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Genetic Characterization and Functional Analysis of the GID1 Gibberellin Receptors in *Arabidopsis* [W]

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We investigated the physiological function of three *Arabidopsis thaliana* homologs of the gibberellin (GA) receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1) by determining the developmental consequences of *GID1* inactivation in insertion mutants. Although single mutants developed normally, *gid1a gid1c* and *gid1a gid1b* displayed reduced stem height and lower male fertility, respectively, indicating some functional specificity. The triple mutant displayed a dwarf phenotype more severe than that of the extreme GA-deficient mutant *ga1-3*. Flower formation occurred in long days but was delayed, with severe defects in floral organ development. The triple mutant did not respond to applied GA. All three *GID1* homologs were expressed in most tissues throughout development but differed in expression level. GA treatment reduced transcript abundance for all three *GID1* genes, suggesting feedback regulation. The DELLA protein REPRESSOR OF *ga1-3* (RGA) accumulated in the triple mutant, whose phenotype could be partially rescued by loss of RGA function. Yeast two-hybrid and in vitro pull-down assays confirmed that GA enhances the interaction between GID1 and DELLA proteins. In addition, the N-terminal sequence containing the DELLA domain is necessary for GID1 binding. Furthermore, yeast three-hybrid assays showed that the GA-GID1 complex promotes the interaction between RGA and the F-box protein SLY1, a component of the SCF^{SLY1} E3 ubiquitin ligase that targets the DELLA protein for degradation.

INTRODUCTION

Gibberellins (GAs) are endogenous growth regulators in higher plants. GA-deficient mutants have been largely instrumental in identifying the myriad of developmental processes in which GAs participate, including seed germination, seedling growth, determination of leaf size and shape, stem and root extension, flower induction and development, pollination, seed development, and fruit expansion (Davies, 2004; Fleet and Sun, 2005). In addition, these mutants have provided the basis for identifying and characterizing many of the GA biosynthetic enzymes (Hedden and Phillips, 2000). These studies have led to a rapid improvement in our understanding of GA biosynthesis. The molecular mechanisms by which bioactive GAs regulate plant growth and development are less understood, although recent genetic and biochemical studies have identified several key components in GA signaling (Gomi and Matsuoka, 2003; Sun and Gubler, 2004). The study of GA response in the model plant *Arabidopsis thaliana*, although fruitful, has been complicated by the high level of functional redundancy within GA signaling components (Dill and Sun, 2001; King et al., 2001; Strader et al., 2004; Nakajima et al., 2006). By contrast, several GA signaling components in rice (*Oryza sativa*) are encoded by single genes, making it a powerful system for studying this pathway. This is emphasized by a recent study by Ueguchi-Tanaka et al. (2005) in which they identified the elusive GA receptor in rice. The identity of the GA receptors provides an important opportunity to dissect the GA signaling cascade in *Arabidopsis* (Nakajima et al., 2006).

The most extensively studied components of the GA signaling cascade are the DELLA proteins, which act as repressors of GA-responsive growth (Peng et al., 1997; Silverstone et al., 1998; Ikeda et al., 2001; Chandler et al., 2002). In the *Arabidopsis* genome, there are five DELLA genes (*Repressor of ga1-3* [*RGA*], *GA-INSENSITIVE* [*GAI*], *RGA-LIKE1* [*RGL1*], *RGL2*, and *RGL3*), whereas rice contains one, *SLENDER RICE1* (*SLR1*; Dill and Sun, 2001; Itoh et al., 2002). In *Arabidopsis*, RGA and GAI are the major repressor of GA-promoted vegetative growth and floral initiation, RGL2 is the primary DELLA during seed germination, and RGA, RGL1, and RGL2 are all involved in controlling flower development (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002; Cheng et al., 2004; Tyler et al., 2004). The discovery that DELLAs are nuclear localized and regulate the expression of GA-responsive genes suggests that they act as transcriptional regulators (Silverstone et al., 1998). Although their mode of action is currently unclear, the mechanisms by which GAs act to relieve the repression on growth exerted by DELLAs are being elucidated. In response to GA, DELLAs are rapidly degraded through the action of an SCF E3 ubiquitin (Ub) ligase (McGinnis et al., 2003; Sasaki et al., 2003). This involves the interaction of DELLAs with the SLEEPY1 (SLY1)/GIBBERELLIN-INSENSITIVE DWARF2 (GID2) F-box component through a direct protein–protein interaction, allowing their polyubiquitination and subsequent degradation

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by the 26S proteasome (Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004). All DELLA proteins contain an N-terminal DELLA domain and a C-terminal GRAS domain. The GRAS domain is conserved among a large family of regulatory proteins, namely the GRAS family (Pysh et al., 1999). This domain is likely to be the functional domain, presumably for transcriptional regulation. Additionally, the GRAS domain in the DELLA proteins was shown to be involved in F-box protein binding (Dill et al., 2004). By contrast, the DELLA domain is only present in the DELLA proteins and is essential for their degradation in response to GA, although it is not required for F-box protein binding. A number of *DELLA* gain-of-function mutations have been described; all except one are located in the DELLA regulatory domain (Peng et al., 1997, 1999; Boss and Thomas, 2002; Chandler et al., 2002; Muangprom et al., 2005). Deletions or specific missense mutations of the conserved motifs (DELLA and/or VHYNP) within the DELLA domain render the mutant proteins resistant to GA-induced degradation, leading to a GA-insensitive dwarf phenotype (Dill et al., 2001; Gubler et al., 2002; Itoh et al., 2002). However, the precise role of the DELLA domain in GA-mediated degradation is not known. In *Brassica rapa*, a novel GRAS domain mutation affects the ability of the encoded DELLA protein to interact with the F-box component of the SCF E3 Ub ligase, resulting in its accumulation and insensitivity to GA (Muangprom et al., 2005).

A major breakthrough in our understanding of the GA signaling cascade was the recent identification of GID1 as a soluble GA receptor in rice (Ueguchi-Tanaka et al., 2005). Genetic analysis of the *gid1* mutants does not support the existence of other GA receptors in rice. The *GID1* gene encodes a protein with similarity to hormone-sensitive lipase that binds preferentially to bioactive GAs in in vitro assays. A direct role for GID1 in the targeting of SLR1 for degradation is indicated by the finding that GA promotes their interaction in yeast two-hybrid assays. Moreover, GA-mediated degradation of SLR1 is abolished in the *gid1* mutants. However, it is not clear whether or how GA-GID1 and SLR1 interaction promotes binding of the GID2 F-box component of the SCF E3 Ub ligase to SLR1. In the *Arabidopsis* genome, there are three *GID1* homologous genes that have been designated: *GID1a*, *GID1b*, and *GID1c* (Nakajima et al., 2006). There is strong evidence to suggest that the proteins they encode function as GA receptors. First, when expressed in *Escherichia coli*, all three *Arabidopsis* GID1 proteins demonstrated GA binding activity with similar affinities to GID1. Second, in rice, overexpression of individual *Arabidopsis GID1* genes rescued the GA-insensitive dwarf phenotype of the *gid1-1* mutant. Third, in yeast two-hybrid assays, GID1a-1c interacted with the five *Arabidopsis* DELLAs in a GA-dependent manner. Currently, there is no genetic evidence demonstrating a role for the *Arabidopsis GID1* genes in GA signaling. However, the three genes display overlapping expression profiles, suggesting that they perform functionally redundant roles (Nakajima et al., 2006).

In this study, we have investigated the role of the *Arabidopsis GID1* genes in GA signaling using a reverse genetics approach. We first identified insertion alleles of *GID1a*, *GID1b*, and *GID1c* and then constructed all double and triple mutant combinations, on which a detailed phenotypic characterization was performed. Our studies demonstrate that GID1a-1c act as positive regulators of GA signaling, consistent with a role in perceiving bioactive GAs. Although they display a high degree of functional redundancy, it appears that they are the predominant GA receptors in regulating GA growth response in *Arabidopsis*. This is illustrated by the severe GA dwarf phenotype of the *gid1* triple mutant, which did not display any detectable GA growth or transcriptional responses in our assays. The GA-insensitive phenotype of this mutant is caused by the accumulation of the growth-repressing DELLA proteins, which are not degraded in response to GA. With a view to understanding how the *Arabidopsis* GID1 receptors regulate GA responses, using pull-down assays, we have provided additional support indicating that GA binding to GID1a promotes its interaction with DELLA proteins. Based on our studies in yeast, the formation of the GA-GID1-DELLA complex enhances the interaction with the SLY1 F-box component and presumably promotes polyubiquitination by the SCF Ub E3 ligase. Furthermore, we demonstrate that the highly conserved N-terminal DELLA and VHYNP motifs in DELLAs are necessary for the interaction with GID1a.

RESULTS

Expression of the *Arabidopsis GID1* Genes

A full understanding of the physiological roles of the *GID1a-1c* genes in regulating *Arabidopsis* growth and development requires knowledge of their expression profiles throughout the life cycle of the plant. It has previously been shown by semiquantitative RT-PCR that the three genes are expressed in flowers, siliques, stems, leaves, roots, and imbibed seeds at similar levels (Nakajima et al., 2006). However, analysis of the AtGenExpress expression atlas data set (Schmid et al., 2005; www.weigelworld.org/resources/microarray/AtGenExpress) reveals large differences in expression levels of the *GID1a-1c* genes, indicating some tissue specificity. Therefore, to define their expression profiles more precisely, we quantified their transcript levels by real-time RT-PCR in a range of tissues throughout development.

The absolute levels of expression of the *GID1a-1c* genes in selected tissues are compared in Figure 1A. Three reference genes (At1g13320, At4g34270, and At2g28390) that are expressed constantly in tissues throughout development (Czechowski et al., 2005) were used as standards. Consistent with the role of GA in regulating growth processes throughout the life cycle of the plant, expression of the *GID1a-1c* genes was detected in all of the tissues analyzed. The overlapping expression profiles of the different *Arabidopsis GID1* genes that we observed are consistent with the findings of Nakajima et al. (2006) and support their suggestion that the receptors play redundant roles in regulating GA-responsive growth. However, in contrast with their results, our data show clear differences in the absolute and relative levels of expression of the individual *Arabidopsis GID1* genes in different tissues, which could indicate distinct roles in regulating different developmental processes. In all tissues analyzed, with the exception of roots, *GID1a* transcript is present at the highest levels, most notably in dry seeds. *GID1c* is expressed at the lowest levels in almost all of the tissues, with the exception of dry seeds and the stem. While the level of *GID1b* transcript is lower than that of *GID1a* and higher than that of *GID1c* in the majority of tissues we analyzed, it is the most highly expressed *Arabidopsis*

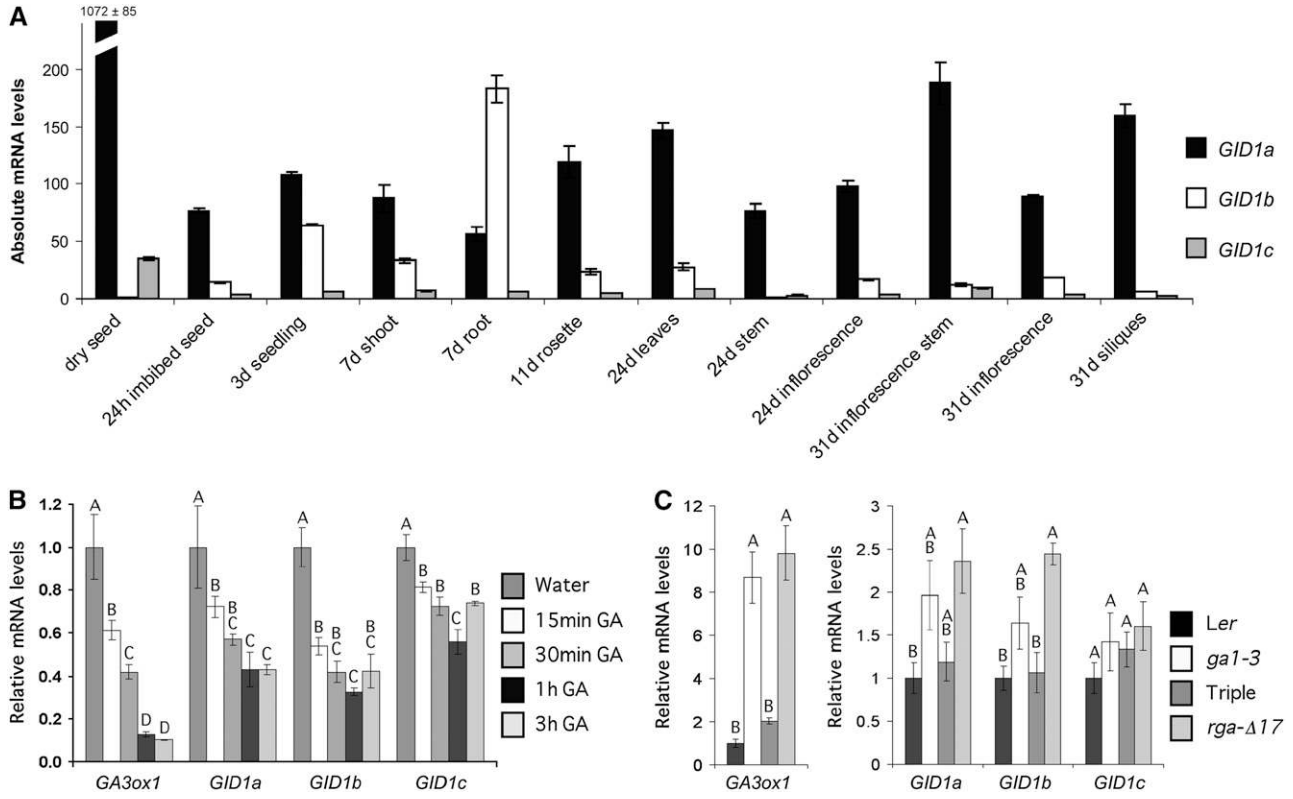

Figure 1. Transcript Levels of *GID1a*, *-1b*, and *-1c* throughout *Arabidopsis* Development and Their Feedback Regulation by GA and DELLA.

(A) Developmental expression profiles of *Arabidopsis GID1s* in Col-0. Absolute transcript levels of individual genes in each tissue sample, as determined by quantitative RT-PCR, were normalized against three stable endogenous control genes (see Supplemental Methods online) and shown relative to the lowest value, that of *GID1b* in dry seed, which is set at 1. The means of three biological replicates ± SE are shown. The value (±SE) for *GID1a* in dry seeds is shown above the bar.

(B) GA treatment downregulates *GA3ox1* and *GID1a-1c* mRNA levels in *ga1-3* (in the L*er* background). The means of three replicates of quantitative RT-PCR ± SE are shown. Relative mRNA levels of individual genes after GA treatment were calculated in comparison to the water-treated control at each time point. Similar results were obtained when quantitative RT-PCR was performed using a second set of samples.

(C) Relative *GA3ox1* and *GID1a-1c* transcript levels in the wild type, *ga1-3*, the triple homozygous mutant *rga-24 gai-t6 ga1-3*, and the transgenic line carrying P_{RGA}:*rga-Δ17* (all lines are in the L*er* background). The means of four replicates of quantitative RT-PCR (using two biological replicates) are shown. Error bars indicate the SE of the mean. The expression level in L*er* was arbitrarily set to 1.0.

For **(B)** and **(C)**, the housekeeping gene *GAPC*, whose expression is not responsive to GA (Dill et al., 2004), was used to normalize different samples (see Supplemental Methods online). One-way analyses of variance were performed with least significant difference (LSD) multiple comparison tests at an α level of 0.01 using SPSS version 10.0 (Chicago, IL). When two samples show different letters (A to D) above the bars, the difference between them is significant ($P < 0.01$).

GID1 gene in the roots. These major differences in their relative levels of expression suggest that the individual *Arabidopsis GID1* genes may play some distinct roles, with *GID1a* potentially encoding the major GA receptor required for most growth processes.

Identification of Insertion Mutations of the *GID1a*, *GID1b*, and *GID1c* Genes

We investigated the physiological roles of the three *Arabidopsis GID1* genes further by identifying and characterizing T-DNA and transposon insertion mutants for each gene (see Supplemental Figure 1A online). We identified two independent insertion alleles of the *GID1a* gene, designated *gid1a-1* and *gid1a-2*. In both cases, the T-DNA is inserted into the second exon. We also identified two independent T-DNA insertion alleles of *GID1c*, similarly designated *gid1c-1* and *gid1c-2*. The T-DNA insertion in *gid1c-2* is located in the second exon, whereas it is in the intron in *gid1c-1*. A single insertion allele (*gid1b-1*) of *GID1b* was identified from the SLAT collection, and the transposon insertion was confirmed to be in the second exon. Using RT-PCR with primers spanning or downstream of the insertion site, we were unable to detect gene-specific transcripts in all five mutants (see Supplemental Figure 1B online; data not shown for *gid1a-2* and *gid1c-2*). This suggests that all the mutations produce null alleles.

Generation of Multiple *gid1* Mutants

The overlapping expression patterns of the *GID1a-1c* genes (Figure 1A) suggest some functional redundancy in regulating

growth processes. This is supported by our findings that none of the single *gid1* mutants exhibited pronounced phenotypic defects when grown under standard growth conditions. To address the issue of functional redundancy, we constructed all double and the triple mutant combinations by genetic crossing. The *gid1a-1*, *gid1b-1*, and *gid1c-1* alleles were used to produce the mutants that are described in the following sections. The *gid1a-1 gid1b-1* double mutant, initially produced by crossing *gid1a-1* and *gid1b-1*, displayed reduced fertility (see below). However, this phenotype did not prevent it being successfully crossed with *gid1c-1*. The F2 population of this cross yielded the remaining *gid1a-1 gid1c-1* and *gid1b-1 gid1c-1* double mutants but not the triple mutant. It has been demonstrated that GAs are necessary for promoting germination in *Arabidopsis* (Koornneef and van der Veen, 1980). If the *GID1a-1c* genes encode the predominant GA receptors that are necessary for controlling GA-dependent growth processes, we hypothesized that the *gid1a gid1b gid1c* triple mutant would exhibit a nongerminating phenotype. To obtain this mutant, we identified a *gid1a-1/GID1a*; *gid1b-1/gid1b-1*; *gid1c-1/gid1c-1* plant, which was self-pollinated and the progeny scored on Murashige and Skoog (MS) agar plates. The self progeny included both germinating and nongerminating seeds. The nongerminating seed phenotype segregated as a single Mendelian recessive trait (see Supplemental Table 2 online). Although severe GA-deficient mutants do not germinate in the absence of GA, this block on germination can be bypassed by separating the embryo from the testa (Silverstone et al., 1997). Therefore, we dissected the embryos from the nongerminating seeds and transferred them to fresh agar plates. The majority of the embryos developed into healthy seedlings, which displayed a characteristic GA dwarf phenotype, initially reminiscent of the *ga1-3* mutant (see below). These mutants were confirmed as the *gid1a-1 gid1b-1 gid1c-1* triple mutant by PCR genotyping using allele-specific primers.

Phenotypes of *gid1* Mutants

We performed an initial phenotypic characterization of all mutant combinations to investigate the roles of each *Arabidopsis GID1* gene in known GA-regulated growth processes, focusing on flowering time, leaf expansion, stem elongation, and reproductive development. However, since the genetic analyses uncovered a high degree of functional redundancy between the three *Arabidopsis GID1* genes (see below), we subsequently focused mainly on the *gid1a-1 gid1b-1 gid1c-1* triple mutant as a means of dissecting the role of this gene family as a whole.

Vegetative Growth and Development

GAs have a well-characterized involvement in promoting vegetative development in *Arabidopsis*. This is clearly illustrated in the severely GA-deficient mutant *ga1-3*, which displays dramatically reduced leaf expansion, and stem and root elongation (Koornneef and van der Veen, 1980; Fu and Harberd, 2003). We compared rosette radius and root length in the single, double, and triple mutants (Table 1). There were only small differences between the single and double mutants compared with wild-type Col-0 plants. By contrast, the triple mutant displayed dramatic reductions in rosette radius and root length of 87 and 74%, respectively (Table 1, Figures 2A, 3B, and 3C). This result implies that the *Arabidopsis GID1* genes play functionally redundant roles in controlling leaf and root elongation under our standard growth conditions. In a separate experiment, we compared leaf and root elongation in the *gid1a-1 gid1b-1 gid1c-1* triple mutant and the *ga1-3* mutant that has been backcrossed six times with Col-0 (Tyler et al., 2004). Although there was no obvious difference in root length after 5 d growth (Figure 3C; see Supplemental Figure 3 online), at 12 d, the root length of the triple mutant was reduced by 27% compared with *ga1-3* (see Supplemental Figure 3 online). Similarly, the rosette radius of the triple mutant was reduced by 47% compared with *ga1-3* (Table 2, Figure 2B). This result indicates that GA signaling, which promotes root and leaf expansion, is not completely abolished in the *ga1-3* mutant.

The *ga1-3* mutant does not undergo stem elongation, indicating an essential role for GAs in this growth process (Koornneef and van der Veen, 1980). In this study, we show that the *gid1a-1 gid1b-1 gid1c-1* triple mutant also does not initiate stem elongation (Table 1, Figure 2A). Based on our findings that all of the single and double *gid1* mutants exhibit some degree of stem elongation (Table 1, Figure 2A), we conclude that the *Arabidopsis GID1* genes play functionally redundant roles also in controlling this growth process. However, they display only partial redundancy because the *gid1a-1 gid1c-1* double mutant exhibits a semidwarf phenotype in which stem elongation is reduced by 76% compared with the wild type (Table 1, Figure 2A). To confirm that the *gid1a-1* and *gid1c-1* mutations are responsible for the defects in stem elongation, we constructed the *gid1a-2 gid1c-2* double mutant. An identical semidwarf phenotype was observed in the *gid1a-2 gid1c-2* mutant (see Supplemental Figure 2A online), providing further support for the *gid1a* and *gid1c* alleles used in our study being nulls. The semidwarf phenotype of *gid1a gid1c* mutants indicates that *GID1a* and *GID1c* are the major GA receptor genes responsible for promoting stem elongation, but *GID1b* has a minor influence. This finding is consistent with our real-time PCR data, which show the levels of *GID1a* and *GID1c* transcripts to be ~60 and 2.4 times higher, respectively, than those of *GID1b* in the elongating stem (Figure 1A). However, the difference in the expression levels of *GID1b* and *GID1c* in the stem is small, and it is likely that factors other than their transcript levels also influence their contribution to promoting stem elongation. In contrast with the reduced stem elongation in *gid1a-1 gid1c-1* plants, the *gid1a-1 gid1b-1* mutant attains a final stem height that is 14% taller than the wild type (Table 1). An identical phenotype was also observed in the *gid1a-2 gid1b-1* mutant (see Supplemental Figure 2B online). This increase in final height is unexpected considering that the *Arabidopsis GID1* genes act as positive regulators of stem elongation. It is conceivable that this increase in stem elongation in *gid1a-1 gid1b-1* is an indirect consequence of the reduced fertility (Hensel et al., 1994) observed in this mutant (see below; Table 1).

Reproductive Growth and Development

GAs are involved in many aspects of reproductive physiology. In *Arabidopsis*, they promote floral initiation and the development of floral organs, seeds, and siliques (Wilson et al., 1992; Singh

Table 1. Phenotypic Characterization of the *gid1* Mutants

Genotype	Flowering Time (d)	Flowering Time (No. of Leaves)	Rosette Radius (mm)	Stem Height (cm)	Silique Length (mm)	Seeds/Silique (No.)	Root Length (mm)
Col-0	18.6 ± 0.3	11.0 ± 0.3	62.8 ± 1.5	51.4 ± 1.6 (3.95)[a]	14.3 ± 0.4	60.2 ± 4.1 (4.06)[a]	18.4 ± 1.0 (2.90)[a]
gid1a-1	18.8 ± 0.2	11.6 ± 0.2	65.1 ± 1.7	50.8 ± 1.0 (3.94)	10.8 ± 0.5*	53.2 ± 3.3 (3.97)	17.4 ± 0.7 (2.85)
gid1b-1	18.8 ± 0.2	11.2 ± 0.2	63.1 ± 1.6	52.9 ± 1.2 (3.98)	14.0 ± 0.6	54.6 ± 5.4 (3.95)	19.4 ± 0.8 (2.95)
gid1c-1	18.8 ± 0.2	11.3 ± 0.3	63.1 ± 1.8	49.7 ± 1.1 (3.92)	13.0 ± 0.5	62.8 ± 1.4 (4.16)	18.2 ± 0.9 (2.88)
gid1a-1 gid1b-1	19.1 ± 0.2	11.5 ± 0.1	67.9 ± 1.1	58.4 ± 0.6 (4.08)*	7.4 ± 0.3*	20.4 ± 5.1 (2.72)*	18.3 ± 0.9 (2.89)
gid1a-1 gid1c-1	18.6 ± 0.2	11.9 ± 0.3	67.5 ± 1.2	12.1 ± 0.2 (2.57)*	8.9 ± 0.2*	45.7 ± 4.3 (3.78)	17.0 ± 1.4 (2.75)
gid1b-1 gid1c-1	19.6 ± 0.3*	10.5 ± 0.3	62.0 ± 1.9	51.7 ± 1.3 (3.96)	13.7 ± 0.5	62.5 ± 3.8 (4.13)	15.9 ± 0.9 (2.80)
gid1a-1 gid1b-1 gid1c-1	39.5 ± 0.2*	21.7 ± 0.6*	8.2 ± 0.2*	NA	NA	NA	4.8 ± 0.3 (1.54)*
LSD (1%) (*df*)	0.82 (82)	1.31 (80)	5.21 (82)	0.077 (44)[a]	2.26 (45)	0.547 (44)[a]	0.235 (110)[a]

See Methods for details of experimental design. The measurements are the means ± SE from 12 plants per line (except for root length). For the primary root length measurements, seeds were dissected to remove the testa and plated on vertical MS agar plates. The measurements were taken after 5 d and are the means ± SE ($n = 10$ to 17). *, Significantly different values from Col-0 ($P < 0.01$).

[a] Log-transformed values (shown in parentheses) were used for statistical analysis (see Methods), and the LSD (1%) corresponds to these values.

et al., 2002; Cheng et al., 2004). To assess the involvement of the *Arabidopsis GID1* genes in these developmental processes, we have characterized reproductive development in all combinations of mutants. Under a long-day (LD) photoperiod, the *gid1a-1 gid1b-1 gid1c-1* mutant flowered at 22 and 8 d after the Col-0 and *ga1-3* controls, respectively (Table 2). Furthermore, using scanning electron microscopy of dissected flower buds at a stage immediately before they senescence, we observed defects in floral organ morphology in the triple mutant that are more severe than those in *ga1-3* (Figures 2E and 2F). However, there do not appear to be defects in floral patterning. The floral organ defects observed in the triple mutant include a dramatic reduction in the length of the pistil, although elongation of the papillae appears to be unaffected, and arrest of the stamens at an earlier stage of development than in *ga1-3* (Figures 2E and 2F). Petal and stamen development do not progress beyond floral stage 10 in *ga1-3* (Cheng et al., 2004). While the petal development in the *gid1a-1 gid1b-1 gid1c-1* mutant appears similar to that in *ga1-3*, the stamens appear to be arrested earlier, before stage 9, based on the lack of differentiation between the filament and the anthers (Smyth et al., 1990). The triple mutant also exhibits a striking reduction in the elongation of the pedicel compared with *ga1-3* (Figures 2E and 2F). As for *ga1-3*, the arrested development of the floral organs in the triple mutant resulted in complete infertility.

In contrast with the triple mutant, all of the single and double mutants flowered at essentially the same time (within 1 d) as wild-type plants (Table 1), indicating that the *Arabidopsis GID1* genes possess functionally redundant roles in promoting floral initiation in LD photoperiods. However, there does appear to be some specificity in their regulation of filament and silique elongation. All of the mutants lacking GID1a displayed reductions in silique length compared with Col-0 (Table 1). Siliques of the *gid1a-1*, *gid1a-1 gid1b-1*, and *gid1a-1 gid1c-1* mutants are reduced in length by 24, 48, and 38%, respectively (Table 1). Even more strikingly, the *gid1a-1 gid1b-1* double mutant exhibits a reduction in seed number per silique of 66% compared with Col-0 (Table 1). The dramatically reduced fertility in the *gid1a-1 gid1b-1* double mutant appears to be a direct consequence of a defect in filament elongation. Compared with wild-type flowers, the filaments of *gid1a-1 gid1b-1* are shorter, such that less pollen released from the anthers is likely to reach the stigmatal surface (Figures 2C and 2D). It is clear that GID1c plays some role in filament elongation because the reduction in length in *gid1a-1 gid1b-1* is not as severe as that observed in the *gid1a-1 gid1b-1 gid1c-1* triple mutant.

The *gid1a-1 gid1b-1 gid1c-1* Triple Mutant Is Insensitive to GA

The phenotype observed in the *gid1a-1 gid1b-1 gid1c-1* mutant provides supporting evidence that the *Arabidopsis GID1* genes act as positive regulators of GA signaling. We confirmed that GA signaling is perturbed in the triple mutant by examining response to applied GA at both the whole plant and molecular levels. We treated *gid1a-1 gid1b-1 gid1c-1*, *ga1-3*, and Col-0 plants throughout their life cycle with or without GA_4 (Figure 3A, Table 2). While the *ga1-3* and Col-0 plants demonstrated a visible growth response to GA, the treated and untreated *gid1a-1 gid1b-1 gid1c-1* mutants were indistinguishable (Figure 3A, Table 2). In the same study, we observed that the semidwarf phenotype of the *gid1a-1 gid1c-1* double mutant was also not rescued by the GA treatment (see Supplemental Figure 2B online). We investigated the GA responsiveness of the triple mutant further by determining the effects of GA treatment on flowering time, leaf expansion, and root and hypocotyl elongation. In all cases, we were unable to detect any effect of GA in promoting growth (Figures 3B to 3D, Table 2), providing clear evidence that *gid1a-1 gid1b-1 gid1c-1* is GA-insensitive, at least for the developmental processes investigated. Furthermore, it provides additional evidence that the *gid1* mutations used in this study produce null alleles.

GA homeostasis is maintained by regulation of its own metabolism via the signaling pathway. This involves downregulation of some of the GA-biosynthetic genes encoding GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) and upregulation of genes encoding GA 2-oxidase (Hedden and Phillips, 2000; Olszewski et al., 2002). We investigated GA responsiveness of the *gid1a-1 gid1b-1 gid1c-1* mutant at the molecular level by examining expression of *GA3ox1* (Chiang et al., 1995) using real-time

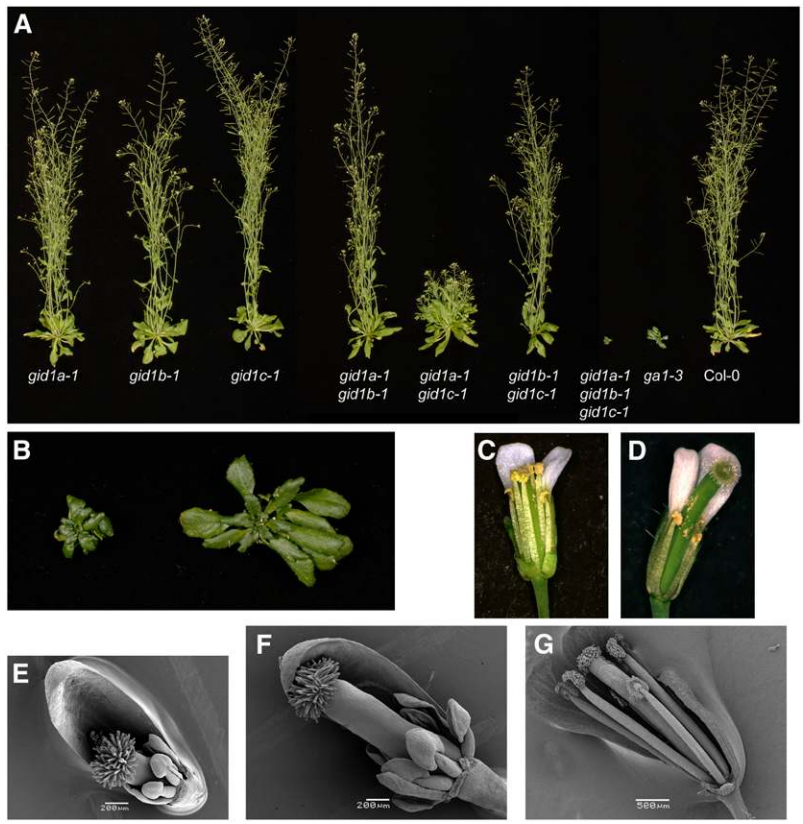

Figure 2. Phenotypes of the *gid1* Mutants.

(A) and **(B)** Aerial portions of 37-d-old wild-type and homozygous mutant plants. Genotypes are indicated in **(A)**. Close-up view of the *gid1a-1 gid1b-1 gid1c-1* (left) and *ga1-3* (right) mutants is shown in **(B)**.
(C) and **(D)** Close-up views of Col-0 **(C)** and *gid1a-1 gid1b-1* **(D)** flowers. Sepals and petals have been removed to reveal the anthers and pistil.
(E) to **(G)** Scanning electron microscopy floral images of *gid1a-1 gid1b-1 gid1c-1* **(E)**, *ga1-3* (Col-0) **(F)**, and Col-0 **(G)**. Sepals and petals have been removed to visualize the anthers and pistil. Bars = 200 μm in **(E)** and **(F)** and 500 μm in **(G)**.

quantitative RT-PCR. Compared with Col-0 seedlings, *GA3ox1* transcript levels were elevated approximately sixfold in the triple mutant (Figure 3E). Application of GA_4 caused an ~2.5-fold reduction in the transcript levels of *GA3ox1* in wild-type seedlings but had no effect in the triple mutant. This result is consistent with GA biosynthesis being regulated through the activity of the GA response pathway to maintain GA homeostasis and provides further evidence that the *gid1a-1 gid1b-1 gid1c-1* mutant is GA insensitive. It has been demonstrated that the loss of feedback regulation of GA biosynthesis in the rice *gid1-2* mutant resulted in the accumulation of the bioactive GA (GA_1) to levels that were 120 times higher than those of wild-type plants (Ueguchi-Tanaka et al., 2005). To determine whether GA content is also altered in the *gid1a-1 gid1b-1 gid1c-1* mutant, we analyzed C_{19}-GA levels in the vegetative rosettes of Col-0 and mutant plants (Table 3). The concentration of GA_4, the major bioactive GA in *Arabidopsis*, was elevated by at least 12-fold in the triple mutant compared with the wild type, whereas that of GA_{34}, its 2β-hydroxylated (inactivated) metabolite, was >20-fold higher in the mutant. Large increases were found also for the concentrations of GA_1 and GA_8, the 13-hydroxylated analogs of GA_4 and GA_{34}, respectively. In this case, the concentration of GA_1 in the wild type was too low to be quantified. There was a much smaller change in the concentration of GA_{20}, the immediate precursor of GA_1, as might be anticipated since both its rate of conversion to GA_1 by GA3ox and its formation by GA20ox are likely to be elevated in the triple mutant. The content of GA_{29}, the 2β-hydroxylated analog of GA_{20}, was too low to be quantified in the wild type and mutant.

A consequence of the elevated GA levels in *gid1a-1 gid1b-1 gid1c-1* is that GA may be saturated for some responses in this mutant. We investigated this possibility by reducing GA content in the triple mutant by growing them in the presence of the GA biosynthesis inhibitor paclobutrazol (PAC) and then determining the extent of root growth in response to GA (see Supplemental Figure 3 online). PAC inhibited the growth of *ga1-3* and *gid1a-1 gid1b-1 gid1c-1* roots to a similar extent. However, when GA was also added to the plates, the roots of *ga1-3* seedlings were approximately four times longer after 5 d, whereas those of the triple mutant did not elongate further. This confirms that roots of the triple mutants are unable to respond to GA. It is interesting to note that PAC can inhibit root growth further in the *gid1a-1 gid1b-1 gid1c-1* mutant. This observation suggests that there are GA-independent pathways responsible for regulating root

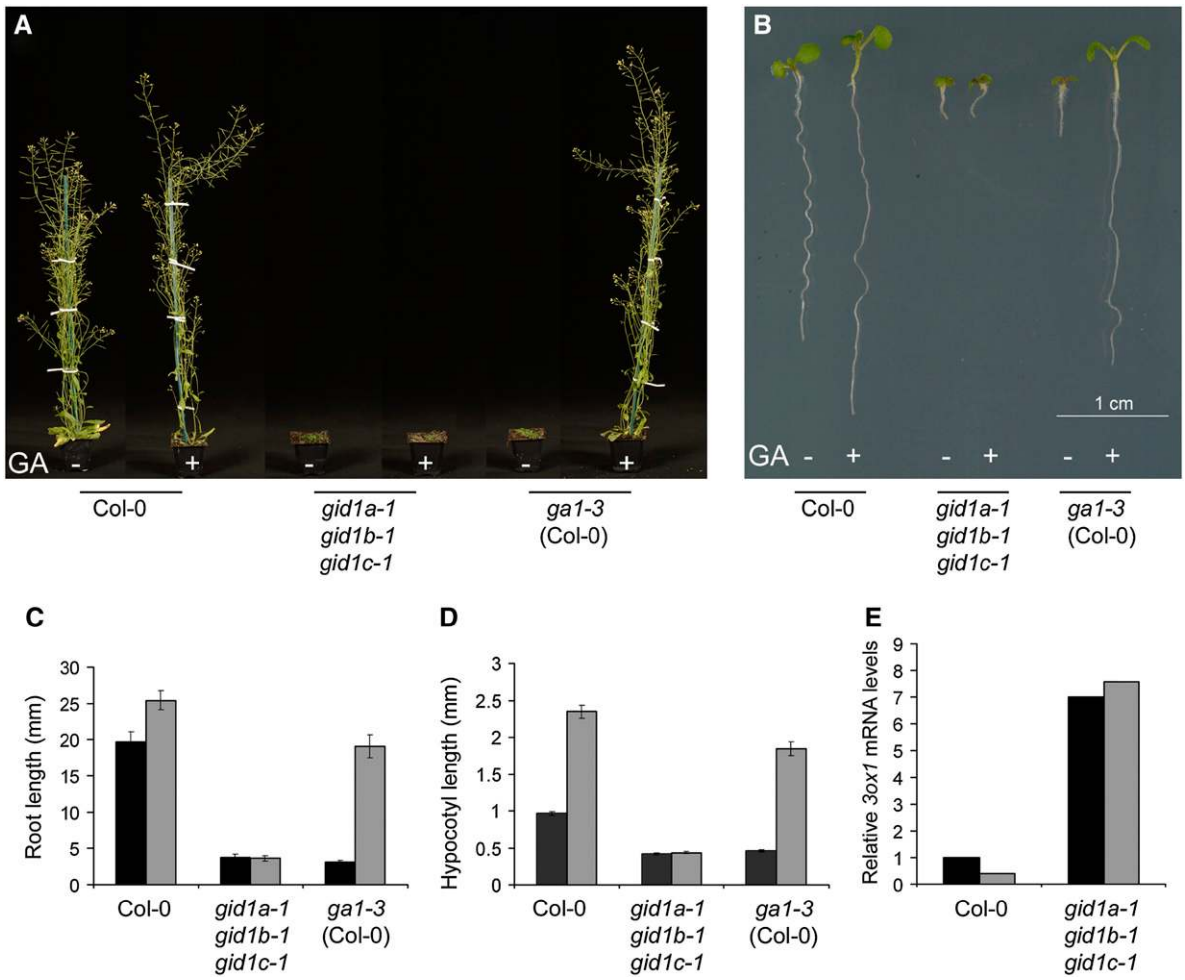

Figure 3. GA-Insensitive Phenotype of *gid1a-1 gid1b-1 gid1c-1*.

(A) Aerial portions of 35-d-old plants treated with (+) or without (−) 100 μM GA_4 as indicated. Genotypes of plants are indicated below.
(B) Representative 5-d-old seedling primary roots of selected genotypes, grown on vertical MS plates and treated with (+) or without (−) 0.2 μM GA_4 as indicated. Bar = 1 cm.
(C) and **(D)** Mean length (±SE; $n = 20$) of 5-d-old primary roots **(C)** and hypocotyls **(D)** of wild-type (Col-0), *gid1a-1 gid1b-1 gid1c-1*, and *ga1-3* (Col-0) grown on vertical MS plates and treated with (gray bars) or without (black bars) 0.2 μM GA_4.
(E) Transcript levels of *GA3ox1*. The relative levels of *GA3ox1* expression in *gid1a-1 gid1b-1 gid1c-1* and wild-type (Col-0) plants treated with 0.2 μM GA_4 (gray bars) or water (black bars) for 2 h as determined by quantitative RT-PCR (see Supplemental Methods online). Total RNA used for quantitative RT-PCR analysis was extracted from 10-d-old seedlings grown on MS plates under continuous light. The means of two technical replicates are shown.

growth that are also inhibited by PAC. For example, brassinosteroid biosynthesis and abscisic acid catabolism also were shown to be affected by PAC and/or related compounds that target cytochrome P450 monooxygenases (Rademacher, 2000).

Feedback Regulation of *Arabidopsis GID1* Transcript Levels by GA and DELLA

Our initial interest in the *Arabidopsis GID1* genes arose from analysis of the AtGenExpress microarray data set (http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm) in which the effect of treating a GA-deficient mutant, *ga1-5*, with bioactive GA was analyzed using Affymetrix ATH1 gene chips. Among the genes shown to be regulated by GA were two subsequently shown to encode the GID1 homologs GID1a and GID1b (data not shown). Furthermore, a recent transcriptomics study by Cao et al. (2006) identified *GID1a* and *GID1b* as DELLA-upregulated genes. We examined this further by quantitative RT-PCR analysis and confirmed that indeed a feedback mechanism, similar to that operating in GA biosynthesis, also appears to modulate transcript levels of *GID1a*, *GID1b*, and *GID1c* (Figures 1B and 1C). As a control, transcript levels of *GA3ox1* were also measured. Similar to changes in *GA3ox1* expression, although less dramatic, GA treatment of *ga1-3* reduced transcript levels of *GID1a-1c* (to ~50% for *GID1a* and *GID1b* and a smaller decrease for *GID1c*; Figure 1B). In addition, *rga-24* and *gai-t6* null mutations

Table 2. GA-Insensitive Phenotype of *gid1a-1 gid1b-1 gid1c-1*

Genotype	Flowering Time (d)		Flowering Time (No. of Leaves)		Rosette Radius (mm)	
	−GA	+GA	−GA	+GA	−GA	+GA
Col-0	16.9 ± 0.1	14 ± 0.2**	13.0 ± 0.44	10.3 ± 0.33**	55.4 ± 2.4 (4.00)[a]	45.7 ± 2.5 (3.81)[a]
ga1-3 (Col-0)	30.8 ± 0.3*	16.6 ± 0.5*/**	20.6 ± 0.63*	13.1 ± 0.42*/**	17.6 ± 0.6 (2.86)*	43.1 ± 2.5 (3.74)**
gid1a-1 gid1b-1 gid1c-1	38.7 ± 0.6*	38.8 ± 0.3*	26.6 ± 0.76*	25.3 ± 0.69*	9.4 ± 0.4 (2.23)*	9.8 ± 0.3 (2.28)*
$LSD_{within\ treatments}$ (1%) (*df*)	1.30 (12)		2.24 (12)		0.240 (12)[a]	
$LSD_{between\ treatments}$ (1%) (*df*)	1.34 (13)		2.13 (15)		0.269 (11)[a]	

See Methods for experimental design. The measurements are the means ± SE from 12 plants per line. Plants were treated with 100 μM GA_3 by spraying at 48-h intervals throughout the course of the experiment (+GA) or kept as control (−GA). *, Significantly different from Col-0 (within a treatment) ($P < 0.01$); **, significantly different from the −GA treatment (within a genotype) ($P < 0.01$).

[a]Log-transformed values (shown in parentheses) were used for statistical analysis (see Methods), and the LSD (1%) corresponds to these values.

(in the triple homozygous mutant *ga1-3 rga-24 gai-t6*) resulted in slightly decreased *GID1a* and *1b* mRNA levels, presumably because the GA signaling pathway is derepressed by these mutations (Figure 1C). Consistent with this hypothesis, the gain-of-function *rga-Δ17* caused an opposite effect on *GID1a* and *1b* expression in comparison with the null DELLA alleles. Although the experiments described in this section were performed in the Landsberg *erecta* (L*er*) background, we have also analyzed the expression of the *GID1a-1c* genes in Col-0 and found that they are expressed at very similar levels and show an equivalent response to GA treatment in the two ecotypes (data not shown).

It is conceivable that the feedback regulation of *GID1a-1c* mRNA levels through the GA signaling pathway has an effect on the phenotype of *gid1* single and double mutants, which contain one of these functional genes. To explore this hypothesis, we analyzed the relative levels of the *GID1a-1c* transcripts in all combinations of *gid1* single and double mutants using real-time PCR. In all of these mutants, we did not detect any obvious changes in the levels of *GID1a-1c* transcripts in 7-d-old seedlings compared with Col-0 (data not shown). This result suggests that the lack of developmental defects observed in the *gid1* seedlings is not due primarily to the increased expression of the remaining *Arabidopsis GID1* genes.

RGA Is Not Degraded in Response to GA in *gid1a-1 gid1b-1 gid1c-1*

In rice, it has been shown that GID1 plays a direct role in targeting SLR1 for degradation (Ueguchi-Tanaka et al., 2005). In the absence of GID1, SLR1 accumulates to high levels and is not degraded in response to GA, leading to a GA-insensitive dwarf phenotype. The role of SLR1 in producing the GA-insensitive phenotype was confirmed by epistasis analysis showing that *slr1* loss-of-function mutations completely rescue the defects in *gid1* (Ueguchi-Tanaka et al., 2005). Considering the highly conserved mechanisms between the GA signaling cascades in *Arabidopsis* and rice, it seems probable that the GA-insensitive dwarf phenotype of the *gid1a-1 gid1b-1 gid1c-1* mutant is caused by the accumulation of DELLAs and absence of GA-mediated degradation. To explore this hypothesis, we analyzed the level and GA sensitivity of the DELLA protein RGA in the *gid1a-1 gid1b-1 gid1c-1* triple mutant by immunoblotting. Similar to RGA accumulation in *ga1-3*, we found that RGA protein levels were elevated in the triple mutant compared with wild-type Col-0 (Figure 4A). In contrast with wild-type Col-0 and *ga1-3*, but similar to the *sly1-2* mutant, GA treatment of the triple mutant did not result in any decrease in RGA protein levels (Figure 4A). Our results in Figure 4A suggest that there is a basal level of GA (and GID1)–independent degradation of RGA mediated by SCF^{SLY1} because the amount of RGA in the *sly1* mutant was much higher than in *ga1-3* or the *gid1* triple mutant.

To confirm that the dwarf phenotype of the triple mutant is caused by the overaccumulation of the DELLAs, we constructed the *rga-28 gid1a-1 gid1b-1 gid1c-1* quadruple mutant by genetic crossing. Figure 4B shows a comparison of the *gid1a-1 gid1b-1 gid1c-1* and *rga-28 gid1a-1 gid1b-1 gid1c-1* mutants and wild-type Col-0 plants after 37 d grown on soil. The *rga-28* mutation partially suppresses many of the growth defects of the *gid1a-1 gid1b-1 gid1c-1* mutant, including leaf expansion and stem elongation. However, some of the defects, including germination and fertility, are not rescued. This result demonstrates that GID1a-1c are responsible for promoting GA-dependent growth in *Arabidopsis*, in part, through their regulation of GA-mediated degradation of RGA. It is likely that the developmental defects still observed in the *rga-28 gid1a-1 gid1b-1 gid1c-1* mutant are caused by the accumulation of the other DELLA proteins, GAI, RGL1, RGL2, and RGL3, in this plant.

DELLA Proteins Interact with *Arabidopsis* GID1 via Their N-Terminal DELLA Domain

Recent studies using yeast two-hybrid assays showed that binding of GA to GID1 promotes direct interaction of GID1 and

Table 3. GA Content of Col-0 and *gid1a-1 gid1b-1 gid1c-1* Rosettes

	GA Content (ng/g Dry Weight)									
	GA_{20}		GA_1		GA_8		GA_4		GA_{34}	
Replicate	1	2	1	2	1	2	1	2	1	2
Col-0	1.2	0.6	0.0[a]	0.0[a]	0.8	0.5	3.7	7.0	4.7	7.7
gid1a gid1b gid1c	1.4	3.0	19.8	28.5	15.3	14.1	83.8	90.8	149.0	174.6

[a] Below the level of detection.

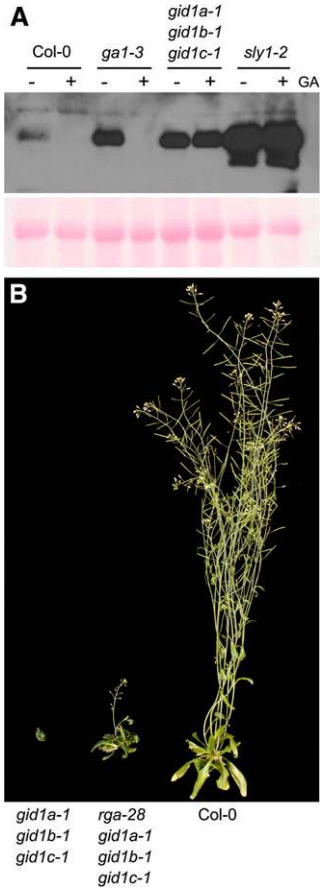

Figure 4. The Triple *gid1* Mutant Accumulates a High Level of RGA and Is Suppressed by an *rga* Null Allele.

(A) Elevated RGA protein levels in *gid1a-1 gid1b-1 gid1c-1* with or without GA treatment. Total proteins were extracted from seedlings of Col-0 and homozygous mutants after treatment with water (−) or 1 μM GA_4 (+GA) for 1 h. Protein samples (50 μg each) were separated by 8% SDS-PAGE gel, and the blot was probed with affinity-purified rabbit anti-RGA antibodies. Ponceau staining was used to confirm equal loading. Experiments using two additional biological replicates showed similar results.

(B) Partial suppression of *gid1a-1 gid1b-1 gid1c-1* by *rga-28*. Col-0 and the homozygous mutants indicated were grown on soil for 37 d under an LD photoperiod. The *rga-28* single mutant is not shown because the phenotype is similar to the wild type (Tyler et al., 2004).

DELLA (Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006). However, the sequence in DELLA that is involved in GID1 binding has not been characterized, although the DELLA domain is a candidate because it is essential for GA-induced proteolysis of the DELLA proteins. To test our hypothesis, we first examined interaction between *Arabidopsis* DELLA proteins and GID1a-1c. RGA and GAI were expressed as Gal4 transactivation domain (AD) protein fusions and the GID1a-1c as LexA DNA binding domain (DB) protein fusions in the yeast strain L40 harboring the *His3* and *LacZ* reporter genes. Interactions of DB and AD fusion proteins in the yeast cells were scored for the relative growth on His^- plates containing 3-aminotriazole (3-AT; 5 to 60 mM) and for β-galactosidase (β-gal) activity. 3-AT serves as an indicator of the strength of interaction between the DB and AD protein fusions because it is a competitive inhibitor of the His3 enzyme. Therefore, the ability to grow on a higher concentration of 3-AT indicates a higher His3 expression level (Durfee et al., 1993). Consistent with a recent report of Nakajima et al. (2006), we observed GA-enhanced interactions between DELLA proteins (RGA and GAI) and GID1a, -1b, and -1c in yeast two-hybrid assays (Figure 5A). Interestingly, GID1b and GID1c showed some degree of GA-independent interactions with DELLA proteins (Nakajima et al., 2006; Figure 5A). To provide additional evidence for a direct, GA-triggered interaction between RGA and GID1a, we performed in vitro pull-down assays. GID1a was expressed in *E. coli* as a glutathione *S*-transferase (GST) fusion protein and purified using glutathione sepharose. The purified GST-GID1a protein was incubated with crude lysates prepared from *sly1-10* and *sly1-10 rga-24* seedlings in the presence or absence of GA_4, washed, and then immunoblotted with anti-RGA antibodies. Only in the presence of GA_4 was the endogenous RGA protein pulled down from a *sly1-10* lysate by GST-GID1a but not by GST (Figure 6A). The RGA pull-down efficiency appears to be dependent on the concentration of GA_4 (Figure 6B). Similar pull-down assays were also performed using GST-GID1b and GST-GID1c to test whether we could detect GA-independent interactions with RGA, as observed in the yeast two-hybrid system. However, both GID1b and GID1c pulled down RGA from *sly1* extracts in a GA-dependent manner, although there might be a very weak interaction between GID1c and RGA in the absence of GA (see Supplemental Figure 4 online). Future studies will be needed to determine whether GA-independent interaction occurs in planta for any of the GID1s.

The conserved motifs DELLA and VHYNP near the N termini of DELLA proteins are essential for GA-induced degradation (Dill et al., 2001; Itoh et al., 2002). However, these motifs are not required for SLY1 binding (Dill et al., 2004), raising the possibility that they are involved in *Arabidopsis* GID1 binding. Figures 5B and 5C show that deletion of the DELLA motif in GAI or RGA abolished interaction with GID1a. Additional assays using a RGA deletion series indicated that the N-terminal 108 amino acids region containing both DELLA and VHYNP motifs (RNT2) is required for GID1a binding, whereas the Poly S/T region or GRAS domain is not.

GA-GID1 Promotes RGA and SLY1 Interaction in Yeast Three-Hybrid Assays

Although it was demonstrated that GA enhances *Arabidopsis* GID1 and DELLA protein interaction (Nakajima et al., 2006) and that the SCF^{SLY1} complex targets DELLA protein for GA-induced degradation (Dill et al., 2004; Fu et al., 2004), the question remains whether the GA-GID1-DELLA complex is recognized directly by the F-box protein SLY1. To address this question, yeast three-hybrid assays were performed. Previously, we showed that DB (LexA)-SLY1 and AD-RGA interact very weakly (with only 0.3 units of β-gal activity) in a yeast two-hybrid assay (Dill et al., 2004). In the current system using DB (Gal4)-SLY1 and AD-RGA, the weak SLY1-RGA interaction appears to be masked by the higher background of the vector controls (Figure 7). Moreover, addition of GA in the media did not enhance SLY1-RGA interaction. Nonetheless,

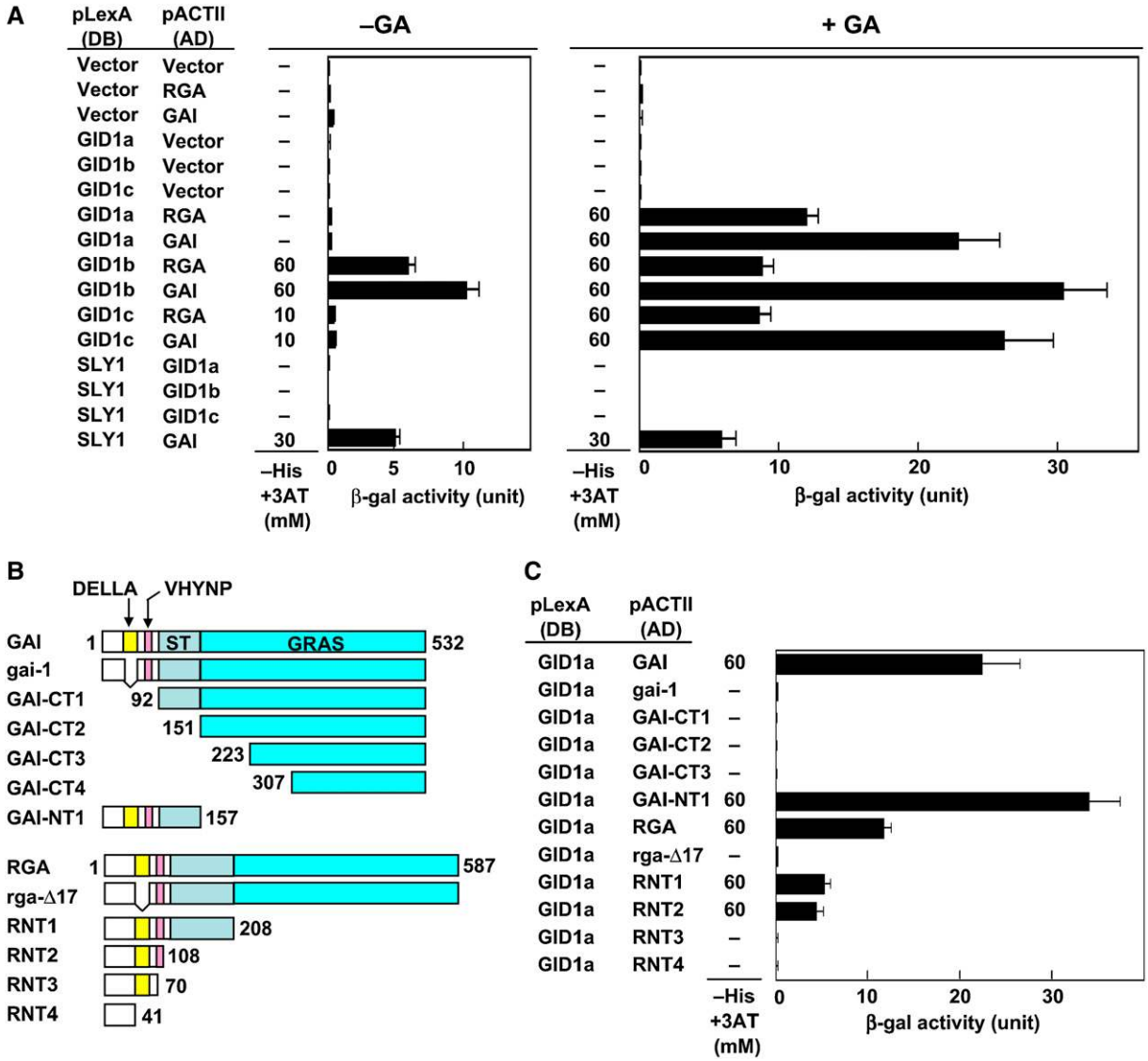

Figure 5. GA-Dependent Interaction between GID1a-1c and DELLA Proteins via the DELLA Domain.

(A) GID1a, -1b, and -1c interact with DELLA proteins but not with SLY1 in yeast two-hybrid assays. The presence of 100 μM GA_3 (+GA) in the media enhanced GID1–DELLA interactions.

(B) A schematic showing the full-length and truncated GAI and RGA used in the yeast two-hybrid assays. The numbers represent amino acid positions of the start and/or end of deletion constructs. ST, Poly S/T region.

(C) The DELLA domain without the Poly S/T region is essential for *Arabidopsis* GID1 interaction.

All assays in **(C)** were performed in the presence of 100 μM GA_3. In **(A)** and **(C)**, interaction of DB and AD fusion proteins in the L40 yeast cells was scored by the relative growth in His^- media containing 3-AT (5, 10, 30, and 60 mM) and β-gal activity (means ± SE; at least nine colonies were assayed for enzyme activity in triplicates). A dash indicates no growth on His^- plates at 5 mM 3-AT.

when GA was added to the cells that coexpressed GID1a with DB-SLY1 and AD-RGA, a strong interaction between SLY1 and RGA was observed. rga-Δ17 (lacking the DELLA motif) or RNT1 (lacking the GRAS domain) failed to interact with SLY1 even in the presence of GA and GID1 (Figure 7B). These observations are consistent with previous results showing that the DELLA domain (Figure 5) and the GRAS domain (Dill et al., 2004) are required for binding to GID1 and SLY1, respectively. We also showed that SLY1 alone does not interact with *Arabidopsis* GID1 in the presence or absence of GA (Figure 5A). These results support the idea that SLY1 binds to a GA-GID1-RGA complex directly and that GA-GID1 dramatically enhances RGA and SLY1 interaction.

DISCUSSION

In this study, we provide genetic evidence that GID1a, GID1b, and GID1c function as positive regulators of the GA-signaling cascade in *Arabidopsis*. These findings, coupled with the

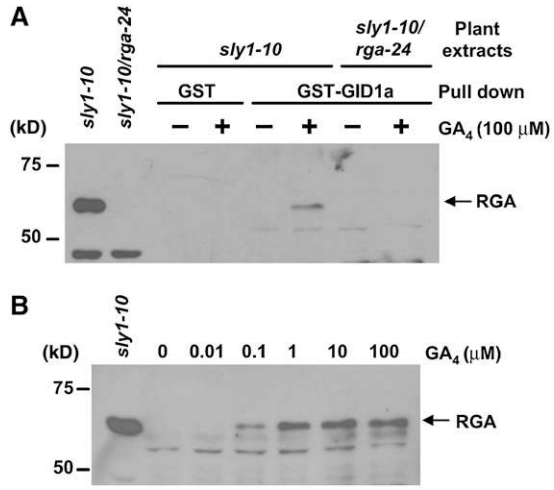

Figure 6. GA-Dependent RGA and GID1a Interaction in Pull-Down Assays.

(A) Recombinant GST-GID1a or GST was used in pull-down assays with lysates prepared from *sly1-10* or *sly1-10 rga-24* seedlings in the absence (−) or presence (+) of 100 μM GA_4. The first two lanes contain 3.5 μg of total proteins from *sly1-10* and *sly1-10 rga-24*.

(B) RGA was pulled down by GST-GID1a in a GA dose-dependent manner. Pull-down assays were performed as described in **(A)**, except with different GA_4 concentrations (as indicated above the blot).

In **(A)** and **(B)**, pull-down samples were separated by 8% SDS-PAGE gel, and the blots were probed with affinity-purified rabbit anti-RGA antibodies. Experiments were repeated once with similar results.

demonstration by Nakajima et al. (2006) that recombinant *Arabidopsis* GID1s bind bioactive GAs, strongly supports their roles as GA receptors that are responsible for regulating GA-responsive growth and development. Furthermore, they appear to function analogously to the rice GA receptor GID1 (Ueguchi-Tanaka et al., 2005) by binding to DELLA proteins in a GA-dependent manner. Our studies in yeast also provide two important findings that help to explain how the GA-GID1 complex targets DELLA protein degradation. First, the N-terminal regulatory domain of the DELLAs is necessary for interacting with the GA-GID1 complex. Second, binding of GA-GID1 to RGA (an *Arabidopsis* DELLA) enhances interaction between RGA and the F-box protein SLY1, presumably promoting ubiquitination by the SCF^{SLY1} Ub E3 ligase and allowing subsequent degradation by the 26S proteasome in planta.

GID1a, GID1b, and GID1c Show Partial Redundancy

Our studies with the *gid1* mutants demonstrate that GID1a, GID1b, and GID1c are required for all GA-regulated developmental processes that we have analyzed, with a high degree of functional redundancy. For example, the *gid1b-1 gid1c-1* double mutant displays an essentially wild-type phenotype, indicating that GID1a is sufficient for all visible aspects of GA-responsive growth and development under our growth conditions. However, GID1a-1c are not completely redundant. The *gid1a* single mutant and both double mutants lacking functional GID1a display phenotypic defects in reproductive development, including stem length, silique length, and fertility. It seems likely from the expression profiles of the *GID1a-1c* genes that the more significant contribution of *GID1a* is due primarily to its higher levels of expression in almost all tissues analyzed compared with the other two genes. For example, the reduction in silique elongation in the *gid1a-1* mutants correlates well with the high levels of expression of *GID1a* in this organ. However, in several other tissues that exhibit significantly higher levels of *GID1a* expression, no phenotypic abnormalities are observed in the *gid1a-1* mutants. We found no evidence to indicate that the lack of these defects in any of the *gid1* single or double mutants was due to the upregulated expression of the remaining functional *Arabidopsis GID1* gene(s) (data not shown). It is therefore possible that other factors, including different affinities of the *Arabidopsis* GID1s for bioactive GAs, or their specificity for individual DELLAs may influence their physiological roles. It has been demonstrated that the *Arabidopsis* GID1s display different affinities in vitro for the tritiated GA_4 derivative 16,17-dihydro-GA_4 (Nakajima et al., 2006). Most notably, GID1b has a higher GA binding affinity than GID1a and GID1c. However, based on the lack of any obvious phenotypic defects in the *gid1b-1* mutant, the physiological significance of this increased affinity is not evident. It is known that the five DELLAs exhibit both overlapping and distinct roles in regulating GA-responsive growth in *Arabidopsis* (Dill and Sun, 2001; King et al., 2001; Lee et al., 2002; Cheng et al., 2004; Tyler et al., 2004). It is conceivable that individual *Arabidopsis* GID1s display specificity for the DELLAs they target, although our data and those of Nakajima et al. (2006) do not support this model; there are no clear differences in binding specificity of individual *Arabidopsis* GID1s to the different DELLAs in yeast two-hybrid assays. Furthermore, GID1a-1c display functional redundancy in developmental processes that are known to be controlled by individual DELLAs, for example, promotion of stem elongation. A better understanding of the physiological roles of the *Arabidopsis* GID1s will require defining expression profiles of GID1a-1c and DELLAs at the cellular level. It would also be important to determine whether the stability and/or activity of *Arabidopsis* GID1s are affected by GA.

The *gid1* Triple Mutant Displays Severe Growth Defects

An important finding of our study is that the *gid1a-1 gid1b-1 gid1c-1* triple mutant exhibits growth defects that are even more severe than those of the highly GA-deficient mutant *ga1-3*. While the *ga1-3* mutant already exhibits severe defects in the development of the leaves, anthers, petals, carpel, and the timing of floral induction, all of these are more pronounced in the triple mutant. Coupled with our finding that all of the phenotypic defects in *gid1a-1 gid1b-1 gid1c-1* are not rescued by GA, this implies that GID1a-1c are the major GA receptors in *Arabidopsis*. Furthermore, the severity of the phenotypic defects observed in *gid1a-1 gid1b-1 gid1c-1* demonstrate that the role of GA signaling in promoting *Arabidopsis* growth and development is even greater than previously described. In *Arabidopsis*, a single gene encodes *ent*-copalyl diphosphate synthase (CPS), an enzyme that catalyzes the first committed step of GA biosynthesis (Sun and Kamiya, 1994). The GA-deficient phenotype of the *ga1-3*

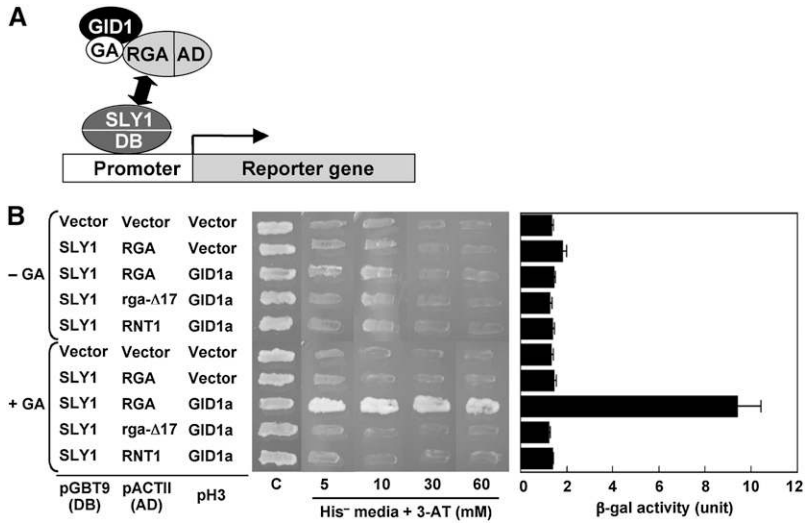

Figure 7. Enhanced SLY1-RGA Interaction by GA-GID1 in Yeast Three-Hybrid Assays.

(A) A diagram showing that binding of the GA-GID1-(AD-RGA) complex with DB-SLY1 allows reporter gene expression in the yeast three-hybrid assays.
(B) SLY1 and RGA interaction was only detected in the presence of both GID1a and 100 μM GA_3 (+GA). Interaction of DB and AD fusion proteins in the PJ69-4A yeast cells was scored by the relative growth in His^- media containing 3-AT (5 to 60 mM; middle panel) and by β-gal activity (means ± SE; at least nine colonies were assayed for enzyme activity in triplicates) (right panel). C, growth in His^+ media.

mutant is caused by a large deletion in the *CPS* gene that produces a null allele (Sun et al., 1992). Although this might be expected to produce a completely GA-deficient plant, there are two reports that *ga1-3* (in the L*er* background) accumulates detectable levels of bioactive GAs (King et al., 2001; Silverstone et al., 2001), which could provide a simple explanation for its increased growth compared with the triple mutant. It has recently been shown that *Arabidopsis* mutants that accumulate high levels of the volatile GA precursor *ent*-kaurene release it to the atmosphere at levels that are sufficient to completely rescue the growth defects of *ga1-3* (Otsuka et al., 2004). It is therefore conceivable that the very low levels of GAs in *ga1-3* detected in the two studies were formed from *ent*-kaurene released from neighboring plants. Another potential explanation for the reduced growth of the *gid1a-1 gid1b-1 gid1c-1* mutant compared with *ga1-3* is that the *Arabidopsis* GID1s promote growth to a small extent through a GA-independent pathway. Potential support for this pathway is suggested by our findings that GID1b interacts with the DELLAs, RGA, and GAI, even in the absence of GA in yeast cells. However, in the pull-down assays, we failed to detect this GA-independent interaction. It should be noted that in similar yeast two-hybrid assays, Nakajima et al. (2006) found that GID1b interacted only with RGL1 in the absence of GA. Future studies will be needed to determine whether a GA-independent *Arabidopsis* GID1 pathway exists.

Despite the gross development defects apparent in the *gid1* triple mutant, it was possible to obtain fertile mutant seeds by self-fertilizing a line that was heterozygous for the *gid1a-1* mutation and homozygous for *gid1b-1* and *gid1c-1*. Indeed, there was no evidence for discrimination against triple mutant seeds in the progeny of this cross. This finding was unexpected in the light of results using GA-deficient mutant and transgenic plants that indicated a requirement for GA in pollen development, pollen tube growth, and seed development (Swain et al., 1997; Singh et al., 2002; Cheng et al., 2004). It is therefore possible that these processes use GA signaling pathways that are independent of the GID1 receptors. The existence of other GA receptors and signaling pathways, perhaps specific to particular developmental processes, cannot be discounted. Indeed, there is considerable, albeit indirect, evidence for the existence of membrane-associated receptors for GA, most notably in the cereal aleurone (Hooley et al., 1991; Gilroy and Jones, 1994; Lovegrove and Hooley, 2000).

Interactions among Components of GA Metabolism, GA Perception, and GA Signaling

Our results demonstrate that GA homeostasis is maintained by a more complex mechanism than previously realized. GA signaling activities not only affect transcript levels of some genes encoding enzymes for GA biosynthesis or deactivation but also regulate expression of the GA receptor genes *GID1a-1c*. It is interesting to find that derepression of GA signaling by the DELLA mutations resulted in a reduced expression of GID1a-1c, whereas DELLAs are targeted by *Arabidopsis* GID1s for degradation in response to GA. These results suggest that GA response is regulated by an intricate circle of interactions (at both transcript and protein levels) among GA promoting components (GA biosynthetic enzymes and GA receptors) and GA signaling repressors (DELLA proteins).

Essential Role of the DELLA Domain in *Arabidopsis* GID1 Binding and GA-Dependent Proteolysis of DELLA

Previous studies showed that both DELLA and VHYNP motifs are essential for GA-induced degradation of DELLA proteins (Dill et al., 2001; Itoh et al., 2002). However, these sequences are not required for binding to the F-box protein SLY1 in yeast

two-hybrid assays (Dill et al., 2004). Based on these observations, we hypothesized that the DELLA domain is involved in perceiving the GA signal, which may enhance SCF^{SLY1} binding through a conformational change in the DELLA protein. The data in Figures 5 and 7 strongly support this model. We demonstrate that the DELLA domain without the Poly S/T region directly interacts with *Arabidopsis* GID1 in a GA-dependent manner and that GA-GID1 and DELLA interaction promotes binding of the DELLA protein to SLY1 via its GRAS domain. As suggested by Nakajima et al. (2006), DELLA and GID1 interaction appears to increase binding affinity to GA. Future structure analysis of the GA-GID1-DELLA complex will reveal how *Arabidopsis* GID1 binds to its ligand GA and how DELLA interaction enhances this binding.

METHODS

Plant Material and Growth Conditions

The wild-type background in this study was Col-0 or L*er* as specified. The *ga1-3* mutant is in the L*er* background unless otherwise specified. All *gid1* mutants are in the Col-0 background. The *gid1a-1* (SALK_142767), *gid1a-2* (SAIL_536_G10), *gid1b-1* (SM_3_30227), and *gid1c-1* (SALK_023529) alleles were obtained from the Nottingham Arabidopsis Stock Centre. *gid1c-2* (GABIKat_639F05) was obtained from the GABI Kat collection (Max Planck Institute for Plant Breeding Research). Plants were grown on Levington F2 soil at 22°C under LD conditions (16 h light/8 h dark). The *ga1-3*, *gid1a-1 gid1b-1 gid1c-1*, and *gid1a-1 gid1c-1* mutants had their seed coats removed to assist germination. All lines grown for phenotypic characterizations had their seed coats manually removed after 3 d of incubation in water to reduce experimental variation. Dissected embryos were plated on 1× MS agar (1% sucrose and 0.8% agar, pH 5.7) for 8 d before transfer to soil. The same MS medium and soil were used for mutant characterization.

The homozygous *Arabidopsis thaliana* mutants *ga1-3*, *rga-24 gai-t6 ga1-3*, *sly1-10*, and *sly1-10/rga-24* (in the L*er* background) and the *rga-Δ17* transgenic line (L*er* carrying P_{RGA}*:rga-Δ17*) were described previously (Dill et al., 2001; Dill and Sun, 2001; McGinnis et al., 2003). The *ga1-3* (Col-0) line used had been backcrossed for six generations to Col-0 (Tyler et al., 2004). The *sly1-2* mutant (backcrossed three times to Col-0) was a gift from Camille Steber (Washington State University). For immunoblot analysis, quantitative RT-PCR (Figures 1B and 1C), and pull-down experiments, seedlings were grown on 1× MS medium (1 to 3% sucrose and 0.7% agar) in constant light at 22°C.

Genotyping of Plant Material

Wild-type *GID1A*, *gid1a-1*, and *gid1a-2* were detected by PCR using the following pairs of primers: GID1A-1F and GID1A-1R for the wild-type *GID1A* allele; GID1A-1R and LBa1 for the *gid1a-1* allele; and GID1A-1R and SAIL-Lb3 for the *gid1a-2* allele. Wild-type *GID1B* and *gid1b-1* were detected by PCR using the following pairs of primers: GID1B-1F and GID1B-1R for the wild-type *GID1B* allele; GID1B-1R and SLAT-3′ for the *gid1b-1* allele. Wild-type *GID1C*, *gid1c-1*, and *gid1c-2* were detected by PCR using the following pairs of primers: GID1C-1F and GID1C-1R for the wild-type *GID1C-1* allele; GID1C-2F and GID1C-2R for the wild-type *GID1C-2* allele; GID1C-1R and LBa1 for the *gid1c-1* allele; and GID1C-2R and GABIKatLB1 for the *gid1c-2* allele. PCR was performed for 20 s at 94°C, for 15 s at 55°C, and for 1 min 30 s at 72°C for 35 cycles. The primers used for genotyping are listed in Supplemental Table 1 online. To analyze *GID1* transcripts in each *gid1* mutant by RT-PCR, total RNA was isolated from the mutants using the RNeasy plant mini kit (Qiagen). DNA was removed via an on-column treatment and a subsequent separate treatment with Turbo RNase-free DNase I (Ambion). One microgram of the resultant RNA was reverse transcribed using a mixture of oligo(dT) pimers and random hexamers with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA was then used in PCR reactions. The following primers (see Supplemental Table 1 online) were used: GID1A-1F and GID1A-1R for *GID1A*, GID1B-F2 and GID1B-R2 for *GID1B*, GID1C-F3 and GID1C-R3 for *GID1C*, and ACT2-1F and ACT2-1R for *ACT2* (At3g18780). The PCR was performed for 20 s at 94°C, for 15 s at 55°C, and for 1 min and 15 s at 72°C for 35 cycles.

Mutant Characterization

Hypocotyl and root length measurements were taken from plants grown vertically on 1× MS agar at 22°C under LD conditions for 5 d in square Petri dishes. Seedlings were grown on both GA-free agar and agar containing 0.2 μM GA_4. For the other measurements, plant lines (12 plants per line) were grown in a randomized grid. Seeds were germinated on MS agar plates and then transferred to soil after 4 d. Flowering time was measured by counting the number of days from sowing until flower buds were visible by the naked eye or by counting the number of rosette and cauline leaves. The rosette radius of each plant was determined by taking the mean of the lengths of the two largest leaves when fully expanded. Silique number was counted on the main stem, and silique length is the average length of siliques in positions 16 to 20 (from the base of the primary stem). Seed number was counted in the 18th silique or the next fertile silique. Plants treated with GA were sprayed with 100 μM GA_4 three times per week.

Real-Time Quantitative RT-PCR Analysis

Transcript levels were measured by real-time quantitative RT-PCR with SYBR Green. The methods are described in detail in the Supplemental Methods online.

Quantitative GA Analysis

Independent duplicate samples of freeze-dried rosettes from 13-d-old Col-0 (~1 g dry weight) and 27-d-old *gid1a-1 gi1b-1 gid1c-1* seedlings (~0.5 g dry weight) were analyzed for GA content as described by Coles et al. (1999) with some modifications. Purified extracts after elution from the C_{18} cartridge were methylated with diazomethane and after taking to dryness under N_2 were dissolved in ethyl acetate (1 mL) and partitioned against water (1 mL). The ethyl acetate phase was passed through a Bond-Elut NH_2 cartridge (100 mg; Varian) that had been preconditioned with ethyl acetate (1 mL). The remaining water phase was partitioned twice more against ethyl acetate, with the organic phases being passed through the NH_2 cartridge. The pooled ethyl acetate phases were evaporated to dryness in vacuo, and then the GA methyl esters were resolved by reverse phase HPLC using conditions described previously (Croker et al., 1990). Pooled fractions were analyzed as methyl ester trimethylsilyl ethers on a ThermoFinnigan GCQ mass spectrometer. Samples in *N*-trimethylsilylfluoroacetamide (10 μL) were diluted with dry ethyl acetate (20 μL) and injected (1 μL) into a TR-1 capillary column (30 m × 0.25 mm × 0.25 μm film thickness; Thermo Electron) at 50°C. The split valve (50:1) was opened after 2 min and the temperature increased at 20°C min^{-1} to 200°C and then at 4°C min^{-1} to 300°C. The instrument was operated in selective ion monitoring mode, with the selected ions for each GA and its $^2H_2$-labeled internal standard as described by Croker et al. (1990).

Examination of Floral Morphology by Scanning Electron Microscopy

Individual flower buds were removed from fresh *ga1-3* (Col-0), *gid1a-1 gid1b-1 gid1c-1*, and Col-0 and dissected; at least two sepal and two petals were removed from each flower to expose the internal structure.

Buds were attached to a mounting stub using OC compound (Tissue TEK), plunged into preslushed LN_2, and then transferred under vacuum to the preparation chamber stage maintained at −180°C. Ice contamination was sublimed from the sample by raising the temperature to −90°C for ~2 min. The temperature was returned to −180°C, and the samples were coated with gold for 1 min before imaging using a Jeol LVSEM 6360 scanning electron microscope in high vacuum cryo mode.

Statistical Analysis of Phenotypic Measurements

A completely randomized design was used for plants from which root length measurements were taken. The design for the other measurements in the characterization experiment was a randomized block with eight blocks, whereas the design for the GA treatment experiment was a split plot in four blocks, pairs of trays making up each block, with one tray treated with GA and the other kept as control (Gomez and Gomez, 1984). Analysis of variance was applied to all phenotypic measurements taken. To correct for heterogeneity of variance of the stem height, seed number, and root length data, a natural log transformation was taken. Note that, due to a common transformation being applied to all observations, the comparative values between genotypes are not altered and comparisons between them remain valid. The analysis provided the overall significance between genotypes for the characterization experiment and the main effect of treatment and the genotype treatment interaction for the GA-insensitivity experiment. LSDs at 1% were then used to assess significance between particular pairs of genotypes or treatment combinations. The GenStat statistical system (version 8.2) was used for all analyses (Lawes Agricultural Trust, Rothamsted, UK).

Immunoblot Analysis of the RGA Protein

Eight-day-old Col-0 seedlings that were grown on MS plates were treated with water or 1 μM GA_4 for 1 h as described previously (Dill et al., 2004). For *ga1-3* (backcrossed six times to Col-0), *sly1-2* (backcrossed three times to Col-0), and the triple *gid1* mutants, embryos were dissected from imbibed seeds and then incubated on MS plates at 22°C for 8 d. The seeds of the triple *gid1* mutant were produced from *GID1a*/*gid1a gid1b*/*gid1b gid1c*/*gid1c* parents because homozygous triple mutant plants are sterile. To identify and allow growth of the triple mutants, embryos were dissected from nongerminating seeds (triple *gid1* mutants) after incubating on MS plates for 4 d at 22°C under 24 h of light. Total plant proteins were isolated and fractionated in a 8% SDS-PAGE gel and analyzed by immunoblot analysis using affinity-purified anti-RGA antibodies raised in rabbit (DU176) as described (Silverstone et al., 2001). Ponceau staining was used to confirm equal loading.

Plasmid Constructs

Sequences of primers used in this study are listed in Supplemental Table 1 online. The PCR-amplified fragments in all constructs were analyzed by DNA sequence analysis to ensure that no mutations were introduced. *GID1a* cDNA was PCR amplified from a SALK cDNA clone (U17384). *GID1b* and *GID1c* cDNAs were amplified from *Arabidopsis* (*Ler*) leaf cDNA. *GID1a-c* PCR fragments were cloned into pCR4Blunt-TOPO (Invitrogen) to generate pCRGID1a (*GID1a*), pCRGID1b (*GID1b*), and pCRGID1c (*GID1c*).

For yeast two-hybrid analysis, pLexA-NLS (containing LexA DB domain; Vojtek et al., 1993) and pACTII (containing transcriptional AD domain; Li et al., 1994) were used as expression vectors. pSLY101 (DB-SLY1), pRG42 (AD-RGA), pRG225 (AD-rga-Δ17), pGAI102 (AD-GAI), pgai-1 (AD-gai-Δ17), and AD fusions with GAI truncations (GAI-NT1 [amino acids 1 to 157], GAI-CT1 [amino acids 92 to 532], GAI-CT2 [amino acids 151 to 532], and GAI-CT3 [amino acids 223 to 532]) were made previously (Dill et al., 2004). The *Bam*HI-*Pst*I fragments from pCRGID1a-c were cloned into *Bam*HI-*Pst*I sites of pLexA-NLS, resulting in pLexAGID1a (DB-GID1a), pLexAGID1b (DB-GID1b), and pLexAGID1c (DB-GID1c). The *Bam*HI-*Eco*RI fragments from pCRGID1a-c were cloned into the *Bam*HI-*Eco*RI site of pACTII, generating pACTGID1a (AD-GID1a), pACTGID1b (AD-GID1b), and pACTGID1c (AD-GID1c). A series of truncated fragments of *RGA* were amplified from pRG42 and cloned into pCR4Blunt-TOPO, generating pCRRNT1-4. pCRRNT1-4 contain the following RGA coding regions: pCRRNT1 (amino acids 1 to 208), pCRRNT2 (amino acids 1 to 108), pCRRNT3 (amino acids 1 to 70), and pCRRNT4 (amino acids 1 to 4). *Bam*HI-*Eco*RI fragments of pCRRNT1-4 were subcloned into *Bam*HI-*Eco*RI sites of pACTII, resulting in pACTRNT1-4 (AD-RNT1-4).

For yeast three-hybrid analysis, pGBT9 (for DB fusion; Clontech), pACTII (for AD fusion), and a third expression vector, pH3 (with the selectable marker *URA3*), were used. The pH3 vector was generated by the following procedures. *Sal*I-*Xmn*I fragment of pIIIEx426 RPR (Good and Engelke, 1994) containing the *URA3* gene was cloned into *Sal*I-*Eco*RV–digested pACT2 (Clontech), generating pACTUra. The *LEU2* gene in pACT2 was destroyed in this step. The AD domain region of pACTUra was removed by *Hin*dIII digestion, and a multicloning site sequence (see Supplemental Table 1 online) was inserted into the *Hin*dIII site of pACTUra to generate pH3. The *Bam*HI-*Eco*RI fragment containing *GID1a* from pCRGID1a was cloned into the *Bam*HI-*Eco*RI site of pH3 to yield pH3GID1a (GID1a). The *Eco*RI-*Bam*HI fragment of pSLY101 containing the *SLY1* coding region was cloned into the *Eco*RI-*Bam*HI site of pGBT9, generating pGBTSLY1 (DB-SLY1).

For pull-down assays, the *Bam*HI-*Eco*RI fragments from pCRGID1a, pCRGID1b, and pCRGID1c were cloned into the *Bam*HI-*Eco*RI sites of pGEX-3X (GE Healthcare) to generate pGEXGID1a (GST-GID1a), pGEX-GID1b (GST-GID1b), and pGEXGID1c (GST-GID1c).

Yeast Two- and Three-Hybrid Assays

For yeast two-hybrid assays, DB (pLexA-NLS derivative; Trp selection) and AD (pACTII derivative; Leu selection) fusion constructs were cotransformed into *Saccharomyces cerevisiae* strain L40 (Vojtek et al., 1993). Yeast transformation, the growth test with 3-AT, and β-gal liquid assay were performed as described previously (Dill et al., 2004). The effect of GA on protein–protein interactions was tested by adding 100 μM GA_3 in the growth media.

For yeast three-hybrid assays, the yeast strain PJ69-4A (James et al., 1996) was cotransformed with three types of plasmids. The first plasmid expressed the DB-SLY1 fusion (in pGBT9; Trp selection), the second type of plasmids encoded the AD fusion proteins for RGA, rga-Δ17, and RNT1 (in pACTII; Leu selection), and the third plasmid expressed GID1a (pH3 derivative; uracil selection). Transformed colonies were selected on synthetic complete medium lacking Leu, Trp, and uracil. Transformants were maintained on the same medium and transferred to synthetic medium without Leu, Trp, uracil, and His supplemented with 3-AT (5, 10, 30, and 60 mM) in the presence or absence of 100 μM GA_3. β-gal liquid assays were performed by growing transformants on the synthetic complete medium lacking Leu, Trp, and uracil in the presence or absence of 100 μM GA_3. Expression of DB and AD fusion proteins were confirmed by immunoblot analysis using anti-LexA and anti-HA antibodies (Santa Cruz Biotechnology).

Pull-Down Assays

Recombinant GST and GST-GID1a were expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene). One-twentieth volume of precultured cells was added to 200 mL (GST) and 1.2 liters (GST-GID1a) of Luria-Bertani medium and cultured at room temperature until OD_{600} 0.3 to 0.6. Induction of recombinant proteins was performed by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4 h. Cells were then

harvested and resuspended with buffer A containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Nonidet P-40, 1× Gamborg's B-5 with Minimal Organics (CAISSON Laboratories), and 1 mM DTT. The cells were lysed by French press (5000 p.s.i.; three times). The lysates were centrifuged at 21,000*g* for 10 min, and the supernatants were mixed with 200 μL of glutathione sepharose 4B beads (GE Healthcare) and agitated for 30 min at 4°C. The beads were washed five times with buffer A.

Seven-day-old *Arabidopsis sly1-10* and *sly1-10/rga-24* seedlings (0.5 g for Figure 6; 2.5 g for Supplemental Figure 4 online) were ground in liquid nitrogen, and proteins were extracted by adding 10 mL of buffer B (same as buffer A, except containing 0.5% Nonidet P-40 and 1× Complete EDTA free [Roche]) with gentle agitation for 10 min at 4°C. The extracts were then centrifuged at 21,000*g* for 10 min at 4°C. For each pull-down assay, 10 μL of beads (containing 30 μg GST or 5 μg GST-GID1a) were mixed with 500 μL of the plant extracts and incubated in the presence or absence of GA_4 for 60 min with gentle agitation at 4°C. The beads were washed three times in the buffer A containing 100 μM GA_4 and resuspended with 50 μL of SDS-PAGE sample buffer. Protein samples (10 μL each) were separated by 8% SDS-PAGE gel and immunoblotting with affinity-purified anti-RGA antibodies (DU176) as described (Silverstone et al., 2001). Signals were detected by SuperSignal West Pico chemiluminescent substrate (Pierce).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *GID1a* (At3g05120), *GID1b* (At3g63010), *GID1c* (At5g27320), *RGA* (At2g01570), *GAI* (At1g14920), *SLY1* (At4g24210), and *GA3ox1* (At1g15550).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Methods. Real-Time Quantitative RT-PCR Analysis.

Supplemental Figure 1. *gid1a*, *gid1b*, and *gid1c* Insertion Mutants.

Supplemental Figure 2. Phenotypes of the *gid1a gid1c* and *gid1a gid1b* Mutants.

Supplemental Figure 3. GA-Insensitive Root Growth of *gid1a-1 gid1b-1 gid1c-1*.

Supplemental Figure 4. GA-Dependent Interactions between RGA and *Arabidopsis* GID1s in Pull-Down Assays.

Supplemental Table 1. Primers for Cloning, Sequencing, and Quantitative PCR.

Supplemental Table 2. Genetic Segregation of Germination Phenotypes in the *Arabidopsis gid1* Multiple Mutants.

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