Gibberellins Are Not Required for Normal Stem Growth in *Arabidopsis thaliana* in the Absence of GAI and RGA

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ABSTRACT

The growth of *Arabidopsis thaliana* is quantitatively regulated by the phytohormone gibberellin (GA) via two closely related nuclear GA-signaling components, GAI and RGA. Here we test the hypothesis that GAI and RGA function as "GA-derepressible repressors" of plant growth. One prediction of this hypothesis is that plants lacking GAI and RGA do not require GA for normal stem growth. Analysis of GA-deficient mutants lacking GAI and RGA confirms this prediction and suggests that in the absence of GAI and RGA, "growth" rather than "no growth" is the default state of plant stems. The function of the GA-signaling system is thus to act as a control system regulating the amount of this growth. We also demonstrate that the GA dose dependency of hypocotyl elongation is altered in mutants lacking GAI and RGA and propose that increments in GAI/RGA repressor function can explain the quantitative nature of GA responses.

THE shoots of plants are composed of organs whose identities are initially defined in the shoot apical meristem (Steeves and Sussex 1989). Organ growth follows the initiation of organ primordia, and most of the cells that comprise the adult shoot arise from cell divisions that occur during this growth phase. During growth, coordinated cell division and expansion determine the final size and shape of each organ. Growth is controlled by the phytohormone gibberellin (GA) and can be largely unidirectional, as in elongating stem internodes, or multidirectional, as in expanding leaves. Studies of plants lacking enzymes in the GA biosynthetic pathway have shown that many different stages of the *Arabidopsis thaliana* life cycle require GA, from seed germination to the growth of hypocotyls, stem internodes, leaves, stamens, and petals (Koornneef and van der Veen 1980; Zeevaart and Talon 1992). In addition, GA is required for the normal flowering response to day length (Wilson *et al.* 1992; Blázquez *et al.* 1998).

The pathway from the perception of GA, probably by a receptor in the plasma membrane (Hooley *et al.* 1991; Gilroy and Jones 1994), to the responses described above is beginning to be understood. In recent years, the genes encoding several GA-signaling components have been identified and cloned, and how these proteins control the response to GA is becoming clearer (Harberd *et al.* 1998). In particular, the proteins encoded by the related Arabidopsis genes *GAI* (Koornneef *et al.* 1985; Peng and Harberd 1993; Peng *et al.* 1997) and *RGA* (Silverstone *et al.* 1997, 1998), and also by orthologs in other plant species such as wheat (*Rht-B1a* and *Rht-D1a*) and maize (*d8*) (Peng *et al.* 1999a), play an important role in GA signaling. The GAI/RGA/Rht/D8 proteins are members of a larger family of putative plant transcription factors called GRAS (Pysh *et al.* 1999). GRAS proteins regulate several different aspects of plant growth and development in addition to GA signaling, including root development (Di Laurenzio *et al.* 1996; Helariutta *et al.* 2000), lateral branch development (Schumacher *et al.* 1999), and phytochrome A signal transduction (Bolle *et al.* 2000). The GRAS proteins contain several features that are found in transcription factors (Di Laurenzio *et al.* 1996; Peng *et al.* 1997; Pysh *et al.* 1999), and it has been suggested that they may be related to the STAT signal transducer proteins found in metazoans and slime molds (Richards *et al.* 2000).

The GAI and RGA proteins are important negative regulators of the GA-signaling pathway, because some aspects of the growth of plants lacking either *GAI* or *RGA* function require less GA than do those of wild-type plants (Peng *et al.* 1997; Silverstone *et al.* 1998). Furthermore, *GAI* and *RGA* share a high degree of sequence homology (Peng *et al.* 1997; Silverstone *et al.* 1998), suggesting that they have substantially overlapping functions in GA signaling. It has been suggested that GAI and RGA are "GA-derepressible repressors" of plant growth (Peng *et al.* 1997; Harberd *et al.* 1998). According to this hypothesis (the GA-derepressible repressor model) GAI and RGA repress growth in the absence of GA. When GA is present, a signal, probably originating from a GA receptor, converts GAI and RGA to nonrepressing forms, thus allowing growth to occur. One prediction of this model is that plants lacking GAI and RGA might exhibit growth that is independent of

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GA. Here, we test this prediction. The *ga1-3* mutation confers very low endogenous GA levels because it blocks an early step in the GA biosynthetic pathway (Sun and Kamiya 1994). As a result, plants homozygous for *ga1-3* are severely dwarfed, exhibit abnormal floral development, and require exogenous GA for germination (Koornneef and van der Veen 1980). We show that plants homozygous for *ga1-3*, and that also contain mutations that result in loss of both GAI and RGA function, are not dwarfed, but grow at least as tall as wild-type plants. Thus, when GAI and RGA are not functional, GA is not required for stem elongation, as predicted by the GA-derepressible repressor model. However, loss of GAI and RGA is not sufficient to restore germination or normal floral development to *ga1-3* in the absence of GA. We discuss the relative roles of GAI and RGA in mediating the response to GA for these and other aspects of plant growth. In addition, plants respond to GA in a quantitative fashion. Here we describe experiments that investigate the function of GAI and RGA in linking the degree of response to the magnitude of GA dose. Our observations lead to an extension of the original GA-derepressible repressor model, which can account for the dose-dependent nature of the GA response.

MATERIALS AND METHODS

Genetic nomenclature: In this article, genotypes are written in italics with the wild-type genotype in capitals (*e.g.*, *GAI*) and the mutant genotype in lowercase letters (*e.g.*, *gai*). Wild-type polypeptide gene products are written in nonitalic capital letters (*e.g.*, GAI) and mutant gene products in nonitalic lowercase letters (*e.g.*, gai).

Plant materials and growth conditions: All mutants described here were derived from the *A. thaliana* laboratory strain Landsberg *erecta*. Throughout this article, Landsberg *erecta* is referred to as wild type (*GAI RGA GA1*). The *ga1-3* and *gai-t6* mutant lines were obtained as described previously (Peng *et al.* 1997; Cowling and Harberd 1999). The *rga-24 ga1-3* and *rga-24 GA1* lines were kindly provided by Tai-ping Sun. For growth in the greenhouse, seeds were allowed to imbibe on moistened filter paper at 4° for 3–5 days and then grown in standard greenhouse conditions as described previously (Peng *et al.* 1999b). The *ga1-3* mutant does not germinate unless supplied with exogenous GA. Thus, for the lines containing the *ga1-3* mutation, seeds were imbibed in the same way as described above, but on filter paper wetted with a 10^{-4} M solution of GA_3 (Sigma, St. Louis). For growth of seedlings in sterile conditions, seeds were surface sterilized as described previously (Cowling *et al.* 1998) and chilled in either water or a 10^{-4} M GA_3 solution for 3–5 days. After chilling, seeds that had been imbibed in GA_3 were washed five times with sterile water before being transferred onto petri dishes containing germination medium (GM) supplemented with appropriate chemicals (see below) as described previously (Cowling *et al.* 1998). Seedlings were grown in a growth room maintained at 22° with a 16-hr/8-hr light/dark cycle of fluorescent light.

Generation of double and triple mutant homozygotes: PCR testing was used to confirm the genotypes of the various double and triple mutants at the *GA1* locus (Silverstone *et al.* 1997). The *gai* mutant allele differs from the *GAI* wild-type allele in that it contains a 51-bp deletion of coding sequence (Peng *et al.* 1997). Since the *gai-t6* allele is derived from *gai* (Peng *et al.* 1997), this allele can be distinguished from *GAI* via a PCR test that specifically amplifies a fragment of DNA from primers that flank the deletion. These primers are N6 (5′-TAG-AAG-TGG-TAG-TGG-3′) and R1 (5′-GTG-AAC-AGT-CTC-AGT-AGC-3′). A test for the GA requirement for germination was used to facilitate identification of individuals that were homozygous for *ga1-3*. Seeds of lines to be tested were surface sterilized, placed onto petri dishes containing GM, and incubated for 3–5 days at 4°. The petri dishes were then transferred to a continuous light growth room (22°) for 4 days. Seeds that had not germinated were transferred to sterile filter-paper discs soaked in 10^{-4} M GA_3, sealed in petri dishes, placed in a continuous-light growth room until germination (1–2 days), and then carefully transferred to fresh GM plates. In this way, individuals with a GA requirement for germination were separated from individuals that did not require GA.

The *gai-t6 ga1-3* double homozygous mutant line was obtained from a *gai-t6* × *ga1-3* cross. The F_2 population of the cross was tested for a GA requirement for germination to separate individuals that were homozygous for *ga1-3* from those that were heterozygous or wild type at the *GA1* locus. The selected individuals were transplanted to soil and grown in standard greenhouse conditions. All individuals selected had a phenotype indistinguishable from that of *ga1-3*, and so PCR testing was used to identify individuals that were homozygous for *gai-t6*.

To generate the *gai-t6 rga-24 ga1-3* triple homozygous mutant, F_1 plants from the *gai-t6* × *ga1-3* cross were crossed with *rga-24 ga1-3*, and the F_1 population obtained was then tested for a GA requirement for germination. The seeds that did require GA for germination (and thus were presumed to be homozygous for *ga1-3*) were given exogenous GA, and, after 14–21 days growth, the seedlings were transplanted to soil and grown in standard greenhouse conditions. PCR genotyping was used to identify the individuals that were heterozygous for the *gai-t6* mutation and to confirm that these individuals were homozygous for *ga1-3*. PCR genotyping was not required to test for the *rga-24* mutation as all individuals in the F_1 of this cross are heterozygous for *rga-24*. The selected individuals were allowed to self-pollinate, and their progeny (F_2) were examined. The F_2 contained individuals that looked like *ga1-3* and *rga-24 ga1-3*. Other individuals from within this F_2 exhibited a phenotype not previously observed: these were tall rather than dwarfed like *ga1-3*. The latter were tested using PCR and found to be homozygous for *gai-t6* and *ga1-3*. Because these tall plants were infertile, exogenous GA was applied (10^{-4} M GA_3) to attempt to correct this defect. The treatment was successful and allowed seed setting. Plants that had been identified as putative *gai-t6 rga-24 ga1-3* were allowed to self-pollinate, and the resulting F_3 seed was grown to check that there was no segregation of the nondwarf phenotype, thus confirming that the line was also homozygous for *rga-24*.

To generate the *gai-t6 rga-24* double homozygous mutant, F_1 plants from the *gai-t6* × *ga1-3* cross were crossed with *rga-24 ga1-3*. The F_1 population obtained from this cross was tested for a GA requirement for germination. Individuals that did not require GA for germination (and thus were heterozygous rather than homozygous for *ga1-3*) were transplanted to soil and grown in standard greenhouse conditions. These plants were allowed to self-pollinate, and the F_2 population was analyzed to identify *gai-t6 rga-24* homozygous individuals. To facilitate this, we used the assumption that, as the *gai-t6* and *rga-24* mutations individually and partially reduce the GA requirement for growth (Peng *et al.* 1997; Silverstone *et al.* 1997, 1998), the combination of both mutations might further reduce this requirement. To test this assumption, we used

resistance to the GA biosynthesis inhibitor Paclobutrazol (PAC; ICI Agrochemicals). When grown on PAC, wild-type plants are smaller than plants grown in the absence of PAC, with darker-green leaves and a reduced rosette diameter (Peng *et al.* 1999b). The F_2 population was grown on GM supplemented with 10^{-6} M PAC and was found to contain a range of PAC phenotypes, ranging from those that were very small and dark green and looked similar to wild type to individuals that were larger and paler green. The large pale-green individuals were selected and genotyped by PCR. Those plants confirmed as being homozygous for *gai-t6* and wild type at the *GA1* locus were allowed to self-pollinate, and the F_3 seeds were collected. To test that the selected lines were not segregating at the *rga-24* locus, they were analyzed for their resistance to PAC. F_3 seeds were grown on 10^{-6} M PAC alongside wild-type and *gai-t6 ga1-3* seeds, and all individuals of the F_3 lines were found to be larger and paler green than wild type or *gai-t6 ga1-3* (data not shown). This identifies that the *rga-24* locus is homozygous in these lines because, if it were not, a proportion of the F_3 population would have wild-type or *gai-t6 ga1-3* phenotypes on PAC. In addition, when grown in standard greenhouse conditions, all F_3 individuals have a phenotype not seen in wild type or *gai-t6* (shorter siliques that produce fewer seed), which further confirms that there is no segregation and that the lines are homozygous for *rga-24*.

All of the lines generated (*gai-t6 ga1-3*, *gai-t6 rga-24 ga1-3*, and *gai-t6 rga-24*) also contain the *tt1* mutation, a marker that confers a transparent testa phenotype.

Promotion of normal flower development by application of GA: *gai-t6 rga-24 ga1-3* has abnormal flower development, which results in infertility. This can be corrected by GA application to allow seed set. To promote normal flower development, plants were sprayed with 10^{-4} M GA_3.

Genetic analysis of segregants: In the F_2 population of the cross to generate *gai-t6 rga-24 ga1-3* triple mutants, we noticed individuals that had a phenotype intermediate between *rga-24 ga1-3* and *gai-t6 rga-24 ga1-3*. These individuals were tested using a PCR-based assay that distinguishes *gai-t6* from *GAI* (as described above), and they were found to be heterozygous (*GAI/gai-t6*). These plants were allowed to self-pollinate, and the progeny were analyzed. The segregation of one such line was assessed, and the intermediate phenotype was shown (by PCR) to segregate with the genotype *GAI/gai-t6*.

Quantification of GAs: Plants were grown in soil at 22° under long-day conditions of a 16-hr photoperiod, with a photon density of 130 μE m^{-2} s^{-1}. After 5 weeks, when the plants were at the eight-leaf stage, the rosette leaves of plants were harvested and immediately frozen in liquid nitrogen. GA levels in samples of 1–2 g fresh weight were analyzed as described previously (Peng *et al.* 1999b) by combined gas chromatography/mass spectrometry (GC/MS)-selected reaction monitoring using a JEOL MStation mass spectrometer (JEOL, Tokyo). [$^2H_2$]GAs (obtained from L. Mander, Research School of Chemistry, Canberra, Australia) were added as internal standard. The amount of internal standard added varied with the genotype.

GA and PAC dose-response curves: To analyze the response to GA for hypocotyl elongation, seeds of the genotypes wild type, *ga1-3*, *gai-t6 ga1-3*, *rga-24 ga1-3*, and *gai-t6 rga-24 ga1-3* were surface sterilized and chilled (4°) in a 10^{-4} M solution of GA_3 for 4 days. The seeds were then washed five times with water to remove any traces of GA and placed onto growth medium containing the appropriate concentration of GA_3. After 7 days in a growth room (22°, 16 hr light), 7–12 seedlings for each line whose aerial parts were growing vertically and were not in contact with other plants or the side of the petri dish were selected for measurement. These were laid flat and photographed using a Zeiss stereomicroscope camera with Kodak slide film (Ektachrome 160-Tung). Each photograph also contained a 1-mm scale. The images were scanned and measured using Sigmascan Pro 5.0 image analysis software.

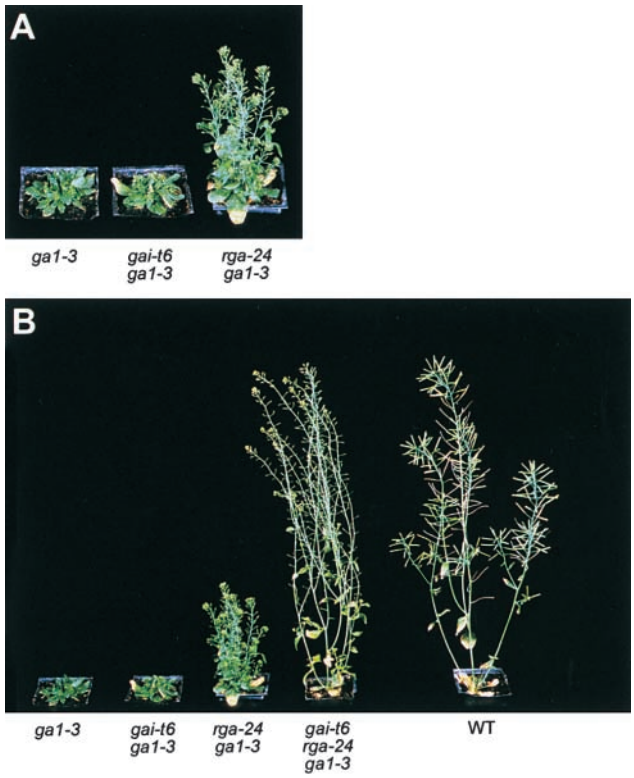

Figure 1.—Loss of GAI and RGA function decreases the GA requirement for normal stem elongation. (A, left to right) *ga1-3*, *gai-t6 ga1-3*, and *rga-24 ga1-3*. (B, left to right) *ga1-3*, *gai-t6 ga1-3*, *rga-24 ga1-3*, *gai-t6 rga-24 ga1-3*, and wild type. Plants were grown for 57 days in standard greenhouse conditions.

To analyze the response of the genotypes to PAC, seeds of the genotypes wild type, *gai-t6*, *rga-24*, and *gai-t6 rga-24* were surface sterilized and chilled (4°) in water for 4 days. The seeds were then placed on growth medium supplemented with the appropriate concentration of PAC. Seedlings were grown for 7 days in the conditions described above and measured in the same way as for the GA dose-response curves.

RESULTS

Genetic analysis of *GAI* and *RGA* loss-of-function alleles: *gai-t6* is a *Ds*-insertion loss-of-function allele of *GAI* (Peng *et al.* 1997). *rga-24* is a fast-neutron-induced deletion allele that abolishes *RGA* function (Silverstone *et al.* 1997). The *ga1-3* mutant contains a mutation that disrupts *GA1*, a gene that encodes a key enzyme in GA biosynthesis (Sun *et al.* 1992; Sun and Kamiya 1994). Because they contain very low levels of GA (Zeevaart and Talon 1992), plants homozygous for *ga1-3* are severely dwarfed and exhibit reductions in petal and stamen elongation (Koornneef and van der Veen 1980; see also Figure 1A). To further analyze the roles of GAI and RGA in GA signaling, we constructed plants carrying different combinations of *gai-t6*, *rga-24*, and *ga1-3*. Plants

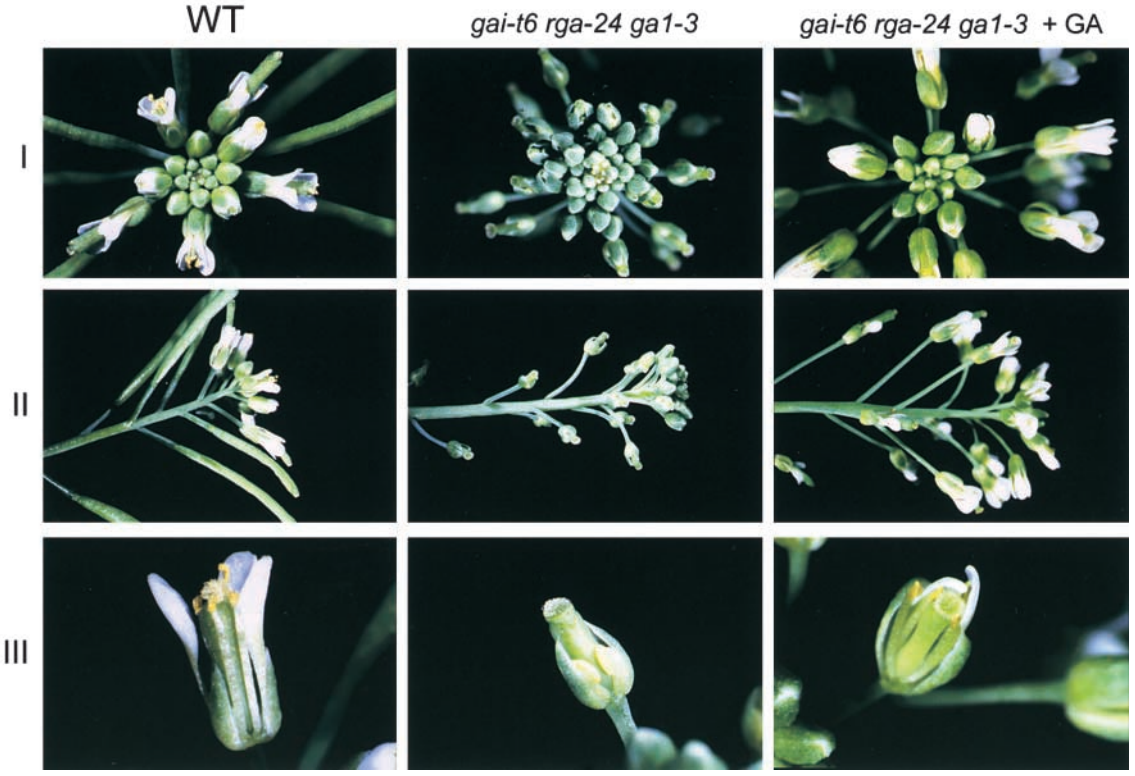

FIGURE 2.—The floral phenotype of *gai-t6 rga-24 ga1-3* flowers is reversible by exogenous GA treatment. Plants were photographed using a Tessovar microscope camera system. All flowers were from plants of the same age (42 days) except those in the +GA panels, which were photographed 7 days after a single GA application (plants were sprayed once with 10^{-4} M GA_3) and thus were 49 days old. All flowers within each row (I, II, or III) were photographed at the same magnification. Where necessary, sepals and petals were removed from flowers in row III to make the stamens visible.

doubly homozygous for both *gai-t6* and *ga1-3* (*gai-t6 ga1-3*) display a phenotype very similar to that of *ga1-3* single mutants, being severely dwarfed (Figure 1A). The *rga-24* mutation was originally isolated during a screen for mutations that suppressed the dwarfism conferred by *ga1-3* (SILVERSTONE *et al.* 1997, 1998). Accordingly, the *rga-24 ga1-3* double mutant is taller than the *ga1-3* single mutant (Figure 1A), but considerably shorter than wild-type plants (Figure 1B). Thus, while lack of GAI function has no clear effect, lack of RGA function results in an obvious partial suppression of the *ga1-3* phenotype (Figure 1A; see also SILVERSTONE *et al.* 1997, 1998).

The effects of GAI and RGA are most clearly demonstrated in plants that lack both *GAI* and *RGA* gene functions. Triple mutant *gai-t6 rga-24 ga1-3* plants grow at least as tall as wild-type (*GAI RGA GA1*) controls, showing that absence of GAI and RGA fully suppresses the stem elongation phenotype of *ga1-3* (Figure 1B).

Loss of GAI and RGA function does not suppress the abnormal flower phenotype of *ga1-3*: While loss of GAI and RGA function can fully suppress the dwarf aspect of the *ga1-3* phenotype, other aspects are not affected. In particular, while *gai-t6 rga-24 ga1-3* plants have an elongated stem similar to that of wild type, they develop abnormal flowers (see Figure 2) that are similar to those of *ga1-3* (KOORNNEEF and VAN DER VEEN 1980). *gai-t6 rga-24 ga1-3* flowers have severely reduced petal and stamen growth and are sterile. Thus, although the stem elongation defect in *ga1-3* is restored when GAI and RGA are not functional, the abnormal flower development of *ga1-3* is not affected. Application of GA restores normal petal and stamen elongation and results in fertile flowers (Figure 2). Additionally, like *ga1-3* seeds, *gai-t6 rga-24 ga1-3* seeds still require GA for germination (K. E. KING, unpublished data). Thus, the absence of GAI and RGA suppresses stem elongation but not the floral or seed germination components of the *ga1-3* phenotype, indicating that, while GAI and RGA regulate stem elongation, other signaling components or pathways are involved in controlling the response to GA for flower development and germination. The Arabidopsis genome contains several other open reading frames (ORFs) with a high degree of sequence similarity to those of *GAI* and *RGA* (SÁNCHEZ-FERNÁNDEZ *et al.* 1998; K. E. KING, unpublished data). It is possible that proteins encoded by these genes may regulate the petal/stamen and seed germination responses to GA.

***gai-t6 rga-24 ga1-3* contains very low endogenous GA levels similar to those of *ga1-3*:** *GA1* encodes CPP synthase, an enzyme that catalyzes an early step in the GA biosynthesis pathway (SUN and KAMIYA 1994; HEDDEN and KAMIYA 1997). The *ga1-3* mutation is a 5-kb deletion

TABLE 1

Quantification of endogenous GA levels in wild type, *ga1-3*, and *gai-t6 rga-24 ga1-3*

	Genotype		
GAs	Wild type	*ga1-3*	*gai-t6 rga-24 ga1-3*
GA_{24}	2235 ± 5.6	ND	ND
GA_9	113 ± 10.1	1 ± 0.12	3 ± 0.6
GA_4	447 ± 40.3	10 ± 0.9	19 ± 2.6
GA_{34}	530 ± 4.6	32 ± 2.3	47 ± 2.3
GA_{53}	736 ± 23.6	4 ± 0.3	5 ± 0.2
GA_{19}	233 ± 36.1	ND	7 ± 0.7
GA_{20}	17 ± 0.9	ND	4 ± 0.4
GA_1	60 ± 8.3	ND	ND
GA_8	89 ± 6.0	61 ± 5.2	29 ± 6.0

GA levels shown are measured in picogram per gram fresh weight. Results shown are the mean of three determinations per genotype, and error shown is standard error of the mean. ND indicates that GA levels were so low that they were not detectable.

FIGURE 3.—*GAI* gene dosage can affect growth. Plants shown are the progeny of a line identified as heterozygous for *gai-t6*. PCR analysis of the *GAI* gene in each of the plants shown was used to confirm the genotypes. The plants were photographed after 43 days of growth in standard greenhouse conditions. (Left to right) *gai-t6/gai-t6 rga-24/rga-24 ga1-3/ga1-3*, *GAI/gai-t6 rga-24/rga-24 ga1-3/ga1-3*, and *GAI/GAI rga-24/rga-24 ga1-3/ga1-3*.

that extends over the entire *GA1* ORF, thus abolishing its function (SUN *et al.* 1992; SUN and KAMIYA 1994). Previous experiments have shown that *ga1-3* plants contain very low levels of GA (ZEEVAART and TALON 1992). We analyzed the endogenous GA levels in *ga1-3* mutant plants using GC/MS and compared them to those in *gai-t6 rga-24 ga1-3* and wild type (Table 1). Our results confirm that *ga1-3* mutant plants contain very low endogenous levels of GAs and show that *gai-t6 rga-24 ga1-3* plants contain GA levels similar to those of *ga1-3*. In particular, levels of the biologically active GA_4 and GA_1 are extremely low compared to those of wild type. Thus, *gai-t6 rga-24 ga1-3* contains very low endogenous GA levels, and yet, unlike *ga1-3*, it is not dwarfed and grows as tall as wild type. As predicted by the GA-derepressible repressor model, when GAI and RGA functions are both missing, stem elongation is independent of GA.

***GAI* affects phenotype in a gene-dose-dependent fashion in the absence of *RGA* and *GA1* function:** GA responses are typically GA dose dependent (COWLING *et al.* 1998) and thus the GA-signaling system must transmit information about GA dose. To investigate the dose dependency of GA responses, we studied the effect of varying *GAI* dose by examining the phenotype of plants that lack RGA function (*rga-24/rga-24*) and *GA1* function (*ga1-3/ga1-3*) and are heterozygous for *gai-t6* (*GAI/gai-t6*). Interestingly, *GAI/gai-t6 rga-24 ga1-3* plants are intermediate in height: taller than *GAI/GAI rga-24 ga1-3*, but shorter than *gai-t6/gai-t6 rga-24 ga1-3* (Figure 3). Thus, in the absence of RGA, *GAI* exhibits haplo-insufficiency: a single dose of *GAI* (in the heterozygote) represses stem elongation growth less powerfully than a double dose (in the *GAI* homozygote). Usually haploinsufficiency is caused by gene-dose-dependent changes in protein levels (LINDSLEY and GRELL 1968). The data presented here suggest that, although usually masked by the near-redundancy of GAI and RGA function, absolute levels of functional GAI may affect stem elongation growth in a quantitative fashion.

GA dose-response analysis of hypocotyl elongation: The above observation suggests that there can be a quantitative relationship between the level of GAI function and the amount of growth. To further investigate the dose-dependent nature of GA responses, we examined GA-regulated hypocotyl elongation in a range of mutant genotypes. Hypocotyl elongation is a simple quantitative measure of GA response in Arabidopsis—it is entirely due to cell elongation and does not involve any *de novo* cell division (GENDREAU *et al.* 1997; COWLING and HARBERD 1999). Hypocotyl growth requires GA, as GA-deficient mutants, such as *ga1-3*, have much reduced cell elongation resulting in very short hypocotyls. If given exogenous GA, *ga1-3* hypocotyls elongate in a GA dose-dependent manner (COWLING *et al.* 1998; COWLING and HARBERD 1999). We investigated how loss of GAI and RGA function in a GA-deficient *ga1-3* background can affect the hypocotyl elongation GA response.

Without exogenous GA, the *ga1-3* mutant has a very short hypocotyl. Progressive increases in GA dose cause corresponding increases in *ga1-3* hypocotyl length, and at high GA doses the length of *ga1-3* hypocotyls is very similar to that of wild type (COWLING *et al.* 1998; COW-

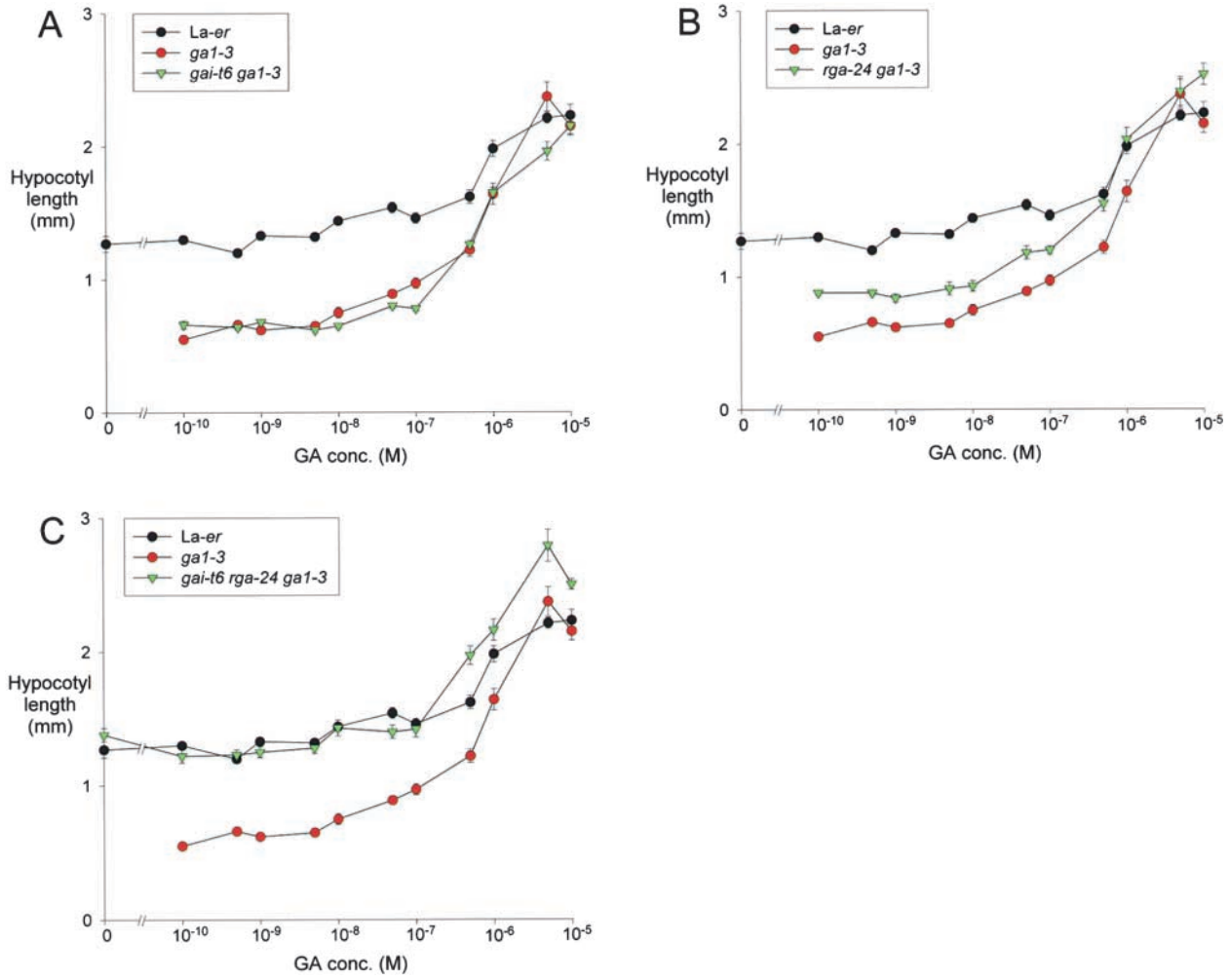

FIGURE 4.—GA is essential only for normal hypocotyl elongation when GAI and RGA are active. (A–C) GA dose-response curves for hypocotyl length. The hypocotyl length of different genotypes at increasing GA concentrations is plotted, using Sigmaplot 4.0 software. For each GA concentration, 7–12 seedlings were measured (see MATERIALS AND METHODS for further details). Seedlings were 7 days old. For all graphs, error bars shown are standard error of the mean.

LING and HARBERD 1999; see also Figure 4A). Loss of GAI, in *gai-t6 ga1-3*, does not appreciably alter this dose-response relationship—the dose-response curve for *gai-t6 ga1-3* is very similar to that of *ga1-3* (Figure 4A). Loss of RGA, however, has a clear effect on the hypocotyl length and GA dose-response relationship. At low exogenous GA concentrations, *rga-24 ga1-3* hypocotyls are longer than *ga1-3* hypocotyls but shorter than wild-type hypocotyls (Figure 4B). As GA dose increases, *rga-24 ga1-3* hypocotyl length also increases, resulting in a dose-response curve that, throughout most of its length, is roughly parallel with that of *ga1-3*. At high GA doses, the wild-type, *rga-24 ga1-3*, and *ga1-3* growth curves converge, with all three genotypes achieving similar hypocotyl lengths (Figure 4B). Thus, while loss of RGA can partially suppress the hypocotyl length phenotype of *ga1-3*, loss of GAI has little effect. In addition, *rga-24 ga1-3* hypocotyls are still GA responsive and exhibit a relatively normal incremental growth response to increases in GA dose.

The effect of loss of both GAI and RGA on the hypocotyl elongation GA response was examined in triple mutant *gai-t6 rga-24 ga1-3* hypocotyls (Figure 4C). In the absence of exogenous GA, *gai-t6 rga-24 ga1-3* hypocotyls are as long as wild-type hypocotyls (Figure 4C). Thus, as for stem elongation, GA is only required for hypocotyl elongation when both GAI and RGA are active. Furthermore, although *gai-t6 rga-24 ga1-3* hypocotyls do not require GA to elongate to a wild-type length, they are still GA responsive. Increasing GA doses elicit progressive increases in the lengths of *gai-t6 rga-24 ga1-3* hypocotyls (Figure 4C). At high GA doses, *gai-t6 rga-24 ga1-3* hypocotyls appear to be even longer than wild type. The significance of this latter observation is not obvious at present. However, it is clear from these experiments that GAI and RGA together play a major role in the mediation of GA-dose-dependent hypocotyl elongation and also that additional factors must be involved.

Loss of both GAI and RGA function in a wild-type background: While lack of both GAI and RGA has a

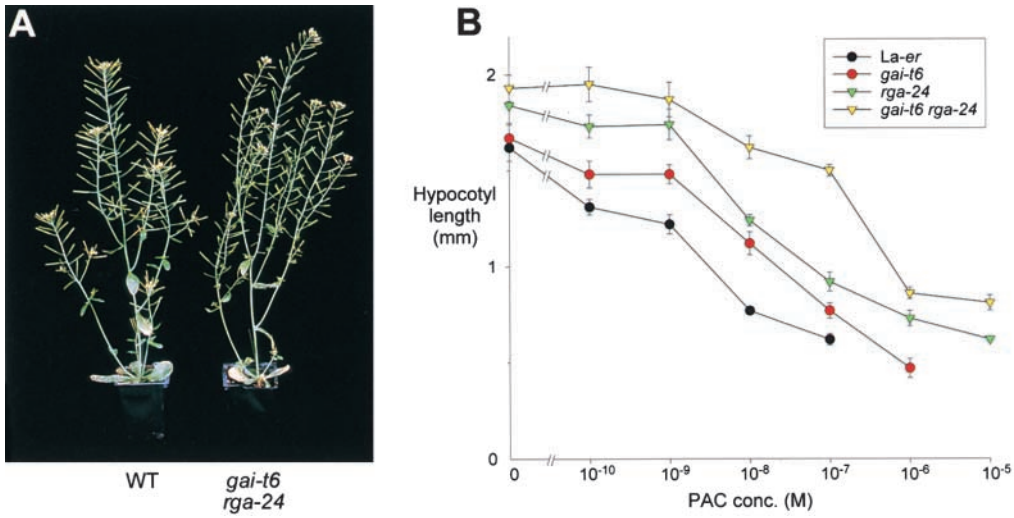

FIGURE 5.—GA deficiency is required to reveal the roles of GAI and RGA. (A) Wild type (WT) and *gai-t6 rga-24*. Plants were grown for 44 days in standard greenhouse conditions. (B) PAC dose-response curve for hypocotyl length. The hypocotyl length of different genotypes at increasing PAC concentrations is plotted using Sigmaplot 4.0 software. For each PAC concentration, 7–12 seedlings were measured (see MATERIALS AND METHODS for further details). Seedlings were 7 days old. For all graphs, error bars shown are standard error of the mean.

dramatic effect on the phenotype of GA-deficient plants, it has a less obvious effect on the stem elongation of plants carrying *GA1*. As shown in Figure 5A, the growth of *gai-t6 rga-24* is similar to that of wild type. However, although *gai-t6 rga-24 GA1* plants grow at least as tall as wild-type plants, there are some differences between them; for example, *gai-t6 rga-24 GA1* plants have shorter siliques and produce fewer seeds (K. E. KING, unpublished data).

In further experiments, we made use of the GA biosynthesis inhibitor PAC (HEDDEN and GRAEBE 1985; DAVIS and CURRY 1991). PAC causes a reduction in hypocotyl length, and its dwarfing effects can be abolished by simultaneous treatment with GA (COWLING *et al.* 1998), indicating that its effects on growth are indeed due to a reduction in GA biosynthesis. The hypocotyl lengths of wild type, *gai-t6*, *rga-24*, and *gai-t6 rga-24* seedlings were examined on a range of PAC concentrations. As expected, wild type hypocotyls become progressively shorter with increasing PAC dose (Figure 5B), as do those of *gai-t6*. However, at each PAC concentration, *gai-t6* hypocotyls are more resistant to PAC than wild-type hypocotyls, *rga-24* hypocotyls are more resistant to PAC than *gai-t6* hypocotyls, and double mutant *gai-t6 rga-24* hypocotyls are the most resistant of all. Although highly resistant, *gai-t6 rga-24* is not completely resistant to PAC, further suggesting that factors additional to GAI and RGA mediate the hypocotyl elongation GA response.

DISCUSSION

Our article describes how combined loss of GAI and RGA function results in plants that do not require GA for normal stem elongation. *gai-t6 rga-24 ga1-3* triple mutant homozygous plants grow as tall as wild type, even though they contain very low GA levels similar to those of *ga1-3*. Loss of either GAI or RGA function results in a partial reduction in the GA requirement for growth (see also PENG *et al.* 1997; SILVERSTONE *et al.* 1998), with loss of RGA having a more obvious effect than loss of GAI. However, the effect of loss of both GAI and RGA is substantially greater than the sum of the effects of the loss of either GAI or RGA alone. Thus, although loss of GAI has no obvious effect on the stem elongation of *ga1-3* (as in *gai-t6 ga1-3*), the tall phenotype of *gai-t6 rga-24 ga1-3* suggests that GAI and RGA act together in the regulation of stem elongation.

Our results also show that, although the *gai-t6 rga-24 ga1-3* triple mutant can grow as tall as wild type, it also exhibits several obvious phenotypic differences from wild type. These differences demonstrate that not all aspects of plant growth and development are affected by the combined loss of GAI and RGA function in the same way. For example, the *gai-t6* and *rga-24* loss-of-function mutations, when combined, can fully suppress the stem and hypocotyl elongation defects of *ga1-3* but have no effect on the GA dependency of *ga1-3* for germination and normal flower development. It seems, therefore, that for certain aspects of Arabidopsis growth such as stem and hypocotyl elongation, the response to GA is mediated largely by GAI and RGA, whereas for other aspects of growth such as germination and flower development, components or pathways other than GAI and RGA are involved.

We have shown that the triple homozygous mutant *gai-t6 rga-24 ga1-3* has a stem length similar to that of wild type and thus does not require GA for normal stem growth. The stem growth of *gai-t6 rga-24 ga1-3* is "normal" in that the internode length of *gai-t6 rga-24 ga1-3* stems is similar to that of wild type and substantially different from that of *ga1-3* (see Figure 1). When plants are allowed to grow for longer than shown in Figure 1, the *gai-t6 rga-24 ga1-3* plants become taller than wild type (K. E. KING, unpublished data). We suggest that this phenomenon may be due to the fact that the infertil-

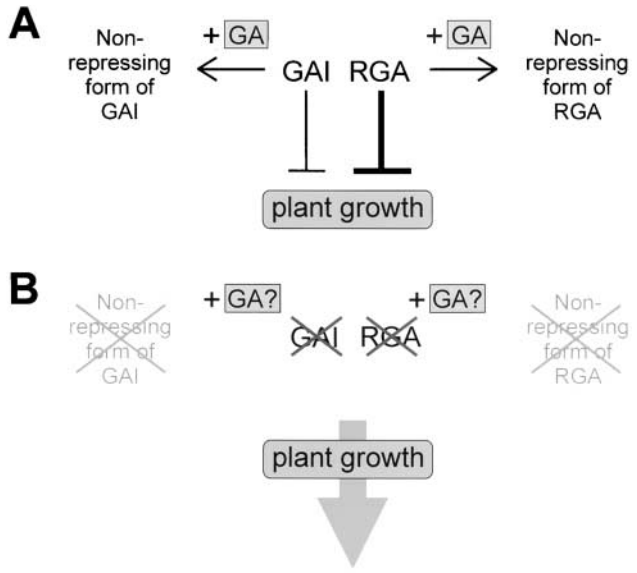

FIGURE 6.—GA-derepressible repressor model for the roles of GAI and RGA in quantitatively modulating the growth response to GA. (A) GAI and RGA are repressors of plant growth, and the action of GA can convert them from the repressing form to a nonrepressing form. The amount of the repressing form, and thus the amount of repression of plant growth, is negatively correlated with GA dose. GAI and RGA do not contribute equally to the repression of growth, with RGA being more important than GAI. Here, plant growth refers to the subset of GA-mediated growth responses mediated by GAI and RGA. (B) When GAI and RGA are not functional, there is no repression of growth, and thus GA is not required for normal growth responses. The shaded arrow indicates that, when GAI and RGA are not functional, plant growth is constitutive regardless of GA dose. In this case, plant growth refers to the subset of GA-mediated growth responses mediated by GAI and RGA when they are active.

ity of *gai-t6 rga-24 ga1-3* is likely to delay senescence and thus permit extra growth in this mutant.

The effects of the combined loss of GAI and RGA function are less obvious in a wild-type (*GA1*) background, as mature *gai-t6 rga-24* plants have a phenotype that is similar to wild type. However, *gai-t6 rga-24* seedlings have a clear phenotype when grown on PAC, as *gai-t6 rga-24* hypocotyl elongation is more resistant to the effects of PAC than is that of wild type. Thus, GA deficiency (such as that conferred by the *ga1-3* mutation or by the GA biosynthesis inhibitor PAC) is required to reveal the full extent of the roles of GAI and RGA in GA signaling.

The *GAI* and *RGA* genes share a high degree of sequence homology, and thus it seems likely that they have overlapping functions. However, our results show that, while loss of *RGA* can partially suppress the effects of GA deficiency conferred by *ga1-3*, loss of *GAI* has little effect on the *ga1-3* phenotype. This indicates that, while it is likely that the roles of *GAI* and *RGA* in GA signaling substantially overlap, they are not identical.

Plant growth is regulated by GA in a quantitative fashion, and thus the GA-signaling system must have a means to transmit information about GA dose. Here we have shown, through varying the dose of the *GAI* gene (in *gai-t6/GAI* heterozygotes), that absolute levels of functional GAI may affect stem elongation growth in a quantitative fashion. We have also shown that decreases in the amount of GAI/RGA in the repressing form—varied both by increasing exogenous GA dose and by changing the *GAI* and/or *RGA* gene dose—can quantitatively affect hypocotyl elongation.

The GA-derepressible repressor model has been proposed to explain the roles of GAI and RGA as repressors of GA-mediated plant growth (PENG *et al.* 1997; HARBERD *et al.* 1998; RICHARDS *et al.* 2001). The model can accommodate our findings if growth is negatively correlated with the amount of GAI/RGA in the repressing form and if this amount is itself a function of GA dose (Figure 6). Growth then becomes a function both of the total amount of GAI/RGA (as varied in our experiments by the *gai-t6* and *rga-24* mutant alleles) and of the proportion of GAI/RGA that is in the repressing form (as varied by changes in GA dose). To account for the differences between the effect of loss of GAI or RGA (*i.e.*, the phenotypic differences between *gai-t6 ga1-3* and *rga-24 ga1-3*), we propose that GAI and RGA do not contribute equally to the repression of growth and that RGA contributes more than GAI (see Figure 6). Thus, we suggest that it is the total amount of GAI and RGA in the repressing form that determines the amount of growth repression and that the amount of the repressing form depends on the GA dose. Our model can only partly explain the results obtained here, as our data also reveal that GAI and RGA do not modulate the GA response for all aspects of GA-mediated growth, suggesting that other components of the GA-signaling pathway are involved. Thus, in our model, "growth" refers only to those aspects of growth and development mediated by GAI and RGA.

We have described here how mutations that abolish the function of GAI and RGA result in a reduced GA requirement for normal growth. However, if *GAI* is mutated in a different way that alters rather than abolishes the function of its product, then constitutive repression of growth independent of GA results (PENG *et al.* 1997; HARBERD *et al.* 1998). This phenomenon can be explained, in terms of our model, if the mutation blocks the conversion of GAI (by the action of GA) from the repressing to the nonrepressing form. The original *gai* mutation is a small in-frame deletion that results in a gai protein lacking 17 amino acids in its N-terminal region (PENG *et al.* 1997). Similar altered-function mutant proteins are encoded by genes orthologous to Arabidopsis *GAI* in wheat and maize mutants that exhibit a reduced response to GA (PENG *et al.* 1999a). Thus the control of GA-mediated plant growth by GAI/RGA and orthologs may be a universal system that is common to all vascular plants. In addition, GA-independent growth,

as described here for *gai-t6 rga-24 ga1-3*, has also been observed in mutants in species other than Arabidopsis, such as the barley and rice *slender* mutants (Chandler 1988; Lanahan and Ho 1988; Chandler and Robertson 1999; Ikeda *et al.* 2001) and the pea *la cry*^{s} mutant (Potts *et al.* 1985). Recent data show that the *slender* mutants of rice and barley contain loss-of-function mutations in their respective *GAI/RGA* orthologs (Ikeda *et al.* 2001; P. Chandler, F. Gubler, A. Marion-Poll and M. Ellis, personal communication).

The data described here support the proposal that GAI and RGA are GA-derepressible repressors of plant growth and that the quantitative nature of the GA response is related to the amount of these proteins in the repressing form. GA is only required for normal growth of stems and hypocotyls when GAI and RGA are active, suggesting that in these organs, in the absence of a GA-signaling system, the default state is growth rather than no growth. When functional, the GA-signaling system represses this growth to a degree that is inversely correlated with GA dose. Thus, the function of GA signaling is as a control system regulating the amount of growth, and the role of GA is to repress a repressor rather than to directly promote growth.

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