 hr (Figure 7).

These results indicate that the behavior of the GFP-RGA fusion protein accurately reflects that of the endogenous RGA protein, and they strongly support the notion that the GA signal inhibits RGA activity by reducing RGA protein level. Thus, the GFP-RGA fusion protein should be a reliable indicator of RGA behavior in planta.

DISCUSSION

GA Homeostasis

There is a growing body of evidence documenting GA homeostasis, that is, that GA signaling is able to modulate GA levels (reviewed in Bethke and Jones, 1998; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). However, the previous studies suggesting homeostasis involved GA treatment of GA-deficient mutants or the gain-of-function mutant *gai-1*. Our data demonstrate that GA levels are able to regulate GA signaling by affecting the level of RGA, a repressor of the GA response pathway. We also show that removing RGA function leads to the downregulation of expression of the GA biosynthetic gene *GA4*.

Our model of GA homeostasis (Figure 8) shows that a sustained environmental or endogenous cue is required to cause changes in GA levels to alter plant growth and development. If there is only a transient input signal, the system is rapidly brought back to the basal homeostatic level through the following mechanism. An increase in the level of active GAs derepresses the GA response through a feed-forward mechanism. The GA signaling pathway, in turn, inhibits GA biosynthesis through a feedback mechanism. The net effect allows GA concentrations to return to the basal level. However, when there is a continuous cue, the system produces more active GAs to induce GA signaling. Because the cue would counter the system's tendency to reduce GA signaling, the strength of the cue would be reflected in the degree of GA-induced growth and development. Once this input signal ceases, the feedback mechanism helps to reset the system.

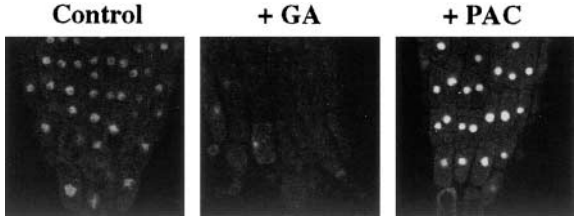

Figure 4. Effects of GA and PAC Treatment on the *RGA* Promoter–Expressed GFP-RGA Protein.

Roots of transgenic plants (L*er* background) expressing the *RGA* promoter::*GFP-RGA* fusion were observed using confocal laser microscopy. Shown are the fluorescent images of root tips that were untreated (Control), treated with 100 μM GA_3 for 2 hr (+GA), or incubated with 100 μM PAC and 0.01% Tween 20 for 48 hr (+PAC).

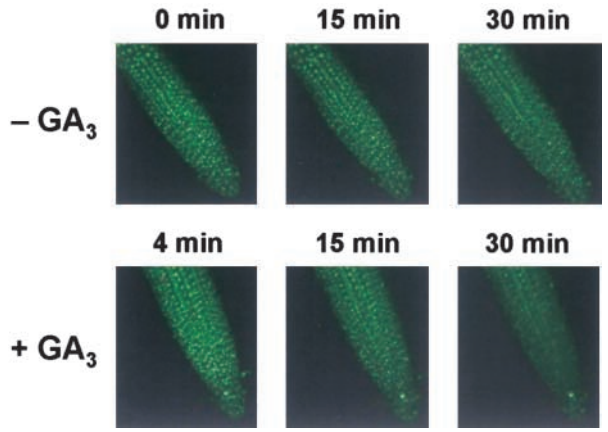

Figure 5. Effect of GA Treatment on the GFP-RGA Protein Expressed by the Cauliflower Mosaic Virus *35S* Promoter.

Roots of transgenic plants (*rga/ga1-3* background) expressing the *35S*::*GFP-RGA* fusion were observed by using confocal laser microscopy. Shown are three-dimensional projections of the fluorescent images of root tips either untreated (top) or treated with 100 μM GA_3 (bottom) for the times indicated.

Feedback Regulation of GA Biosynthesis by GA Response

Previously, the semidwarf phenotype of the *rga/ga1-3* and *spy/ga1-3* mutants led us to propose that mutations in RGA or SPY partially derepress GA signaling in Arabidopsis (Silverstone et al., 1997). This hypothesis is supported by the similar GA content between these mutants and *ga1-3* (Table 1). The *GA1* locus encodes the copalyl diphosphate synthase (CPS) that catalyzes the first committed step in GA biosynthesis (Sun and Kamiya, 1994). Interestingly, the *ga1-3* mutant still accumulates a very low level of GAs (Table 1; Zeevaart and Talón, 1992), even though it has a null mutation at the *GA1* locus. There might be another CPS gene that is expressed at very low levels, although the Arabidopsis sequencing project has not uncovered it. Alternatively, another diterpene cyclase may have weak CPS activity. This raises the possibility that a mutation that completely eliminates GA production is lethal.

Although there were no dramatic differences in GA levels, we did observe changes in the expression of a GA biosynthetic gene, *GA4*, by the *rga* and *spy* mutations. A feedback control mechanism has been suggested to play a role in the regulation of GA biosynthesis by the activity of the GA response pathway (reviewed in Bethke and Jones, 1998; Hedden and Proebsting, 1999; Yamaguchi and Kamiya, 2000). The reduced *GA4* expression caused by *rga* could be explained by this feedback mechanism; that is, partial derepression of the GA signaling pathway by *rga* could cause the downregulation of *GA4* expression. However, the *spy/ga1-3* mutant showed an increase in *GA4* mRNA level, which is opposite to what we would predict. This could be attributable to the indirect effect of *spy* on other cellular processes, because *SPY* likely encodes an OGT, which might function in multiple pathways (Thornton et al., 1999).

Although the *rga* mutation altered *GA4* gene expression, in the *ga1-3* background there were no dramatic changes in GA concentrations. One possible explanation for this finding is that because *ga1* is blocked at an early step in GA biosynthesis, very little metabolite flows through the pathway compared with that of the wild type. Therefore, alterations of downstream steps might not affect the levels of GAs. It is also possible that the expression of other biosynthetic genes is altered by the perturbation of homeostasis caused by the downregulation of *GA4*. The latter hypothesis is supported by the fact that we did not find a significant change in GA levels in the wild-type background.

The *GA4* mRNA level in *spy-8/ga1-3* was sixfold higher than that in *spy-8* (Figure 1). This differs from the results of Cowling et al. (1998), who found that *spy-5* grown in 0.1 μM PAC for 2 weeks (which should mimic the effect in the *ga1-3* background) contained a much lower level of *GA4* mRNA than did untreated *spy-5*. The mutations in *spy-8* and *spy-5* are in different regions of the SPY protein (A.L. Silverstone, T.-s. Tseng, N.E. Olszewski, and T.-p. Sun, unpublished results). If SPY is a multifunctional enzyme, these mutations may have different effects on the feedback mechanism.

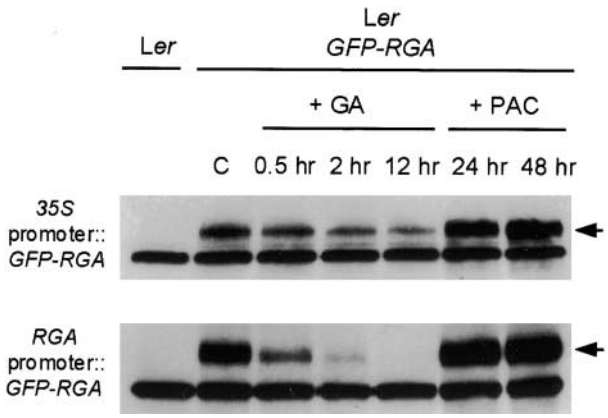

Figure 6. Immunoblot Analysis of GFP-RGA Levels.

The blots contained 50 μg of total protein extracted from L*er* and transgenic seedlings carrying either the *35S*::*GFP-RGA* (top) or the *RGA* promoter::*GFP-RGA* (bottom) fusion gene. Lane C, water-treated control. The times after GA or PAC treatment were as labeled. A rat anti-GFP antiserum and a peroxidase-conjugated goat anti-rat IgG were used as primary and secondary antibodies, respectively. The arrows indicate the GFP-RGA fusion protein (91 kD). The additional lower band in all lanes represents nonspecific background protein because it is present in L*er* as well.

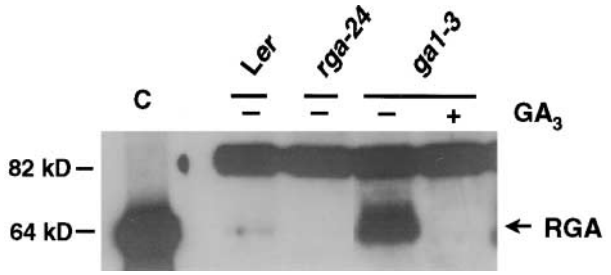

Figure 7. GA Treatment Reduces the Level of the Endogenous RGA Protein.

The blot contained 25 μg of total protein extracted from seedlings of L*er* and mutant plants as labeled. The leaves of the *ga1-3* plants were treated (+) or not treated (−) with GA_3 for 2 hr. Lane C, 2 ng of Ni column–purified 65-kD His-tagged RGA protein. A rabbit anti-RGA antiserum and a goat anti-rabbit IgG were used as primary and secondary antibodies, respectively. The extra upper band in each lane represents nonspecific background protein because it is present in *rga-24* as well.

GA Control of RGA Protein Levels

Using the *GFP-RGA* fusion gene and anti-RGA antibodies, we demonstrated that the level of RGA protein in Arabidopsis is reduced rapidly by GA treatment. The ubiquitin/proteosome pathway appears to play a regulatory role in a number of plant growth processes, including photomorphogenesis, auxin and jasmonic acid signaling, and flower development (reviewed in Callis and Vierstra, 2000; Karniol and Chamovitz, 2000). The rapid disappearance of RGA in response to the GA signal suggests that ubiquitin-mediated proteolysis might be involved in controlling the level of RGA protein in the cell. Future studies using anti-ubiquitin antibodies and proteosome inhibitors will help to determine whether this proteolytic pathway plays a role in GA signaling.

In this study, we found that the GFP-RGA fusion protein in transgenic Arabidopsis is functional and that its response to the GA signal is similar to that of the endogenous RGA protein. The transgenic lines expressing the *RGA* promoter::*GFP-RGA* fusion gene will be a powerful tool to analyze the response of the RGA protein to environmental and developmental cues by visualizing GFP fluorescence in living cells. In mammalian cells, *N*-acetylglucosamine modification of proteins could increase their nuclear localization or stability or affect their activity (Snow and Hart, 1998; Comer and Hart, 2000). Because SPY is predicted to be an OGT and also functions as a repressor in GA signaling (Thornton et al., 1999), we hypothesized that SPY might modify and activate RGA and its homolog GAI (Sun, 2000). The GFP-RGA fusion protein will be useful for monitoring the level and localization of the RGA protein in the *spy* mutant background.

Model of the GA Signal Transduction Pathway

To date, the major components of the GA signaling pathway identified have been negative regulators (SPY, RGA, and GAI). It was hypothesized that the ground state in the GA signaling pathway is repressive (reviewed in Bethke and Jones, 1998; Harberd et al., 1998; Thornton et al., 1999; Sun, 2000). During the growth and development of wild-type plants, different cells in different tissues should have varying degrees of GA response. Appropriate GA response is achieved by the balance between the levels of the GA signal and the repressor proteins (RGA and GAI). Our current working hypothesis of GA signaling in Arabidopsis first considers two extreme conditions. When the GA signal is completely absent as a result of mutations in GA biosynthetic genes or in wild-type cells that are deficient in GA, the fully active transcriptional regulators (RGA and GAI) would directly or indirectly repress the expression of GA-induced genes. In contrast, when a high level of the GA signal is present, these repressors would be inactivated, allowing for derepression to occur and thus GA-mediated growth. Our

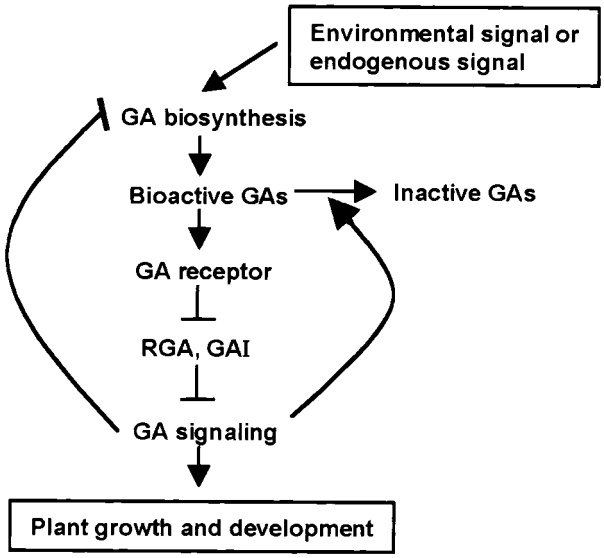

Figure 8. Proposed Role of RGA and GAI in GA Homeostasis.

In the ground (GA-deficient) state, RGA and GAI would repress GA signaling. After the synthesis of bioactive GAs, RGA and GAI would be inactivated (presumably by proteolysis), leading to the induction of GA response. The GA signaling pathway then would reduce bioactive GAs through the inhibition of GA biosynthesis and the induction of GA catabolism. An environmental or endogenous signal would keep the level of bioactive GAs above the homeostatic mean and allow for GA-stimulated growth and development. After the input signal stopped, the system would return to its basal level. Arrows and T-bars indicate positive and inhibitory effects, respectively.

data indicate that protein degradation plays an important role in modulating RGA activity by the GA signal.

One can imagine that the GA response in many cells within a plant lies between these two extremes. This hypothesis also explains a quantitative control in GA-regulated growth. The amount of the GA signal would be reflected in the amount of repressor degraded, which would then control the degree of derepression and consequent growth. It will be interesting to determine if GAI protein levels are controlled by a similar mechanism. In addition to RGA and GAI, three other predicted GRAS proteins in the Arabidopsis database also contain the DELLA region. Future studies using a reverse genetics approach will reveal whether these homologous genes have an overlapping function in controlling GA response.

METHODS

Plant Growth Conditions

Arabidopsis thaliana seed were stratified for 3 days at 4°C before planting. To induce germination, *ga1-3* and *rga/ga1-3* mutant seed were treated with 100 μM GA_3 during the stratification period in all experiments, except for the studies on the level of endogenous RGA protein in *ga1-3* (50 μM GA_4 was used instead). Afterward, seeds were rinsed thoroughly with water before sowing. The plants were grown on soil under a 16-hr-light/8-hr-dark cycle or on medium with Murashige and Skoog (1962) (MS) salts and 2% sucrose under continuous light at 22°C with a light intensity of 150 μE.

Gibberellin Analysis

Plants were grown on soil, and aerial portions of the plants were harvested just before flowering. The tissue was frozen in liquid nitrogen and stored at −80°C. Approximately 100 g (fresh weight) of plant tissue from each line was lyophilized to yield 10 g dry weight. Gibberellin (GA) analysis was performed as described previously (Gawronska et al., 1995; Furukawa et al., 1997). Briefly, 3 g dry weight of plant tissue was extracted in 80% methanol with 1 ng each of ^2H-labeled GAs (17-$^2H_2$-GA_1, 2,2,6-$^2H_3$-GA_4, 17-$^2H_2$-GA_8, 2,2,6-$^2H_3$-GA_9, 17-$^2H_2$-GA_{12}, 17-$^2H_2$-GA_{15}, 17-$^2H_2$-GA_{19}, 17-$^2H_2$-GA_{20}, 17-$^2H_2$-GA_{29}, 17-$^2H_2$-GA_{24}, 17-$^2H_2$-GA_{34}, 20-$^2H_2$-GA_{44}, and 17-$^2H_2$-GA_{53}) as internal standards. After a series of organic extractions, the extracts were purified by HPLC and then analyzed by gas chromatography–selected ion monitoring (GC-SIM; Hewlett-Packard [Palo Alto, CA] 5890 series II gas chromatograph with a J&W Scientific [Folsom, CA] DB-1 column and a JEOL [Peabody, MA] JMSAM150 mass spectrometer with Lucy version 2.0 software). Each sample was measured twice for GA analysis, and the average of the values from each experiment is reported.

Plasmid Construction for GFP-RGA Expression

A 3.6-kb PstI DNA fragment containing the cauliflower mosaic virus *35S* promoter::*GFP-RGA* fusion was isolated from PstI-digested pRG34F (Silverstone et al., 1998). This DNA was ligated with PstI-BamHI adaptors, digested with BamHI, and ligated into the BamHI-digested binary vector pDHB321.1 (a gift from David Bouchez, Institut National de la Recherche Agronomique, Versailles, France) to create pRG36. Multiple cloning steps were performed to place the green fluorescent protein (GFP) coding sequence at the 5′ translational start site of the *RGA* gene in a large genomic clone to create the *RGA* promoter::*GFP-RGA* fusion gene. A 15-kb AvrII DNA fragment from genomic clone λRG2 was ligated into the AvrII site of a modified pUC19 vector in which NotI-AvrII-XhoI-NotI polylinkers were inserted into the SalI site, and the KpnI, SacI, and EcoRI sites were removed (pMH5025a; a gift from Mary Honma, Duke University, Durham, NC). This plasmid was named pRG103. To create a KpnI site immediately 5′ upstream of the ATG start site of the *RGA* coding sequence, a 4-kb SalI-SacI DNA fragment spanning the *RGA* locus from pRG103 was first subcloned into SalI and SacI sites in pUC18 to create pRG102. Polymerase chain reaction (PCR)–based "overlap extension" mutagenesis (Ho et al., 1989) was performed to generate a KpnI site in front of the *RGA* coding sequence in pRG102.

The first round of PCR used two sets of primers: universal M13-20 forward primer and primer 227 (5′-CTCTCTTCATAGGTACCGCTATGAGTTT-3′; KpnI site underlined) and reverse primer and primer 228 (5′-AAACTCATAGCGGTACCTATGAAGAGAG-3′; KpnI site underlined). The second round of PCR used the universal M13-20 forward and reverse primers. The resulting 4-kb PCR DNA was cut with SalI and SacI and ligated with the SalI- and SacI-digested vector pHXKc (pHXK with the KpnI site removed) to generate pRG108. A 0.75-kb KpnI mGFPS65T DNA fragment was amplified from pRTL2-ΔNmGFPS65T (von Arnim et al., 1998) using forward primer GFP1 (5′-ATGCGGTACCTGATCCATGGGTAAGGAGAAGA-3′; KpnI site underlined) and reverse primer GFP2 (5′-TAGCGGTACCAGAGATCTGTATAGTTCATCCAT-3′; KpnI site underlined) and cut with KpnI. This GFP DNA fragment was then inserted into the KpnI site in pRG108 to create pRG49, which contains the *GFP-RGA* fusion. The 4.7-kb SalI-SacI DNA fragment (containing the *GFP-RGA* fusion) was isolated from pRG49 and used to replace the 4-kb SalI-SacI DNA fragment in pRG103, which contains the 15-kb AvrII genomic DNA fragment around the *RGA* locus. The resulting plasmid (pRG50) was digested with AvrII, and the 15.7-kb insert DNA fragment was cloned into the XbaI site of the binary vector pOCA28 (a gift from Neil Olszewski, University of Minnesota, St. Paul) to create pRG51.

Once the *RGA* and *GFP* DNA fragments were cloned into appropriate vectors, the DNA sequences of the coding regions were determined by DNA sequence analysis to ensure that no mutations were introduced during PCR.

Plant Transformation

pRG36 and pRG51 were introduced into wild-type Arabidopsis, ecotypes Landsberg *erecta* (L*er*) and Columbia (Col-0), and the null *rga/ga1-3* mutants via *Agrobacterium tumefaciens*–mediated transformation using the vacuum infiltration method (Bechtold et al., 1993). Transformants were selected on MS medium containing either 10 μg/mL glufosinate ammonium (Cresent Chemical Co., Happauge, NY) (for pRG36) or 50 μg/mL kanamycin (for pRG51). The number of T-DNA insertion loci was determined by scoring resistant and sensitive plants in the T_2 generation. Those that showed a 3:1 ratio (resistant:sensitive) were tested in the T_3 generation to identify homozygous transgenic lines. Two to four independent lines in the L*er*, Col-0, and *rga/ga1-3* backgrounds were isolated for each construct, and they showed a consistent pattern of GFP fluorescence in roots. For complementation tests, confocal microscopy, and immu-

noblot analysis, homozygous lines in the T_3 or T_4 generation were used. Two independent homozygous lines per construct (in both the L*er* and *rga*/*ga1-3* backgrounds) were examined in detail by fluorescence microscopy and immunoblot analysis.

Expression of RGA Protein in *Escherichia coli* and Production of Anti-RGA Antibodies

A full-length *RGA* cDNA fragment was amplified using primer 224 (5′-ACGCGGATCCGAATGAAGAGAGATCATCACC-3′; BamHI site underlined) and primer 217 (5′-ATTAAGATCTTCAGTACGCCGCCG-TCGAGA-3′; BglII site underlined) with pRG20 as the template. After BamHI and BglII digestion, this PCR DNA fragment was ligated into the BamHI site of pLexA-NLS to create pRG29. The *RGA* coding region in pRG29 was sequenced to ensure that no mutations were introduced during PCR. The 1.8-kb BamHI-SalI DNA fragment containing the *RGA* cDNA was then isolated from pRG29 and ligated into the BamHI and SalI sites of pQE-32 (Qiagen, Valencia, CA) to create pRG48. This plasmid encodes the 64-kD truncated RGA protein with the 6xHis tag at its N terminus (65-kD His-tagged RGA fusion protein).

To induce the production of this 65-kD His-tagged RGA protein, *E. coli* XL1-Blue cells containing pRG48 were treated with 0.2 mM isopropylthio-β-galactoside at $A_{600} = 0.7$ for 3 hr at 37°C. Cell cultures (50 mL) were then harvested by centrifugation, washed, and resuspended in 1 mL of 1 × binding buffer (His·Bind Kit; Novagen, Madison, WI). The cells were lysed using a French press (American Instrument Co., Silver Spring, MD) at 18,000 p.s.i. The lysate was centrifuged at 21,000*g* for 5 min at 4°C, and the pellet was resuspended in 1 mL of 1 × binding buffer containing 6 M urea and incubated for 1 hr at 4°C to dissolve the proteins in the inclusion bodies. The 65-kD His-tagged RGA fusion protein was purified using 0.5 mL of a 50% slurry of His·Bind Resin in the presence of 6 M urea as described in the His·Bind Kit protocol. Polyclonal antibodies were raised by immunization of a rabbit using the purified 65-kD protein (Cocalico Biologicals, Reamstown, PA), and the anti-RGA antibodies were purified by affinity chromatography (S.G. Thomas and T.-p. Sun, unpublished results).

Detection of the GFP-RGA Fusion Protein and the Endogenous RGA Protein by Immunoblot Analysis

L*er* and transgenic plants containing *GFP-RGA* fusion genes were grown on MS agar plates (35 × 10 mm) for 8 days (for control and GA treatments) or 7 days (for paclobutrazol [PAC] treatments). Whole seedlings were treated with either 1 mL of sterile water for 30 min (control) or 1 mL of 100 μM GA_3 or 1 mL of 100 μM PAC and 0.01% Tween 20 per plate for various times; then they were harvested and frozen in liquid N_2. Total plant proteins were extracted by grinding the tissues in 4% SDS, 25 mM Tris, pH 8.8, and 2.5% glycerol, boiled for 5 min, and centrifuged in a microcentrifuge for 5 min at room temperature. The supernatant fractions were transferred to new tubes, and protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA). Each sample then was adjusted to be in 1 × sample buffer and boiled again for 3 min. The proteins were separated by 8% SDS-PAGE and analyzed on immunoblots (Sambrook et al., 1989) using a 1000-fold dilution of anti-GFP polyclonal antibodies from rat (a gift from Maki Asano, Duke University) and a 10,000-fold dilution of peroxidase-conjugated goat anti-rat IgG (Pierce Chemical Co., Rockford, IL). The blots then were incubated in Supersignal Dura Reagent (Pierce), and the signals were detected by chemiluminescence.

To examine the level of the endogenous RGA protein, whole seedlings of 8-day-old L*er*, *rga-24*, and *ga1-3* mutants (with or without GA treatment) were harvested, and total proteins were extracted and fractionated as described above. The RGA protein was detected by immunoblot analysis using a 500-fold dilution of affinity-purified anti-RGA polyclonal antibodies from rabbit and an 8000-fold dilution of peroxidase-conjugated goat anti-rabbit IgG (Pierce). The signals on blots were detected as described above.

Confocal Laser Microscopy

A Zeiss (Jena, Germany) LSM410 inverted confocal laser microscope with 40× and 63× oil objectives was used in these studies. To detect GFP fluorescence, the excitation wavelength was 488 nm, and a bandpath filter of 510 to 525 nm was used for emission. For the short time point experiments, root tips from 6-day-old transgenic plants expressing *35S*::*GFP-RGA* were mounted on standard microscope slides in the presence of water or 100 μM GA_3. The slide was sealed using nail polish, the root tips were scanned through a Z series, and three-dimensional images of roots were reconstructed using the three-dimensional projection software.

In separate experiments, 7- or 8-day-old transgenic seedlings expressing *GFP-RGA* under the control of the *35S* or the *RGA* promoter were treated with GA or PAC on MS plates as described for the immunoblot experiments. At different times, the root tips were mounted on microscope slides and GFP fluorescence was detected using the confocal microscope.

RNA Gel Blot Analysis

Thirteen-day-old seedlings grown on MS agar plates (100 × 15 mm) were either harvested (−GA sample) or treated with 3 mL of 100 μM GA_3 per plate for 8 hr and then harvested and frozen in liquid N_2. RNA was isolated as described (Ausubel et al., 1990). The *GA4* mRNA levels were examined by RNA gel blot analysis using a *GA4*-specific antisense RNA probe as described previously (Yamaguchi et al., 1998). Radioactive signals were quantified using a phosphorimager as described (Silverstone et al., 1998). As a loading control, a 5′ end ^{32}P-labeled oligonucleotide (5′-TGAAGGGATGCCTCCAC-3′) corresponding to the Arabidopsis 18S rDNA sequence was used as a probe. The blot was prehybridized for 2 hr at 42°C in 10 × Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% BSA), 5 × SSPE (0.75 M NaCl, 50 mM sodium phosphate, and 5 mM EDTA, pH 7.4), 1% SDS, and 100 μg/mL salmon sperm DNA. ^{32}P-labeled 18S oligonucleotides were added, with the final concentration of oligonucleotides at 15 nM and 5 × 10^5 cpm/mL, and hybridized overnight at 42°C. The filters were washed four times (10 min each wash) in 6 × SSC (0.9 M NaCl and 0.09 M sodium citrate) and 0.1% SDS at 48°C and analyzed using a phosphorimager as described (Silverstone et al., 1998).

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