The DELLA motif is essential for gibberellin-induced degradation of RGA

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Communicated by Jan A. D. Zeevaart, Michigan State University, East Lansing MI, October 9, 2001 (received for review August 13, 2001)

***RGA* and *GAI* are homologous genes that encode putative transcriptional regulators that repress gibberellin (GA) signaling in *Arabidopsis*. Previously we showed that the green fluorescent protein (GFP)-RGA fusion protein is localized to the nucleus in transgenic *Arabidopsis*, and expression of this fusion protein rescues the *rga* null mutation. The GA signal seems to derepress the GA response pathway by degrading the repressor protein RGA. The GA-insensitive, semidominant, semidwarf *gai-1* mutant encodes a mutant protein with a 17-amino acid deletion within the DELLA domain of GAI. It was hypothesized that this mutation turns the gai protein into a constitutive repressor of GA signaling. Because the sequences missing in gai-1 are identical between GAI and RGA, we tested whether an identical mutation (*rga-Δ17*) in the *RGA* gene would confer a phenotype similar to *gai-1*. We demonstrated that expression of *rga-Δ17* or *GFP-(rga-Δ17)* under the control of the *RGA* promoter caused a GA-unresponsive severe dwarf phenotype in transgenic *Arabidopsis*. Analysis of the mRNA levels of a GA biosynthetic gene, *GA4*, showed that the feedback control of GA biosynthesis in these transgenic plants was less responsive to GA than that in wild type. Immunoblot and confocal microscopy analyses indicated that rga-Δ17 and GFP-(rga-Δ17) proteins were resistant to degradation after GA application. Our results illustrate that the DELLA domain in RGA plays a regulatory role in GA-induced degradation of RGA. Deletion of this region stabilizes the rga-Δ17 mutant protein, and regardless of the endogenous GA status rga-Δ17 becomes a constitutively active repressor of GA signaling.**

Bioactive gibberellins (GAs) are important plant hormones that regulate many aspects of plant growth and development (reviewed in refs. 1–3). In *Arabidopsis thaliana* they promote seed germination, leaf expansion, flowering, stem elongation, and flower development. Mutants that are severely defective in GA biosynthesis show defects in all the growth processes mentioned above (4, 5). For example, the *Arabidopsis* mutant *ga1-3*, which does not produce the enzyme for the first committed step of GA biosynthesis, contains extremely low levels of bioactive GAs and cannot germinate without GA treatment (6, 7). It is a small, dark green dwarf that does not bolt and is male-sterile (4). The GA-deficient phenotypes of the *ga1-3* mutant can be rescued by applications of GA.

Recent studies have identified *SPY*, *RGA*, and *GAI* as negative regulators of GA signaling in *Arabidopsis* (reviewed in refs. 8–10). *SPY* is likely to encode an *O*-linked *N*-acetylglucosamine (GlcNAc) transferase, which regulates target protein function by GlcNAc modification of Ser or Thr residues (8, 11). Recessive *spy* mutations partially suppress all phenotypes of the *ga1* mutants (12, 13). RGA and GAI are 82% identical in their amino acid sequences, and both contain structural features of transcription regulators including homopolymeric Ser and Thr motifs, Leu heptad repeats, and an Src homology 2-like domain (14–16). RGA and GAI also contain nuclear localization signals, and a green fluorescent protein (GFP)-RGA fusion protein was shown to be localized in the nucleus of stably transformed *Arabidopsis* (7). Loss-of-function *rga* mutations partially suppress defects of *ga1-3* in leaf expansion, stem growth, and flowering time (13). The *rga-24/gai-t6* double null mutations completely restore all processes that are partially rescued by *rga* (17, 18). However, the *gai-t6* null allele alone has little effect in suppressing the phenotype of *ga1-3* (17). Therefore, RGA and GAI have similar functions in repressing GA signaling, but RGA plays a more dominant role than GAI.

RGA and GAI are members of the GRAS family (*G*AI, *RGA*, and *S*CARECROW) of plant regulatory proteins (19). GRAS members are highly homologous in their C-terminal regions but show great variation in their N termini (19). Therefore, the N-terminal regions may be responsible for specifying GRAS protein function in particular pathways. RGA and GAI have a conserved N-terminal domain named DELLA after an amino acid motif contained therein (14, 15). The semidominant *gai-1* allele (20) contains a 51-bp in-frame deletion in the region encoding the DELLA sequence, which results in a 17-amino acid deletion in the gai-1 protein (15). The *gai-1* mutant shows a semidwarf phenotype that resembles GA-deficient mutants but cannot be rescued by GA treatment (20). Interestingly, *gai-1* is not completely insensitive to GAs but rather is saturated in the GA response and contains high levels of bioactive GAs (20, 21). *gai-1/ga1-1* is a severe dwarf, which can be rescued partially by applied GAs to become a semidwarf (20). Peng *et al.* (15) hypothesized that the GA signal may inhibit GAI function by interacting directly or indirectly with the DELLA sequence. They also proposed that deletion of the DELLA sequence turned the gai-1 protein into a constitutively active repressor of GA signaling. Recently, the functional orthologs of *RGA* and *GAI* in several crops such as *Rht* in wheat (16), *d8* in maize (16), and *SLR* in rice (22, 23) have been isolated. Deletions of the DELLA region in these genes also confer a similar semidominant dwarf phenotype in these crops.

The above data suggest that GA may induce the GA signal transduction pathway by inhibiting the repressor proteins GAI and perhaps RGA. Our recent results further demonstrated that expression of the *RGA* gene is regulated mainly at the protein level by the GA signal. Transcript accumulation of the *RGA* and *GAI* genes in *Arabidopsis* seedlings are affected only slightly in different GA response mutant backgrounds or by GA treatment (14). In contrast, the levels of both the endogenous RGA protein and the GFP-RGA protein are reduced dramatically after application of GA for 0.5–2 h (7). Therefore, the GA signal seems to derepress the GA signaling pathway by causing degradation of the repressor protein RGA.

The 17 amino acids (DELLAVLGYKVRSSEMA) deleted in gai-1 are identical between GAI and RGA. In this report, we tested whether the same mutation in *RGA* (named *rga-Δ17*)

Abbreviations: GA, gibberellin; GFP, green fluorescent protein; *PRGA*, *RGA* promoter; L*er*, Landsberg *erecta*; *35S*, cauliflower mosaic virus *35S* promoter; MS, Murashige and Skoog.

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would cause a phenotype similar to that of *gai-1*. The *rga-Δ17* mutant gene and a *GFP-(rga-Δ17)* fusion gene were expressed under the control of the endogenous *RGA* promoter (*PRGA*). We showed that both *rga-Δ17* and *GFP-(rga-Δ17)* conferred a semidominant GA-unresponsive dwarf phenotype in transgenic *Arabidopsis*. We further demonstrated that both rga-Δ17 and GFP-(rga-Δ17) proteins were resistant to GA-induced degradation, indicating that the DELLA sequence is important for the degradation of RGA induced by the GA signal.

It has been shown that GA biosynthesis is affected by the activity of the GA response pathway through a feedback mechanism (reviewed in refs. 24 and 25). The *GA4* gene in *Arabidopsis* encodes GA 3β-hydroxylase, which catalyzes the conversion of GA precursors to bioactive GAs (26). This gene has been used as a reporter to gauge feedback regulation of GA biosynthesis. In this work, we showed that the *rga-Δ17* and *GFP-(rga-Δ17)* transgenic plants accumulated higher levels of the *GA4* mRNA than Landsberg *erecta* (L*er*), and GA treatment did not downregulate the elevated *GA4* transcript levels in these plants.

Materials and Methods

Plasmid Constructions. pRG38 was created by inserting the *RGA* coding region between the cauliflower mosaic virus *35S* promoter (with translational enhancer) and the *35S* terminator, and then this *35S*::*RGA* fusion gene was placed into the *Bam*HI site of the binary vector pDHB321.1 (a gift from David Bouchez, Institut National de la Recherche Agronomique, Versailles, France).

PCR-based "overlap extension" mutagenesis (27) was performed to generate the 51-bp deletion (identical to the mutation in *gai-1*) in the *RGA* gene in pRG102 (7). This *rga* allele, *rga-Δ17*, was fused to *PRGA* to create *PRGA*::*(rga-Δ17)*, and this fusion gene was placed into the *Xba*I site of the binary vector pOCA28. The resulting plasmid was named pRG41. Another plasmid, pRG59, was generated that contained *PRGA*::*GFP-(rga-Δ17)* in the *Xba*I site of pOCA28.

Once the *RGA* DNA fragments were cloned into appropriate vectors, the coding regions were analyzed by DNA sequence analysis to ensure that no mutations were introduced during PCR. More detailed information on plasmid construction is published as supporting information on the PNAS web site, www.pnas.org.

Transformation and Isolation of Transgenic Lines. By using *Agrobacterium*-mediated transformation (28), L*er* was transformed with pRG38, pRG41, and pRG59, *rga-24/ga1-3* [a semidwarf (17)] was transformed with pRG38, and *rga-24* [phenotypically similar to L*er* (17)] was transformed with pRG41. pRG41 and pRG59 transformants were selected on Murashige and Skoog (MS) medium (29) containing 50 μg/ml kanamycin. pRG38 transformants were selected on MS medium containing 10 μg/ml gluphosinate ammonium (Crescent Chemical, Happauge, NY). The number of T-DNA (portions of the tumor-inducing plasmid that are transferred to plant cells) insertion loci was determined in the T_2 generation based on segregation ratio on MS medium containing 40 μg/ml kanamycin (for pRG41 and pRG59) or 10 μg/ml gluphosinate ammonium (for pRG38). Transformants with an $\approx$3:1 ratio of resistant/sensitive were tested in the T_3 generation to identify lines homozygous for the transgene.

Plant Growth Conditions. Plants were grown under Cool White fluorescent bulbs (photon flux of 140 μmol m^{-2}·s^{-1}) in long day conditions (16 h light and 8 h dark) at 22°C. To determine whether the plants were responsive to GA treatment, the plants grown on soil were sprayed with 100 μM GA_3 weekly starting from 18 days after sowing. The experiment to determine the GA response curve for hypocotyl growth was performed as described (13) except that the seedlings were grown in long day conditions.

Immunoblot Analysis. Seeds of L*er*, *rga-24*, *ga1-3*, and different transgenic lines were sterilized and imbibed for 3 days in 50 μM GA_4 (*ga1-3*-containing lines) or water (*GA1*-containing lines) at 4°C. The *ga1-3*-containing lines then were rinsed thoroughly with sterile water. All seeds were plated on MS plates (100 × 15 mm) and grown under continuous light of 100 μmol m^{-2}·s^{-1} at 22°C. The seeds of the *rga-24/PRGA*::*(rga-Δ17)* line were produced from a hemizygous plant. The seedlings that did not contain the transgene had a wild-type phenotype (longer hypocotyls and larger leaves) and were discarded from the plate after 7 days. Seedlings were harvested after 8 days or treated with 2 ml of 100 μM GA_3 for 2 h before harvesting. Total plant proteins were extracted and analyzed by immunoblot analysis using anti-GFP or affinity-purified anti-RGA antibodies as described (7).

Confocal Laser Microscopy. The transgenic *Arabidopsis* plants were grown on MS plates in continuous light for 8 days and treated with water or with 100 μM GA_3 for 2 h as described (7). The root tips were excised with razor blades, and GFP fluorescence was detected by using a Zeiss LSM-410 confocal laser microscope as described (7).

Measurement of *GA4* mRNA Levels. Seedlings (13 days old) grown on MS plates were treated with water or 100 μM GA_3 as described (17). One modification was that wild-type seedlings were removed from the plates containing the segregating transgenic plants carrying *PRGA*::*(rga-Δ17)* after 7 days. Total RNA was isolated, and *GA4* mRNA was detected by using an antisense *GA4* RNA probe as described (30). As a loading control 18S rRNA levels on the same blot were determined by using a labeled oligonucleotide probe as described (17).

Results

To investigate the role of the DELLA sequence of RGA in response to the GA signal, we generated transgenic *Arabidopsis* that contains a mutant *rga* gene (*rga-Δ17*) with the deletion identical to that found in *gai-1*. To ensure that *rga-Δ17* was expressed properly, this mutant gene was flanked by 8 kb of 5′-upstream and 5.8 kb of 3′-downstream sequences around the *RGA* locus to generate the *RGA* promoter *(PRGA)*::*(rga-Δ17)* fusion gene. A *PRGA*::*GFP-(rga-Δ17)* fusion gene also was expressed in transgenic *Arabidopsis* to examine the subcellular localization of *rga-Δ17* by using epifluorescence and confocal laser microscopy. Previously we showed that the GFP-RGA fusion protein was functional in transgenic plants to rescue the *rga* null mutant phenotype (7). This GFP-RGA fusion protein has been a powerful tool to monitor the rapid effect of GA on the RGA protein level in the nucleus.

Transgenic Plants Expressing *rga-Δ17* or *GFP-(rga-Δ17)* Are GA-Unresponsive Dwarfs. By using *Agrobacterium*-mediated transformation (28, 31), L*er* and the *rga-24* null mutant plants were transformed with *PRGA*::*(rga-Δ17)*. Six and four kanamycin-resistant T_1 plants in L*er* and the *rga-24* mutant backgrounds, respectively, were isolated. Five of the L*er* lines and two of the *rga-24* lines showed a semidwarf phenotype that could not be rescued by GA_3 treatment, indicating that *rga-Δ17* constitutively represses plant growth similar to *gai-1*. In the T_2 generation, all the seven semidwarf lines segregated $\approx$3:1 (resistant/sensitive) ratio for kanamycin selection, indicating that they each contain a single T-DNA (portion of the tumor-inducing plasmid that is transferred to plant cells) insertion locus. We found that the kanamycin-resistant T_2 plants of each of these lines segregated into two phenotypic groups, i.e., semidwarfs with reduced fertility or extreme dwarfs that were sterile. The semidwarf and extreme dwarf phenotypes were very similar among the seven lines examined (data not shown). We predicted that the semidwarfs were hemizygous for the *rga-Δ17* transgene, whereas the

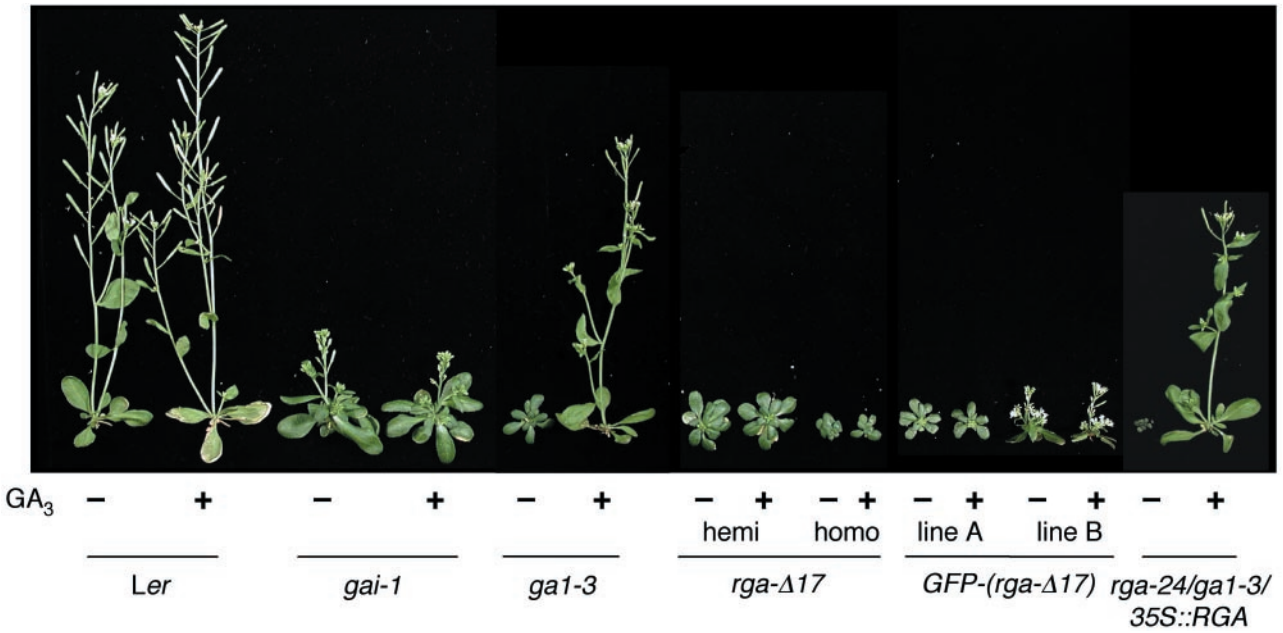

Fig. 1. Effect of GA_3 treatment on the phenotypes of control and transgenic lines. All lines are 36 days old and have been treated (+) or not treated (−) with GA_3 as indicated. All the lines except the hemizygous *rga-Δ17* line are homozygous for the mutation and/or the transgene as labeled. homo, plants are homozygous for the transgene; hemi, plants are hemizygous for the transgene.

extreme dwarfs were homozygotes. To verify that the sterile dwarfs were homozygotes, we grew T_3 progeny of a semidwarf T_2 plant on soil without kanamycin selection and found 48 plants with a wild-type phenotype, 87 semidwarfs, and 41 extreme dwarfs ($\approx$1:2:1, $\chi^2 = 0.57, P > 0.7$). These results confirmed that the sterile dwarfs are homozygous for the *rga-Δ17* transgene and that *rga-Δ17* is semidominant.

We also generated transgenic L*er* plants that carry *PRGA*::*GFP-(rga-Δ17)*. Eight independent kanamycin-resistant T_1 plants were isolated, and all showed the semidwarfed phenotype, indicating that the GFP-(rga-Δ17) fusion protein has a similar effect on plant growth as rga-Δ17. Further analysis of these lines in the T_2 and T_3 generations indicated that *GFP-(rga-Δ17)* is semidominant, and all eight lines contain a single insertion locus. Interestingly, homozygous plants of all eight lines were reduced in fertility but were not completely sterile. Because these lines varied in their final heights, two of the homozygous lines (A and B) with different severity in their phenotypes were chosen for further studies.

Fig. 1 shows the phenotypes of L*er*, *gai-1*, and *ga1-3* in comparison to transgenic lines that contain hemizygous or homozygous *PRGA*::*(rga-Δ17)* (in the *rga-24* background) or homozygous *PRGA*::*GFP-(rga-Δ17)* (in L*er* background). In the remainder of this paper, *rga-24/PRGA*::*(rga-Δ17)* and L*er*/*PRGA*::*GFP-(rga-Δ17)* will be referred to as the *rga-Δ17* and *GFP-(rga-Δ17)* lines, respectively. Unlike the GA biosynthetic mutant *ga1-3* but similar to *gai-1* the *rga-Δ17* or *GFP-(rga-Δ17)* containing dwarf plants did not respond to GA treatment in leaf expansion or stem growth (Figs. 1 and 2). All three transgenic lines are dwarfed more severely than *gai-1* (Figs. 1 and 2). The homozygous *rga-Δ17* plant had the most severe phenotype, with a smaller rosette than untreated *ga1-3*. It did not bolt and died before the hemizygous *rga-Δ17* reached its final height (Fig. 2). Line A of the *GFP-(rga-Δ17)* expressing plants had a smaller rosette and shorter final height than line B (Figs. 1 and 2).

GA Response Curve for Hypocotyl Growth. To examine quantitatively the effect of *rga-Δ17* on the GA responsiveness in shoot growth, we measured the hypocotyl length of *rga-Δ17*, *GFP-(rga-Δ17)*, and control *ga1-3* and *rga-24/ga1-3* seedlings in the presence of different concentrations of GA_3. Consistent with our previous results (13), the hypocotyl growth response of *ga1-3* and *rga-24/ga1-3* is linear from 0.1 to 20 μM GA_3 (Fig. 3). An inhibitory effect on hypocotyl elongation was observed at 50 μM GA_3. In contrast, the hypocotyls of *rga-Δ17* and *GFP-(rga-Δ17)* (both A and B lines) were insensitive to GA treatment and had a similar length as in the absence of GA (Fig. 3*A*).

The severe dwarf phenotype of transgenic plants expressing the rga-Δ17 or GFP-(rga-Δ17) mutant protein is similar to *rga-24/ga1-3* carrying the cauliflower mosaic virus *35S* promoter::*GFP-RGA* fusion gene (*35S*::*GFP-RGA*; ref. 7). In this study, we also generated transgenic *Arabidopsis* that overexpressed *RGA* under the control of the constitutive cauliflower

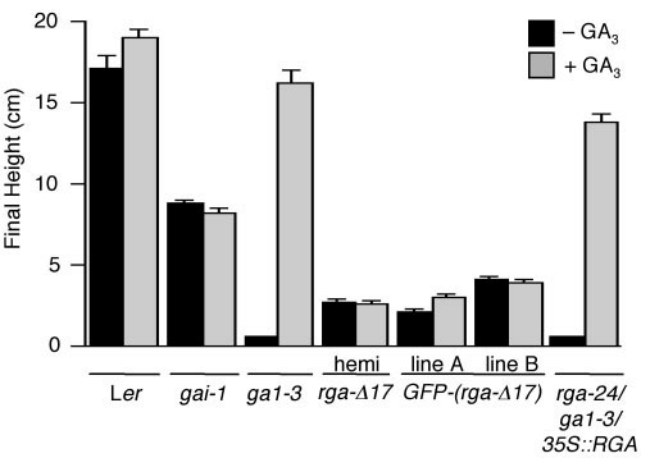

Fig. 2. Final heights of plants in response to repeated applications of GA_3. All the lines except the hemizygous *rga-Δ17* line are homozygous for the mutation and/or the transgene as labeled. Final heights of untreated (−) and GA-treated (+) plants are shown in black and gray, respectively. hemi, plants are hemizygous for the transgene. Means ± SE were measured for 8–12 plants per line.

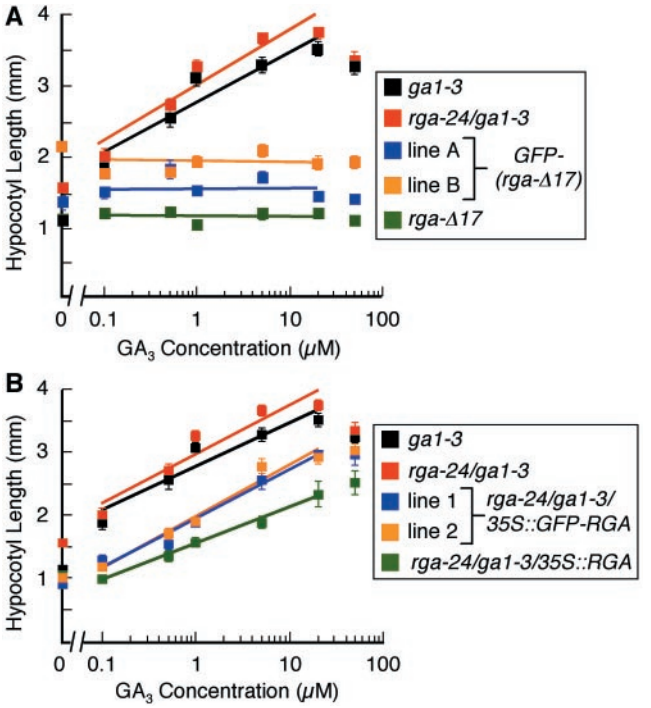

Fig. 3. Hypocotyl growth response to GA_3. All the lines except the hemizygous *rga-Δ17* line are homozygous for the mutation and/or the transgene as labeled. Hypocotyl lengths of *ga1-3* and *rga-24/ga1-3* were compared with *rga-Δ17* and *GFP-(rga-Δ17)* lines (*A*) and *RGA* and *GFP-RGA* overexpression lines (*B*). The curves are shown for GA_3 concentrations that give a linear response. The values plotted are the means ± SE of 12 seedlings measured. Some error bars are too small to be seen.

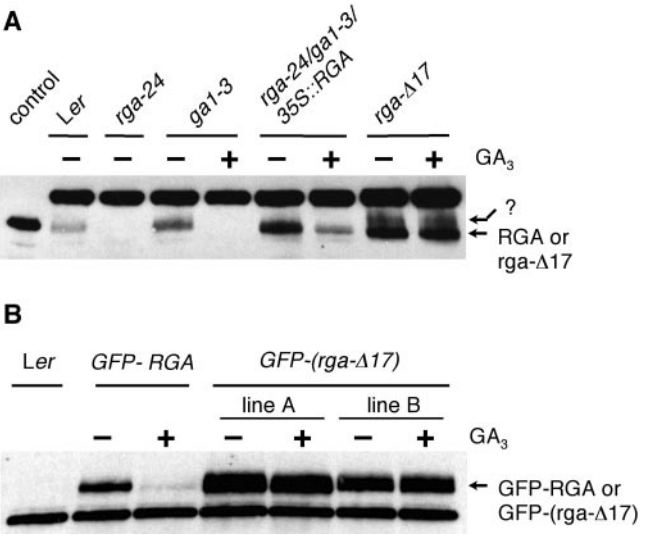

Fig. 4. The rga-Δ17 protein is resistant to GA treatment. The blots contain total plant proteins (25 μg in *A* and 50 μg in *B*) extracted from 8-day-old seedlings after treatment with water (−) or GA (+) as labeled. (*A*) Affinity-purified rabbit anti-RGA polyclonal antibodies and a peroxidase-conjugated goat anti-rabbit IgG were used to detect the RGA (64-kDa) and rga-Δ17 (62-kDa) proteins. Control lane, 0.5 ng of nickel column-purified 65-kDa His-tagged RGA protein. The upper arrow with a question mark indicates the unknown protein that is present only in plants expressing the rga-Δ17 protein. (*B*) Rat anti-GFP polyclonal antibodies and a peroxidase-conjugated goat anti-rat IgG were used to detect GFP-RGA and GFP-(rga-Δ17) fusion proteins (91- and 89-kDa, respectively). The extra upper band in *A* and the additional lower band in *B* are nonspecific background proteins.

mosaic virus *35S* promoter in L*er* and *rga-24/ga1-3* backgrounds. We found that overexpression of the RGA protein not only rescued the phenotype caused by the *rga-24* mutation in *rga-24/ga1-3* but also made the plant even smaller than *ga1-3* (Fig. 1). Repeated GA_3 treatment was able to overcome the effect of overexpression of RGA and resulted in plants similar to GA-treated *ga1-3* (Figs. 1 and 2). However, the hypocotyl growth in *rga-24/ga1-3* carrying *35S*::*RGA* or *35S*::*GFP-RGA* was ≈10-fold less sensitive to GA_3 than that in *rga-24/ga1-3* (Fig. 3*B*).

In L*er* background, however, *35S*::*RGA* did not cause as dramatic a phenotype as in the *rga-24/ga1-3* background (data not shown). These results are consistent with our previous findings that RGA is a more active repressor in a GA-deficient background than in a wild-type GA background (17). Also, *35S*::*GFP-RGA* represses GA-induced rosette growth more efficiently than the endogenous RGA protein, but this effect was only detected in the *ga1-3* background (7).

The rga-Δ17 Protein Is Resistant to GA-Induced Degradation. We demonstrated previously that the RGA protein is accumulated at a higher level in the GA-deficient *ga1-3* mutant than in L*er* (7). In addition, the levels of RGA and GFP-RGA are reduced rapidly in response to GA treatment (ref. 7; also see Fig. 4). Because expression of *rga-Δ17* and *GFP-(rga-Δ17)* conferred the GA-unresponsive dwarf phenotype, we examined whether the rga-Δ17 and GFP-(rga-Δ17) proteins are resistant to GA-induced degradation. For the analysis of the rga-Δ17 protein level, the seedlings that were hemizygous or homozygous for the *rga-Δ17* transgene were pooled together from a segregating population during protein extraction because only hemizygous *rga-Δ17* plants produced seeds. Immunoblot analysis using anti-RGA antibodies showed that the level of the rga-Δ17 mutant protein in transgenic *rga-Δ17* plants was much higher than the level of RGA in L*er* (Fig. 4*A*). After 2 h of GA treatment, the rga-Δ17 protein level remained very similar. In contrast, the high levels of the RGA protein in *ga1-3* and the RGA overexpression line (*rga-24/ga1-3/35S*::*RGA*) were reduced dramatically by the application of GA (Fig. 4*A*). Similarly, immunoblot analysis using anti-GFP antibodies demonstrated that the levels of the GFP-(rga-Δ17) fusion protein in both A and B transgenic lines carrying *PRGA*::*GFP-(rga-Δ17)* remained almost constant after GA treatment (Fig. 4*B*). The level of GFP-(rga-Δ17) in line A being higher than in line B is consistent with our finding that line A has a more dwarfed phenotype than line B (Fig. 2). Our results indicate that the rga-Δ17 and GFP-(rga-Δ17) proteins are resistant to GA-mediated degradation.

Visualization of GFP-(rga-Δ17). To determine whether deleting the DELLA motif affects the subcellular localization of the rga mutant protein, we examined GFP fluorescence in the root tips of transgenic plants expressing GFP-(rga-Δ17) by using confocal laser microscopy (Fig. 5). Similar to the transgenic plant expressing *PRGA*::*GFP-RGA*, the plants containing *PRGA*::*GFP-(rga-Δ17)* showed GFP fluorescence mainly in the nuclei, suggesting that the DELLA motif does not play a major role in facilitating the localization of RGA to the nucleus. Consistent with the results of immunoblot analysis, the GFP fluorescence in the nuclei of root tips of the *GFP-(rga-Δ17)*-expressing plants (both lines A and B) was not affected by the GA treatment, whereas the nuclear fluorescence was not detectable in the *GFP-RGA*-expressing plant after GA treatment (Fig. 5). These results further support that the DELLA motif is important for GA-mediated degradation of RGA. We expected that the transgenic line A would show higher GFP fluorescence than line B, because line A accumulated a higher amount of GFP-(rga-Δ17) determined by immunoblot analysis (Fig. 4*B*).

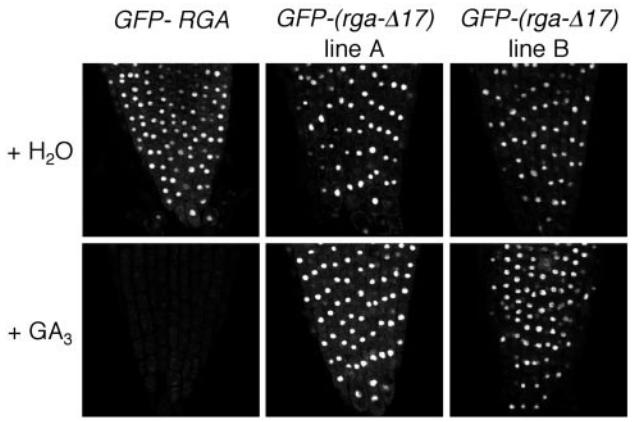

Fig. 5. Effect of GA on the fluorescence in the roots of transgenic plants expressing the GFP-RGA and GFP-(rga-Δ17) fusion proteins. Transgenic seedlings were incubated for 2 h with water (+ H_2O) or 100 μM GA_3 (+ GA_3), and then fluorescence in root tips was visualized by confocal laser microscopy under an identical setting for all images.

However, we did not detect any difference in the fluorescence level between these lines, probably because the fluorescence was saturated under the imaging settings that were used.

***rga-Δ17* Is Less Responsive to GA in the Feedback Inhibition of *GA4* Expression than L*er* and *ga1-3*.** It is known that expression of the GA biosynthetic gene *GA4* is affected by the activity of GA response pathway via a feedback mechanism (reviewed in refs. 24 and 25). Previous studies showed that the *gai-1* mutant contained a higher level of *GA4* mRNA, which was not reduced by GA treatment (32). The GA-unresponsive dwarf phenotype of the transgenic plants expressing *rga-Δ17* or *GFP-(rga-Δ17)* suggests that these plants have reduced GA responses, which may affect the feedback inhibition of *GA4* expression. RNA blot analysis was performed to examine the *GA4* mRNA levels in transgenic *rga-Δ17* and *GFP-(rga-Δ17)* plants in comparison to L*er*, *gai-1*, and *ga1-3* with or without GA treatment (Fig. 6). The RNA sample for the *rga-Δ17* line was extracted from a pool of hemizygous and homozygous seedlings. In agreement with Cowling *et al.* (32), the *GA4* mRNA level in *gai-1* was 2-fold higher than in L*er* without GA treatment and was not reduced by GA treatment. *GA4* transcript levels in untreated *rga-Δ17* or *GFP-(rga-Δ17)* transgenic plants were 3–2-fold higher than L*er*, and GA treatment only reduced the *GA4* mRNA levels by 10–20% in these transgenic lines (Fig. 6). In contrast, *GA4* expression is down-regulated dramatically by GA in L*er* and *ga1-3*.

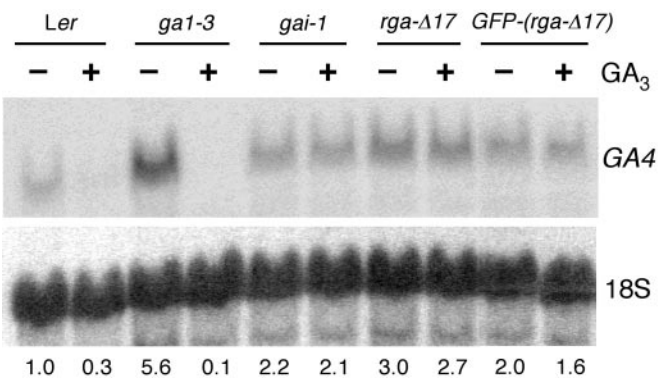

Fig. 6. Levels of *GA4* mRNA in L*er*, *ga1-3*, *gai-1*, *rga-Δ17*, and *GFP-(rga-Δ17)* line A. Shown is an autoradiogram of RNA blots containing 9 μg of total RNA isolated from 13-day-old seedlings with (+) or without (−) GA_3 treatment as labeled. The blot was hybridized with a labeled *GA4* antisense RNA probe and then reprobed with a labeled 18S rDNA probe. The values under the blots indicate the relative amounts of *GA4* mRNA after standardization using 18S rRNA as a loading control. The value of L*er* (−GA_3) was arbitrarily set to 1.0.

Discussion

The Role of the DELLA Motif in RGA. Previous genetic and molecular studies using the *gai-1* mutant had led to the GA-derepressible repressor model (15). Our biochemical analyses of the effect of GA on the stability of RGA (7) and rga-Δ17 protein (this study) not only support this model but also are beginning to uncover the molecular mechanism involved. The GA signal seems to derepress the GA signal transduction pathway by rapidly reducing the level of the repressor protein RGA (7). GA may affect the RGA protein translationally and/or posttranslationally. However, it is more likely that the GA signal triggers the degradation of the RGA protein, because as demonstrated in this paper, GA-inducible RGA protein disappearance depends on the presence of the amino acid sequence in the DELLA motif. Deletion of the 17 amino acid residues within the DELLA sequence turns the rga-Δ17 and GFP-(rga-Δ17) proteins into constitutive repressors of GA signaling, apparently by making these mutant proteins resistant to GA-induced degradation and increasing their stability. Consistent with this hypothesis, the transgenic plants expressing rga-Δ17 or GFP-(rga-Δ17) proteins confer the GA-unresponsive dwarf phenotype and are less responsive to GA-mediated feedback inhibition of the *GA4* gene expression.

We believe that the effect of GA on RGA and rga-Δ17 proteins presented in this paper are physiologically relevant because a 30-min treatment with 0.5 μM GA_4 (the major bioactive GA in *Arabidopsis*) also dramatically reduced the RGA protein level in the *ga1-3* mutant (F. Marsolais, A.D., and T.-p.S., unpublished results).

The molecular mechanism by which the GA signal induces RGA protein degradation is unclear, although ubiquitin-mediated proteolysis is a possibility. The ubiquitin-proteosome pathway plays a regulatory role in a number of cellular processes in eukaryotes including plants (reviewed in refs. 33 and 34). Recent advances in understanding auxin signaling has revealed that the stability of putative transcriptional regulators encoded by the *AUX/IAA* gene family is controlled by ubiquitin and COP9 signalosome-mediated proteolysis (34–37). Furthermore, auxin mediates the degradation of AUX/IAA proteins and domain II in these proteins is necessary for this degradation (38). Mutations in the domain II of the *AUX/IAA* genes confer an increased half-life of the AUX/IAA proteins and cause a reduced response to auxin (39–41).

The sequence around the DELLA motif of RGA might interact directly with the unidentified regulatory protein that mediates the GA signal, or this sequence might be the target of protein modification such as phosphorylation and/or GlcNAcylation. Many target proteins of the ubiquitin pathway need to be phosphorylated to have normal degradation rates (42). Alternatively, the effect of *rga-Δ17* mutation on protein stability could be indirect. For example, deleting the DELLA sequence might alter the conformation of the rga protein such that it can no longer be modified or interact with the regulatory protein. We are in favor of the former possibility for the following reason. Among the 27 independent *rga* mutants, we have identified six missense mutations, and none are located in the N-terminal DELLA domain (A. Silverstone and T.-p.S., unpublished results). This finding suggests that the C-terminal region of RGA is likely to be the functional domain as the repressor of GA signaling, whereas the N terminus is probably a regulatory domain to sense the GA signal. Future studies on the posttranslational modification of RGA and isolation of RGA interactors will help to elucidate how GA regulates RGA function.

Expression of *rga-Δ17* Inhibits GA-Regulated Flower Development. The *gai-1* mutant is a semidwarf, whereas transgenic plants containing the homozygous *PRGA::(rga-Δ17)* transgene exhibit a more severe dwarf phenotype than *ga1-3*. This finding is not surprising, because our previous results indicated that *RGA* is a more active repressor of GA-regulated stem growth than *GAI* (17). Therefore, the constitutively active rga-Δ17 protein would have a more dramatic effect in inhibiting plant growth than the gai-1 protein. The *rga-24/gai-t6* double null mutations did not suppress the nongerminating and sterile phenotypes of *ga1-3*, suggesting that RGA and GAI do not play a major role in repressing GA-induced seed germination and flower development (17). Seeds carrying the homozygous *PRGA::(rga-Δ17)* transgene are able to germinate without GA treatment. This observation supports that RGA is not a major repressor in seed germination. However, RGA and GAI may have a minor function in repressing flower development. We showed previously that the *rga-24/gai-t6* double null mutant had reduced fertility, possibly because of elevated GA signaling during flower development (17). In this work we found that expression of constitutively active rga-Δ17 under the control of the endogenous *RGA* promoter resulted in the sterile phenotype, further supporting that RGA is involved in regulation of flower development. Similarly, a minor role of GAI in flower development is illustrated by reduced fertility of the gain-of-function *gai-1* mutant (T.-p.S., unpublished results).

On average, the phenotype of the *GFP-(rga-Δ17)* lines is less severe than plants expressing *rga-Δ17*. It is possible that the GFP-(rga-Δ17) fusion protein is less active than rga-Δ17, although both function as constitutive repressors of GA signaling. The extra GFP domain at the N-terminal end of the fusion protein may affect its conformation and/or interfere with interaction between RGA and its interactors. Additionally, our results indicate that there are dosage effects in the *rga-Δ17* lines, because we saw that *GFP-(rga-Δ17)* line A, which contains more protein than line B, has a more severe phenotype. Also, the hemizygous *rga-Δ17* plants are fertile and semidwarfed, whereas their homozygous siblings are sterile dwarfs. These data support our model (7) that the exact amount of active RGA protein (modulated by the GA signal) reflects the degree of repression of GA signaling and GA-mediated growth. It will be interesting to see whether the activity of GAI is controlled by a similar protein degradation mechanism.

We thank Maki Asano (Duke University) for providing anti-GFP antibodies, Steve Thomas for help in affinity purification of anti-RGA antibodies, and Aron Silverstone for critical reading of the manuscript. This work was funded by National Science Foundation Grants IBN-9723171 and IBN-0078003.

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