The Plant Cell, Vol. 13, 1791–1802, August 2001, www.plantcell.org © 2001 American Society of Plant Biologists

Expression of Arabidopsis GAI in Transgenic Rice Represses Multiple Gibberellin Responses

Xiangdong Fu, Duraialagaraja Sudhakar,[1] Jinrong Peng,[2] Donald E. Richards, Paul Christou, and Nicholas P. Harberd[3]

John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, United Kingdom

Bioactive gibberellins (GAs) are essential endogenous regulators of plant growth. GA signaling is mediated via GAI, a nuclear member of the GRAS family of plant transcription factors. Previous experiments have suggested that GAI is a GA-derepressible repressor of plant growth. Here we test this hypothesis by examining the effects of the expression of Arabidopsis GAI in transgenic Basmati rice. High-level expression of GAI caused dwarfism and reduced GA responses, and the strength of this effect was correlated with the level of transgene expression. In particular, the expression of GAI abolished the GA-mediated induction of rice aleurone α-amylase activity, thus implicating GAI orthologs in the well-characterized cereal aleurone GA response. The GA derepressible repressor model predicts that high-level expression of GAI should confer dwarfism, and these observations are consistent with this prediction.

INTRODUCTION

The gibberellins (GAs) are tetracyclic diterpenoid hormones that are essential for normal plant growth and development (Hooley, 1994). GA-related mutants are known in a variety of species and can be subdivided into GA-deficient mutants (in which genes that regulate GA biosynthesis are affected; Hedden and Kamiya, 1997) and mutants in which the GA response is affected (Harberd et al., 1998; King et al., 2001). In the last few years, molecular genetic analysis of the Arabidopsis GA response mutant *gai* has resulted in significant advances in our knowledge of how the GA signal is transmitted through the plant (Richards et al., 2001). *gai*, like the numerous GA deficiency mutations of Arabidopsis (Koornneef and van der Veen, 1980), confers a dark green, dwarfed plant phenotype (Koornneef et al., 1985; Peng and Harberd, 1993, 1997a, 1997b; Wilson and Somerville, 1995). However, unlike the GA-deficient mutants, *gai* mutants are not GA deficient (in fact, they contain increased levels of endogenous GAs; Talon et al., 1990; Peng et al., 1999a), and they exhibit reduced growth responses to exogenous GAs (Koornneef et al., 1985; Wilson and Somerville, 1995).

gai was cloned via *Ds*-transposon insertional mutagenesis (Peng et al., 1997). *GAI* (the wild-type gene) encodes a protein (GAI) that belongs to the GRAS family of putative plant transcriptional regulators (Peng et al., 1997; Pysh et al., 1999; Richards et al., 2000, 2001). GAI is related closely to another GRAS family member, RGA, an Arabidopsis protein that also is involved in GA signaling (Harberd et al., 1998; Silverstone et al., 1998). *gai* is a dominant mutation, suggesting that it encodes a product that is altered in structure and function (Peng and Harberd, 1993; Wilson and Somerville, 1995; Peng et al., 1997). Comparisons of the DNA sequences of *GAI* and *gai* showed that *gai* encodes a mutant protein that lacks a segment of 17 amino acids from close to the N terminus of the protein (Peng et al., 1997). Recent observations have shown that GAI/RGA orthologs in dominant reduced GA response mutants of maize and wheat carry mutations that also cause deletions or truncations in the N-terminal regions of the proteins that they encode (Peng et al., 1999b). Loss or alteration of this region of the protein apparently causes the genetically dominant reduced GA responses that are characteristic of these various mutants.

The close sequence similarities between GAI and RGA suggest that they may play very similar roles in GA signaling. This suggestion is supported by observations of the phenotypic properties of mutant plants that lack GAI or RGA. Mutations that confer RGA deficiency cause partial suppression of the dwarf, dark green, GA deficiency phenotype conferred by the *ga1-3* mutation (which substantially blocks GA biosynthesis; Silverstone et al., 1997, 1998). Similarly, mutations that confer GAI deficiency partially suppress the phenotypic effects of paclobutrazol, an inhibitor of

[1] Current address: Rice Transformation Laboratory, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore 641 003, India.
[2] Current address: The Institute of Molecular Agrobiology, 1 Research Link, The National University of Singapore, Singapore 117604.
[3] To whom correspondence should be addressed. E-mail nicholas.harberd@bbsrc.ac.uk; fax 44-1603-450025.

GA biosynthesis (Peng et al., 1997). Thus, both GAI and RGA act as negative regulators of GA responses. Furthermore, it has been proposed that GAI and RGA are GA derepressible repressors of GA responses and that the gai mutant protein is a repressor that is less responsive to the derepressive effects of GA (Peng et al., 1997; Harberd et al., 1998; King et al., 2001; Richards et al., 2001).

The observations with Arabidopsis, wheat, and maize also suggested that GAI/RGA orthologs play conserved roles in GA signaling across the plant kingdom. Here we describe experiments in which we expressed the Arabidopsis *GAI* and *gai* open reading frames (ORFs) in rice. Previously, we showed that Basmati rice carrying constructs with the potential to express *gai* was dwarfed and had altered GA responses (Peng et al., 1999b). Here we extend these observations, showing that even a relatively low level of expression of Arabidopsis *gai* causes these effects, thus indicating that *gai* is highly active in rice, despite the millions of years since the divergence of the Arabidopsis and rice lineages. We also show that high-level expression of the wild-type Arabidopsis *GAI* ORF also causes dwarfism and reduced GA responses in rice and that the strength of this effect correlates with the level of gene expression. This observation is actually a prediction of the GA derepressible repressor model of GAI action, as described in Discussion.

The response of the aleurone cells of cereals to GA is one of the best understood hormone responses in plants. In essence, GA, released by the embryo during germination, is perceived by receptors that are thought to reside in the plasma membranes of the aleurone cells (Hooley et al., 1991; Gilroy and Jones, 1994). This signal then initiates α-amylase synthesis (via transcriptional up regulation of α-amylase genes) and secretion of α-amylase from aleurone cells into the endosperm. In the endosperm, the α-amylase digests the starch reserves, making sugars available to the germinating seedling. Many factors appear to influence the transmission of the GA signal through the cell after the primary perception event, including heterotrimeric G proteins, lipids (inositol triphosphate and diacylglycerol), Ca^{2+}, calmodulin, cyclic GMP, cytosolic pH, and protein phosphorylation (Bethke et al., 1997). The expression of *GAmyb*, a gene identified originally in barley, is up regulated by GA, and its product, a myb-type transcription factor, appears to regulate α-amylase gene expression (Gubler et al., 1995). Our transgenic rice lines enabled us to determine whether GAI/gai, identified originally on the basis of their effects on plant stem elongation, also affect the cereal aleurone GA response. Here we show that the expression of *gai* and *GAI* in rice inhibits the GA-regulated production of α-amylase by the rice aleurone layer. This is an important observation because it links two significant areas of GA research: the genetics of plant stature mutants and the biochemistry of the GA-mediated cereal aleurone α-amylase response (Harberd et al., 1998). In short, our observations show that GAI/RGA and orthologs are central to GA signaling in a wide variety of species and GA responses.

RESULTS

Recovery of Primary Transformant Basmati Rice Plants Expressing *GAI* and *gai*

DNA constructs expressing the Arabidopsis *GAI* and *gai* ORFs were developed as described in Methods. These constructs allow the expression of *GAI* or *gai* from the cauliflower mosaic virus (CaMV) 35S and maize ubiquitin (Ubi) promoters. Four constructs were made (CaMV35S::*GAI*, Ubi::*GAI*, CaMV35S::*gai*, and Ubi::*gai*; Figure 1A). These DNAs then were used in transformation experiments, using the particle bombardment transformation method (Christou et al., 1991; Sudhakar et al., 1998). We regenerated 41 independent transgenic rice plants and performed polymerase chain reaction (PCR) analyses to confirm the presence of the *GAI* or *gai* ORFs in each of them (data not shown). In total, we obtained eight independent Ubi::*GAI* plants, 16 independent Ubi::*gai* plants, nine independent CaMV35S::*GAI* plants, and eight independent CaMV35S::*gai* plants. Genomic DNA gel blot analyses further confirmed the presence of the transgenes in the genomes of each of these plants (data not shown). All of these primary transformants were allowed to set seed by self-pollination, and the resulting (R1) seed were collected for further analysis. Many of the primary transformants exhibited an obvious dwarf phenotype (data not shown), and this article is concerned with the molecular, physiological, and visual characterization of this heritable phenotype in subsequent generations. The pedigree of the different lines described in this article is summarized in Methods.

Molecular Characterization of R1 Plants

We investigated transgene segregation and expression in the R1 plants derived from each of the 41 primary transformant lines. PCR analysis of 40 R1 plants from each line (using primers gai-1 and gai-2; Figure 1A) showed that in each case the *GAI* or *gai* transgene segregated with a ratio that approximated to three plants carrying the transgene for every one plant that did not (data not shown). These results indicated that, as expected from previous observations (Kohli et al., 1998; Maqbool and Christou, 1999; Fu et al., 2000), the transgenes in these lines were integrated at single genetic loci. Genomic DNA gel blot analyses of a representative sample of 11 of these R1 plants (previously shown to be carrying a transgene by PCR analysis), using a radiolabeled *GAI* ORF probe, are shown in Figure 1B. Digestions were with BamHI (which cuts once within the transgenes; Figure 1A) or were double digestions using both BamHI and EcoRI. This double digestion released an $\sim$1.6-kb DNA fragment containing the *GAI* or *gai* ORF (note that this fragment is slightly smaller in constructs containing *gai* than in constructs containing *GAI*; Figure 1A). In most cases, sibling

pairs were assayed from lines transformed with the CaMV35S::*GAI* or the Ubi::*gai* construct (e.g., plants a and b are sibling R1 progeny of a primary transformant carrying the CaMV35S::*GAI* construct; see Figure1B).

In the BamHI digests, each transformant line showed a different pattern, indicating that in each case the transgene was inserted into a different part of the genome. Furthermore, the signal intensity varied from line to line, indicating some difference in the number of transgenes at a locus (Figure 1B). Sibling plants exhibited similar patterns, although the intensity of the pattern varied from sibling to sibling (Figure 1B). This variation in intensity may reflect segregation of the transgene, with homozygous plants yielding a more intense signal than hemizygous plants (although unequal

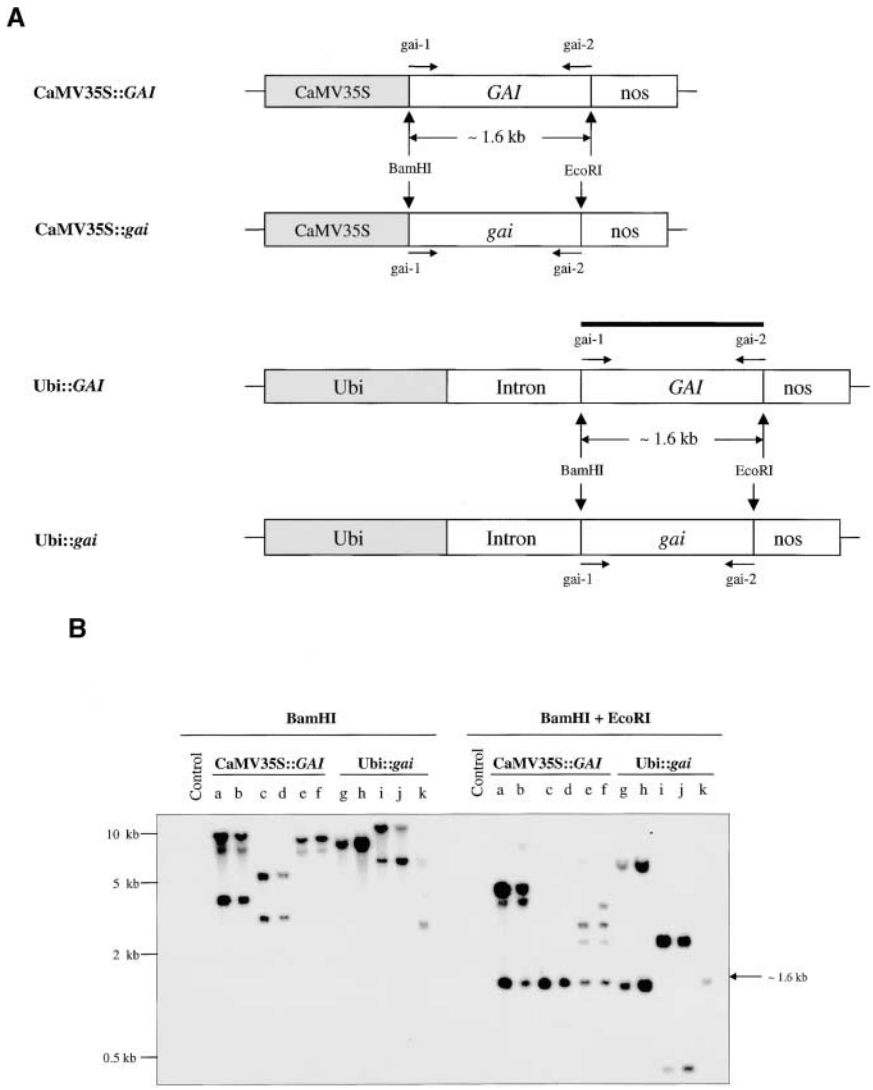

Figure 1. Generation and Preliminary Characterization of Transgenic Rice Plants Expressing Arabidopsis *GAI* or *gai*.

(A) Maps of the constructs showing transcription units, relevant restriction sites, and primers used for PCR and RT-PCR (arrows). The *GAI* and *gai* ORFs are ~1.6 kb in size; the *gai* ORF is slightly smaller than the *GAI* ORF as a result of its characteristic 51-bp internal deletion (Peng et al., 1997). nos, nopaline synthase. Bar represents hybridization probe used in **(B)**.

(B) DNA gel blot analysis of R1 transgenic plants after digestion of genomic DNA with BamHI or with BamHI and EcoRI using a radiolabeled Arabidopsis *GAI* ORF as probe. There is no detectable hybridization of the probe to digests of DNA from untransformed Basmati rice plants (Control). Plants were G38-4 (a), G38-17 (b), G39-3 (c), G39-8 (d), G41-22 (e), G41-26 (f), g51-1 (g), g51-6 (h), g58-1 (i), g58-2 (j), and g59-1 (k). The arrow marks the position of the intact ~1.6-kb ORF.

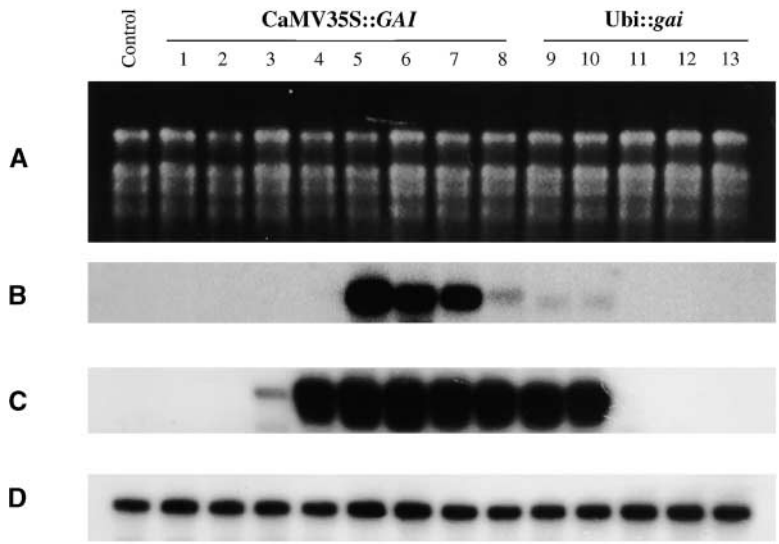

Figure 2. RNA Gel Blot and RT-PCR Analyses of the Expression of *GAI* or *gai* Transcripts in Transgenic Rice.

(A) UV fluorescence of ethidium bromide–stained gel containing RNA from plants (nontransgenic control and 1, G36-1; 2, G36-2; 3, G38-4; 4, G38-17; 5, G39-3; 6, G39-8; 7, G41-22; 8, G41-26; 9, g51-1; 10, g51-6; 11, g58-1; 12, g58-2; and 13, g59-1) used for the hybridization shown in **(B)**.
(B) RNA gel blot analysis of RNA shown in **(A)** using a radiolabeled *GAI* ORF as probe.
(C) RT-PCR analysis of *GAI*/*gai* transcript levels in RNA from R1 plants. RT-PCR amplification was for 25 cycles using the gai-1 and gai-2 primers (see Methods and Figure 1A).
(D) RT-PCR control showing that actin transcripts are detected consistently in all of the samples used for the *GAI*/*gai* RT-PCR experiment shown in **(C)**.

sample loading also might contribute to this variation in signal intensity). In the BamHI–EcoRI double digests, most of the lines displayed the ~1.6-kb *GAI*/*gai* ORF fragment (Figure 1B, arrow), indicating the existence of at least one intact coding region in the transgenes that these lines contain. In several cases, additional fragments were seen, indicating the existence of additional, rearranged copies of the *GAI*/*gai* ORF. In sibling plants i and j (Figure 1B), no ~1.6-kb BamHI–EcoRI fragment was observed, indicating that there was no intact copy of the *gai* ORF in the g58 transformant line. Plants c and d showed a relatively simple integration pattern and appeared to carry only a single insertion with an intact *GAI* ORF. Plant k also showed a relatively simple integration pattern but contained an incomplete *gai* ORF (as shown by DNA sequencing; data not shown).

Expression of *GAI*/*gai* Transcripts in R1 Plants

GAI/*gai* transcripts in selected R1 plants were visualized using RNA gel blot and reverse transcriptase (RT)–mediated-PCR analyses (Figure 2). These analyses revealed a wide variation in the degree of *GAI*/*gai* expression in the different lines. *GAI*/*gai* transcripts were not detected in plants g58-1, g58-2, g59-1 (samples 11, 12, and 13 in Figure 2) or in the nontransgenic control. As shown above (Figure 1B), these plants did not carry intact *gai* ORFs, consistent with the lack of detectable *gai* expression. Plants G36-1 and G36-2 (Figure 2, samples 1 and 2) carried an intact *GAI* ORF (data not shown), and the lack of detectable transcripts in these plants may be attributable to transgene silencing. Two plants from line G39 (G39-3 and G39-8; samples 5 and 6 in Figure 2) exhibited a high level of expression of *GAI* (seen in both the RNA gel blot and RT-PCR assays; Figure 2). Plants from line G41 (G41-22 and G41-26; samples 7 and 8 in Figure 2) differed with respect to *GAI* transcript levels. G41-22 had high levels of *GAI* transcripts that were clearly detectable in both RNA gel blot and RT-PCR analyses, whereas G41-26 had lower levels of *GAI* transcripts that were barely detectable by RNA gel blotting and were clearly detectable only in the more sensitive RT-PCR assay. Plants G38-4 and G38-17 (samples 3 and 4 in Figure 2) and g51-1 and g51-6 (samples 9 and 10 in Figure 2) also had relatively low transcript levels, with transcripts being clearly detectable only by RT-PCR (Figure 2).

Expression of Arabidopsis *gai* Causes Reduced Height and Altered GA Response in Transgenic Rice

We showed previously that the growth and GA responses of Basmati rice can be altered by the Ubi::*gai* transgene (Peng

et al., 1999b). Segregant seedlings carrying this transgene exhibited reduced GA responses, whereas segregants lacking the transgene responded normally to GA. Adult plants carrying the transgene were dwarfed with respect to control segregants lacking the transgene (Peng et al., 1999b). These results suggested that overexpression of Arabidopsis *gai* can cause reduced height and GA response in rice.

We measured the heights of 2-month-old R2 plants derived from transformation experiments using the Ubi::*gai* and CaMV35S::*gai* constructs (Figure 3A). These results confirmed that dwarfism was conferred by expression of the *gai*-containing transgenes. As shown above, plant g51-1 expressed levels of *gai* transcript that were detectable by RT-PCR (Figure 2C, sample 9). R2 progeny of this plant were uniformly dwarfed (Figure 3A). In contrast, plant g58-2 lacked detectable *gai* transcripts and lacked an intact *gai* ORF (Figure 1B, sample j; Figure 2, sample 12). R2 progeny of plant g58-2 were not dwarfed (Figure 3A). In addition, Figure 3A shows that the R2 progeny of two additional plants that contained an intact *gai* ORF (g56-9 and g48-3) were dwarfed with respect to the control, with the g56-9 plants being more severely dwarfed than the g51-1 plants and the g51-1 plants being less severely dwarfed than the g48-3 plants. In further experiments, we investigated a possible cause of this variation in dwarfing severity. For example, as shown in Figure 3B, plant g56-9 was more severely dwarfed than plant g51-1, which was itself dwarfed compared with the untransformed control. The relative heights of these plants were mirrored by *gai* transcript levels (as revealed by RNA gel blot analysis; Figure 3B), such that the plant expressing higher *gai* transcript levels was more severely dwarfed than the plant expressing lower *gai* transcript levels.

We also tested the GA responsivity of seedlings and 2-month-old plants. In seedling tests, performed as described previously (Peng et al., 1999b), R2 progeny of plant g58-2, which did not express detectable levels of *gai* transcripts, exhibited normal seedling GA responses (data not shown). R2 progeny of plant g51-1 (which expressed *gai* transcripts at a relatively low level; Figure 3B) and plant g56-9 (which expressed *gai* transcripts at a higher level; Figure 3B) did not respond to exogenous GA (data not shown). The reduced GA responses of plants expressing *gai* were maintained into adulthood, because 2-month-old Ubi::*gai*-expressing plants did not elongate in response to exogenous GA, whereas control plants did (Figure 3C).

High-Level Expression of Arabidopsis *GAI* Causes Reduced Height and Altered GA Responses in Transgenic Basmati Rice

Because transgenic expression of Arabidopsis *gai* conferred dwarfism and reduced GA responses in Basmati rice, we sought to determine the effect of expression of the wild-type *GAI* ORF on rice phenotype. We analyzed transgenic plants carrying *GAI* under the control of either the CaMV 35S promoter or the Ubi promoter. As shown in Figure 4A, the R2 progeny of plant G36-2 (which did not contain detectable levels of *GAI* transcript; Figure 2, sample 2) were the same height as control, nontransgenic plants. Plants G38-17, G39-8, G41-22, and G41-26 expressed detectable levels of *GAI* transcript from the CaMV 35S promoter (Figure 2, samples 4, 6, 7, and 8), and the R2 progeny of these plants all were dwarfed compared with the control (Figure 4A). Plants in which *GAI* expression was driven by the ubiquitin promoter also exhibited dwarfism (Figure 4A).

Interestingly, there was significant variation (between line variation) in the severity of the dwarfism observed in each of the *GAI*-expressing lines. Additional experiments (Figure 4B) revealed a correlation between *GAI* transcript level and plant height. Plants that contained relatively high levels of *GAI* transcript were severely dwarfed, and plants that contained relatively low levels of *GAI* transcript were less dwarfed.

We also analyzed the GA responses of transgenic Basmati rice plants expressing *GAI*. For example, plant G41-26 exhibited a relatively low level of *GAI* transcripts (Figure 2, sample 8). R2 progeny of G41-26 showed a clear growth response to GA (Figure 4C). In contrast, as shown above, plant G39-3 exhibited fairly high *GAI* transcript levels and substantially reduced height (Figure 2, sample 5, and Figure 4B). R2 progeny of G39-3 showed a greatly reduced growth response to GA (Figure 4C).

GA 20-Oxidase Transcript Levels Are Upregulated by Expression of *gai* and by High-Level Expression of *GAI* in Transgenic Rice

GA 20-oxidase enzymes catalyze the penultimate step in the biosynthesis of biologically active GAs (Hedden and Kamiya, 1997), and a cDNA sequence encoding a 20-oxidase from japonica rice has been identified. Using this and other 20-oxidase sequences, we designed degenerate primers for the amplification of a 20-oxidase–encoding fragment from Basmati (indica) rice genomic DNA and then extended this fragment (to obtain a full-length gene) using 5′ and 3′ rapid amplification of cDNA ends (see Methods). DNA sequence analysis revealed that this gene was ~98% identical to the reported 20-oxidase gene from japonica rice (data not shown).

In many plants, endogenous GA levels are subject to negative feedback regulation (Hedden and Croker, 1992). For example, in Arabidopsis, the levels of transcripts encoding enzymes that catalyze relatively late steps in the GA biosynthesis pathway (20-oxidase, 3β-hydroxylase) are downregulated by active GAs (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995; Cowling et al., 1998) and upregulated in the *gai* mutant (Xu et al., 1995; Peng et al., 1997, 1999a; Cowling et al., 1998). To determine if this is the case in Basmati rice, the Basmati 20-oxidase probe was used to investigate 20-oxidase transcript levels in young seedlings (1 week old). 20-Oxidase transcripts were clearly detectable in these

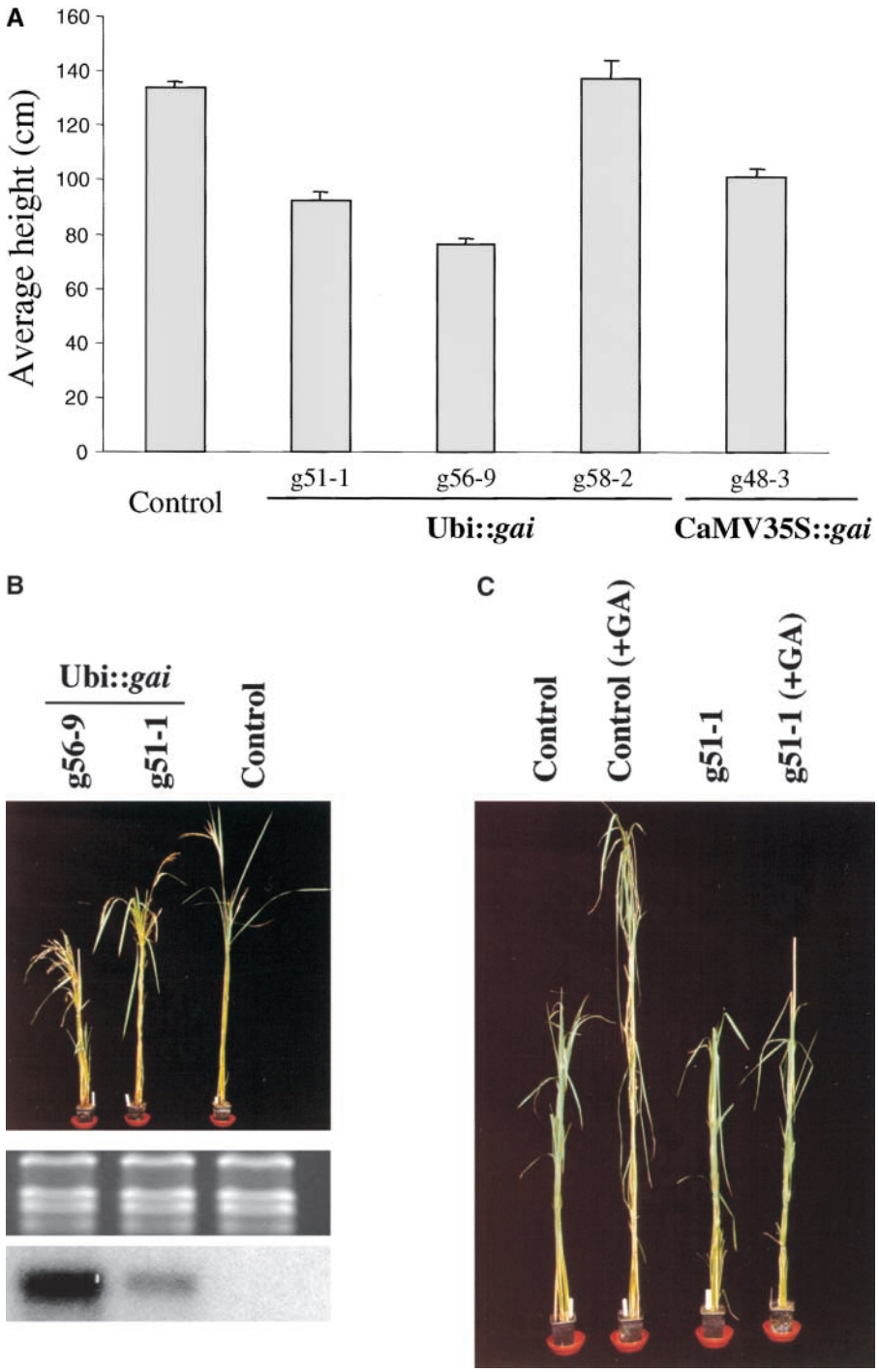

Figure 3. Phenotypic Analysis of Transgenic Basmati Rice Plants Expressing *gai*.

(A) Histogram showing heights of 2-month-old R2 plants. Mean heights (error bars represent ±SE) of 20 plants derived from self-pollination of R1 plants g51-1, g56-9, g58-2, g48-3, and control (untransformed) Basmati 370 rice plants are shown.

(B) Five-month-old plants derived from self-pollination of g56-9, g51-1, and control (untransformed) Basmati 370 rice plants. Beneath the plants, the UV fluorescence of an ethidium bromide–stained gel containing RNA prepared from each plant is shown. Beneath the UV fluorescence, an RNA gel blot hybridization from this gel, using a radiolabeled *GAI* ORF probe, is shown.

(C) Responses to GA treatment in Ubi::*gai* plants. Two-month-old R2 plants derived from self-pollination of g51-1 were sprayed with 100 μM GA_3 (+GA) or water once per day for 1 week and photographed 1 week after the final sprays.

seedlings, but they were greatly reduced within 8 hr and no longer detectable within 16 hr of being treated with GA (Figure 5A). 20-Oxidase transcript levels were unaffected by water control treatments (data not shown). Thus, 20-oxidase transcript levels were negatively regulated by GA in Basmati rice.

We also investigated 20-oxidase transcripts in transgenic rice plants expressing *GAI* or *gai*. All of the plants investigated in these experiments were dwarfed (data not shown), all contained *GAI* or *gai* transcripts (Figure 5B), and all showed increased 20-oxidase transcript levels compared with a control, nontransgenic rice plant (Figure 5B). Thus, expression of *GAI* or *gai* caused increased 20-oxidase levels in Basmati rice.

GA-Mediated Induction of Rice Aleurone α-Amylase Activity Is Inhibited in Transgenic Rice Expressing *GAI* and *gai*

Treatment of cereal aleurone cells with exogenous GA results in increased secretion of α-amylase by those cells, one of the best characterized GA responses of plants (Bethke et al., 1997). We sought to determine if this GA response is altered in transgenic Basmati rice expressing Arabidopsis *GAI* or *gai* using a rice distal half-grain α-amylase assay (see Methods). Distal half-grains lack an embryo (the source of the GA that activates α-amylase production in aleurone cells) and therefore produce very low levels of α-amylase in the absence of exogenous GA. A 60-hr incubation of wild-type distal rice half-grains in 1.0 μM gibberellic acid (GA_3) resulted in a substantial induction of α-amylase activity with respect to control incubations in the absence of GA_3 (Table 1). However, distal half-grains expressing Arabidopsis *GAI* or *gai* exhibited clear differences in secreted α-amylase activity. First, in all cases, half-grains from lines expressing *GAI* or *gai* and incubated in the absence of GA_3 secreted less α-amylase activity than control wild-type half-grains. Second, the GA-mediated induction of α-amylase activity exhibited by control (wild-type) half-grains was blocked completely or almost completely in half-grains from all of the *GAI*- or *gai*-expressing lines (Table 1). These results show that the expression of either Arabidopsis *GAI* or *gai* in transgenic Basmati rice can block the GA-induced secretion of α-amylase by rice aleurone cells.

DISCUSSION

The cloning of the Arabidopsis *GAI* and *RGA* genes was a major step forward in our understanding of how GA regulates plant growth and development (Peng et al., 1997; Harberd et al., 1998; Silverstone et al., 1998). More recently, the cloning of *GAI* orthologs from cereals and other plants has shown that GAI/RGA sequence and function are highly conserved, despite the wide evolutionary divergence between monocots and dicots (Peng et al., 1999b; Richards et al., 2001). This functional conservation suggested that it would be possible to study various aspects of GAI/gai function in transgenic rice plants expressing these proteins.

In this study, we have shown that the dwarf phenotype and reduced GA responses of transgenic Basmati rice plants carrying constructs containing Arabidopsis *gai* were associated with the expression of the transgene at the transcript level. Plants expressing *gai* exhibited a dwarf phenotype, whereas plants lacking detectable *gai* transcripts did not. In addition, the degree of dwarfism exhibited by these plants was related to the amount of *gai* transcript that the plants contained.

Furthermore, we have shown that expression of the wild-type (*GAI*) gene in Basmati rice also causes dwarfism and reduced GA response in an expression level–dependent manner. Low-level *GAI* expression causes relatively mild dwarfism and reduced, although still detectable, elongation in response to GA. High-level *GAI* expression results in more severe dwarfism and completely abolishes any detectable growth response to exogenous GA. The effect of varying *GAI* expression in rice is very different from the effect of varying *gai* expression. Even relatively low levels of *gai* expression cause clear dwarfism and an abolished GA growth response.

Our experiments have shown that overexpression of Arabidopsis *GAI* or *gai* in Basmati rice affects several different aspects of rice growth and development that are GA responsive. Thus, adult plant height, seedling and adult growth response to exogenous GA, regulation of 20-oxidase transcript levels, and induction of α-amylase secretion by aleurone cells are affected by the expression of *GAI* or *gai* in rice. These findings suggest that all of these processes are regulated by a *GAI*/*RGA* ortholog in normal rice, quite possibly the product of the rice equivalent of the maize *d8* and wheat *Rht-B1a*/*Rht-D1a* genes (Peng et al., 1999b; Ogawa et al., 2000).

The Arabidopsis *gai* (mutant) allele encodes a protein that lacks a 17–amino acid N-terminal segment and confers dwarfism (Peng et al., 1997). The *GAI* (wild-type) allele encodes a normal protein that does not confer dwarfism (Peng et al., 1997). Given this, the observation that expression of the wild-type *GAI* gene in rice causes dwarfism may seem surprising. However, these new observations actually are compatible with the derepressible repressor model of GAI function. According to this model (described by Peng et al., 1997, 1999b; Harberd et al., 1998; King et al., 2001; Richards et al., 2001), GAI functions as a repressor of plant growth whose action is opposed by GA, whereas gai is a repressor that is relatively resistant to GA action. One possible prediction of this model is that plants in which GAI is accumulated to higher levels than normal (e.g., as when *GAI* is "overexpressed" in transgenic rice) might be dwarfed, because the normal balance between GA and GAI would be upset.

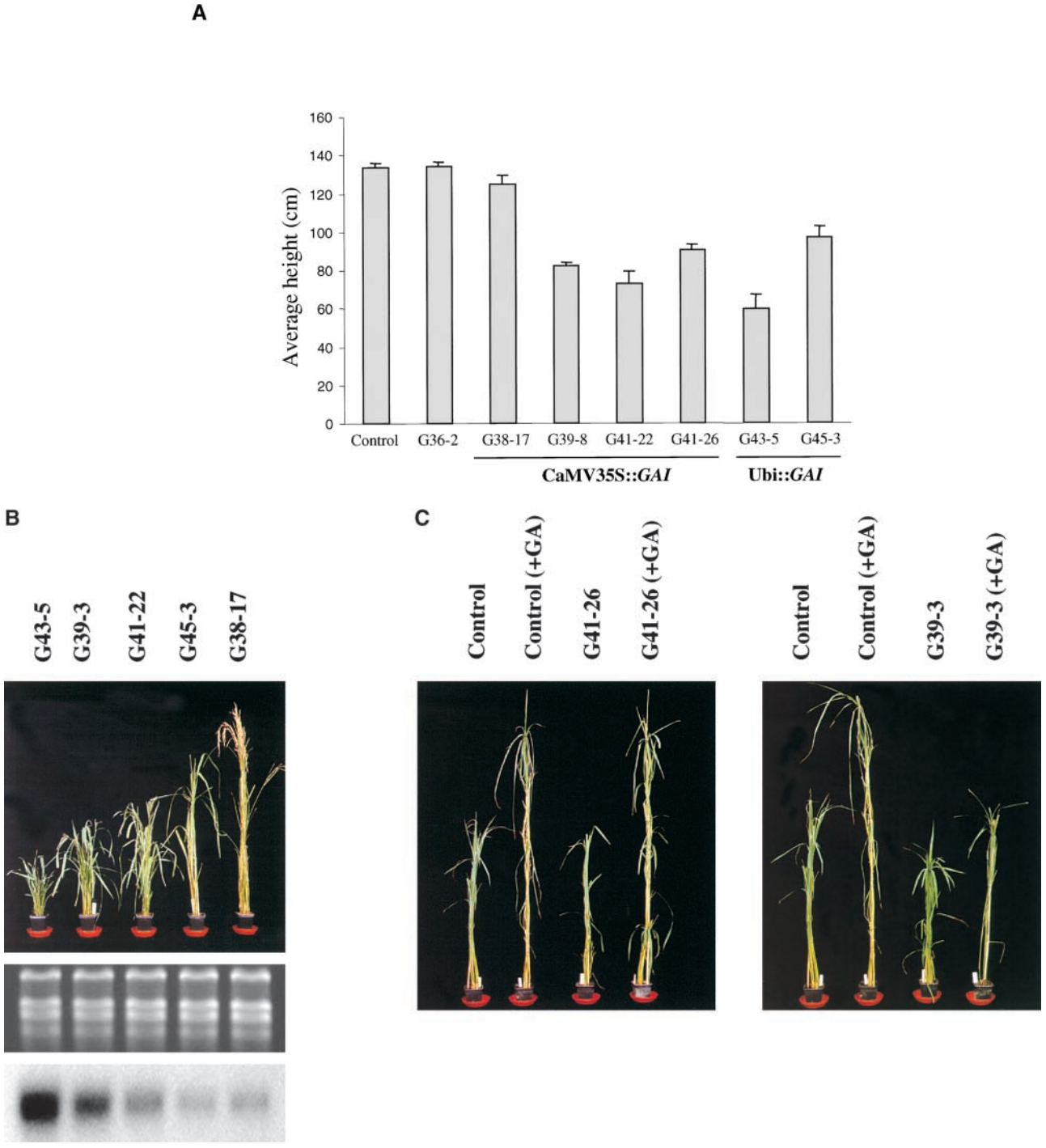

Figure 4. Phenotypic Analysis of Transgenic Basmati Rice Plants Expressing *GAI*.

(A) Histogram showing mean heights of 2-month-old R2 plants. Mean heights (error bars represent ±SE) of 20 plants derived from self-pollination of a control plant and of plants G36-2, G38-17, G39-8, G41-22, G41-26, G43-5, and G45-3.

(B) Five-month-old R2 plants expressing *GAI* and derived from R1 plants G43-5, G39-3, G41-22, G45-3, and G38-17. Beneath the plants, the UV fluorescence of an ethidium bromide–stained gel containing RNA prepared from each plant is shown. Beneath the UV fluorescence, RNA gel blot hybridization of RNA from this gel, using a radiolabeled *GAI* ORF probe, reveals a range of *GAI* transcript levels.

(C) Responses to GA treatment in R2 plants derived from plant G41-26 (homozygous for a CaMV35S::*GAI* transgene and containing relatively low *GAI* mRNA levels) and from plant G39-3 (homozygous for a CaMV35S::*GAI* transgene and containing relatively high levels of *GAI* mRNA). The 2-month-old plants were sprayed with 100 μM GA_3 (+GA) or water once per day for 1 week and photographed 1 week after the final sprays.

Low-level expressors might be dwarfed but correctable by GA treatment; high level expression of GAI might cause severe dwarfism that is not recoverable by GA. This is precisely what is seen, providing further evidence that the derepressible repressor model provides a useful framework for understanding GAI/RGA function in GA signaling.

At the biochemical level, the cereal aleurone α-amylase response is the best understood GA response in plants, and experimental studies of it have implicated a number of different key signaling systems in its mediation (Bethke et al., 1997). Here we have shown that the expression of Arabidopsis *GAI* and *gai* in rice blocks the GA-induced secretion of α-amylase by rice aleurone tissue. This observation is important because it provides a link between the α-amylase response and GAI, a protein originally identified via stature mutant genetics. Such a link had been suggested by the observation that wheat plants carrying the *Rht-B1c* allele (a severely dwarfing allele of the *Rht-B1a* gene, a wheat ortholog of *GAI*; Börner et al., 1996; Peng et al., 1999b) exhibits a reduced aleurone α-amylase response to GA (Gale and Marshall, 1973). Our experiments show directly that GAI (and perhaps RGA) are key components of the α-amylase response regulation by GA. Interestingly, transient assay experiments have shown that expression of the barley ortholog (HvSPY) of another GA signaling component originally identified via Arabidopsis genetics (SPY) largely abolishes the GA-induced activation of an α-amylase promoter (Robertson et al., 1998). Future work will reveal how GAI/RGA and SPY interact with the various biochemically identified components of GA signaling in aleurone, in particular GAmyb (a transcription factor thought to mediate GA-regulated α-amylase gene transcription; (Gubler et al., 1995) and the putative plasma membrane–associated gibberellin receptor (Hooley et al., 1991; Gilroy and Jones, 1994). However, it seems likely that GAI/RGA influences GAmyb levels, because *GAmyb* transcript levels are increased in the barley *sln* mutant (Gómez-Cadenas et al., 2001) and the barley *SLN* gene is an ortholog of Arabidopsis *GAI*/*RGA* (P. Chandler, F. Gubler, A. Marion-Poll, and M. Ellis, personal communication).

In addition to its significance for the understanding of GA signaling, the observation that *GAI* or *gai* expression causes dwarfism in transgenic rice may have important consequences for the future genetic improvement of rice. Although dwarf rice lines already have been used extensively in world agriculture on account of their increased grain yields and resistance to lodging, these lines often are not suited to particular growing environments or to local taste and texture preferences. We have shown that transgenes expressing *GAI* or *gai* can be transferred easily to locally favored rice varieties, with the potential of creating a range of dwarfed derivative lines, all of which can be tested further for yield increases.

Table 1. GA-Mediated Induction of α-Amylase Activity in Deembryonated Half-Grains

	α-Amylase Activity (milliunits/g)	
Source of R2 Seed[a]	Incubated in Buffer Alone	Incubated in Buffer + 1.0 μM GA_3
Control plant	11.39 ± 0.41	1029.06 ± 12.11
G38-2	1.66 ± 0.14	1.65 ± 0.17
G39-3	1.94 ± 0.11	3.53 ± 0.17
G41-26	2.25 ± 0.08	2.67 ± 0.07
g51-1	0.39 ± 0.06	0.34 ± 0.03

[a] Ten R2 seed in each line derived from homozygous R1 plants (G41-26, G38-2, G39-3 [all CaMV35S::*GAI*] and g51-1 [Ubi::*gai*]) were deembryonated and then treated with GA_3 or left untreated, as appropriate.

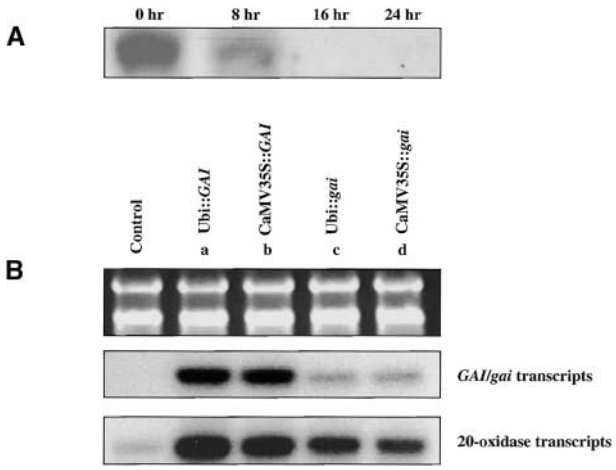

Figure 5. Rice 20-Oxidase Transcripts Are Upregulated by Expression of *GAI* or *gai*.

(A) RNA gel blot analysis of 20-oxidase transcripts in GA-treated Basmati rice seedlings. One-week-old seedlings were sprayed with 100 μM GA_3. Leaf RNA samples were prepared 8, 16, and 24 hr after treatment.
(B) The relationship between 20-oxidase and *gai*/*GAI* transcripts in transgenic rice plants. Top, UV fluorescence of an ethidium bromide–stained gel containing RNA prepared from plants G43-5 (a), G39-8 (b), g51-1 (c), g48-3 (d), and a control, untransformed Basmati rice plant. Beneath the UV fluorescence is an RNA gel blot hybridization from this gel, using a radiolabeled *GAI* ORF probe. Beneath this is shown a further hybridization, using a radiolabeled 20-oxidase probe.

METHODS

Plant Material, Gene Constructs, Transformation, and Notation of Lineages

Mature seed–derived rice callus (*Oryza sativa* cv Basmati 370) was used for particle bombardment and regenerated under 50 mg/L

hygromycin selection as described previously (Christou et al., 1991). The *GAI* and *gai* open reading frames (ORFs) were cloned into constructs under the control of maize ubiquitin (Ubi) or cauliflower mosaic virus (CaMV) 35S promoters (Figure 1A). Cobombardment with an *hpt*-containing plasmid (which confers hygromycin resistance; Christou et al., 1991) permitted the selection of cotransformants. A preliminary analysis of Ubi::*gai* R1 plants has been published elsewhere (Peng et al., 1999b).

Transgenic lineages derived from 41 independent transformation events are described in this article. Individual plants are denominated as follows. Lines G36, G38, G39, and G41 were derived from transformation experiments using the CaMV35S::*GAI* transgene. Line g48 was derived from transformation experiments using the CaMV35S::*gai* transgene. Lines G43 and G45 were derived from transformation experiments using the Ubi::*GAI* transgene. Lines g51, g56, g58, and g59 were derived from transformation experiments using the Ubi::*gai* transgene. Thus, G denotes lines derived from transformation with constructs containing the wild-type (*GAI*) gene, and g denotes lines derived from transformation with constructs containing the mutant (*gai*) gene. R1 generation plants are the self-pollination–derived progeny of primary transformants. Thus, for example, plant G38-4 is plant number 4 of a batch of plants obtained from planting R1 seed from line G38. R2 generation plants were obtained from self-pollination of R1 plants. In all cases, the R2 plants described came from families that were not segregating for the transgene. Thus, all R2 plants were homozygous for the transgene that they contain.

DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from leaves using the Nucleon Phytopure Plant DNA Extraction Kit (Amersham). Five-microgram DNA aliquots were digested overnight using BamHI and/or EcoRI, fractionated by electrophoresis on 0.8% agarose gels, and transferred onto Hybond N^+ membranes (Amersham). Total RNA was extracted using Trizol reagent (Gibco BRL), and 15-μg aliquots were fractionated by electrophoresis through denaturing 1.2% agarose gels. The fractionated RNA was transferred to Hybond N membranes (Amersham). Probes were synthesized by random primer labeling in the presence of α-^{32}P-dCTP, and hybridization and high stringency washing were performed as described previously (Fu et al., 2000). The 20-oxidase probe identifies a single band in DNA gel blot experiments with Basmati rice DNA (data not shown).

PCR and RT-PCR Analyses

DNA was isolated from single leaflets ~2 cm in length according to the method of Edwards et al. (1991). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RNA samples were treated with RQ1 RNase-free DNase (Promega), and reverse transcriptase–mediated polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR system (Promega) in 50-μL reaction volumes. The primer pairs used for PCR and RT-PCR were gai-1 (5′-GACGACGGTAACGGCATGGA-3′) and gai-2 (5′-TGCAGCCGCAAACCCAGCAG-3′), which were derived from N-terminal and C-terminal coding sequences, respectively, of the *GAI* ORF (Peng et al., 1997; Figure 1A). For each plant, rice actin-1 transcripts also were amplified as a constitutive expression control.

α-Amylase Assays

The activity of α-amylase secreted from distal deembryonated half-grains of rice was measured. Rice seed were surface-sterilized by washing with sodium hypochlorite for 15 min and then washed with sterile distilled water. Then, half-grains were deembryonated and transferred aseptically to 5 mL of aqueous buffer (0.02 M calcium chloride, 0.05 M sodium citrate, and 10 mg/L streptomycin sulfate, pH 6.2) containing (or lacking) 1.0 μM gibberellic acid (GA_3). Incubation was at 25°C on an orbital shaker at 100 rpm for 60 hr. After incubation, half-grains were ground in their buffer with a Polytron blender and centrifuged at 1600*g* for 15 min. Two separate 2-mL aliquots (experimental and blank) of the supernatant were incubated at 37°C for 10 min, after which a Phadebas tablet (Pharmacia and Upjohn) was added to one of the aliquots (experimental). The aliquots were incubated at 37°C for another 30 min and vortexed every 10 min. To stop the reaction, 0.5 mL of 0.5 M NaOH was added to both aliquots, which were then vortexed and centrifuged at 700*g* for 10 min. α-Amylase activity then was calculated from the difference in OD_{620} measured from the experimental and blank aliquots.

Cloning and Sequence Analysis of Basmati 370 GA 20-Oxidase

Total RNA was isolated from young seedlings of wild-type Basmati 370 rice. RT-PCR was performed using primer pairs 5′-TTCATATGGCCCGGCGGANNN-3′ and 5′-GTCGGAGAAGGCCTGAAANN-3′ derived from the japonica rice 20-oxidase sequence (GenBank accession number OSU50333) and other plant 20-oxidase sequences. PCR products were cloned in pGEM-T vector (Promega). Sequencing was performed using the ABI Prism Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (PE–Applied Biosystems, Foster City, CA). Full-length 20-oxidase sequence was obtained using 5′ and 3′ rapid amplification of cDNA ends methods (Gibco BRL).

GA Treatments

GA_3 was dissolved in methanol and then in sterile water. A 100-μM solution of GA_3 was used to spray the leaves of rice plants.

ACKNOWLEDGMENTS

We thank P.T. Hang for assistance with molecular and expression analysis of transgenic plants, J. Flintham for assistance with the α-amylase assay, and R.M. Twyman for help with the figures. X.F. and D.S. were supported by training grants from the Rockefeller Foundation. Training activities in the Molecular Biotechnology Unit were supported by a grant to P.C. from the Rockefeller Foundation. This work also was supported by the Biotechnology and Biological Science Research Council (Grant 208/P08211 to N.P.H; core strategic grant to the John Innes Centre).

Received January 16, 2001; accepted May 24, 2001.

REFERENCES

Bethke, P.C., Schuurink, R., and Jones, R.L. (1997). Hormonal signalling in cereal aleurone. J. Exp. Bot. **48,** 1337–1356.

Börner, A., Plaschke, J., Korzun, V., and Worland, A.J. (1996). The relationships between the dwarfing genes of wheat and rye. Euphytica **89,** 69–75.

Chiang, H.-H., Hwang, I., and Goodman, H.M. (1995). Isolation of the *Arabidopsis GA4* locus. Plant Cell **7,** 195–201.

Christou, P., Ford, T.L., and Kofron, M. (1991). Genotype-independent stable transformation of rice (*Oryza sativa*) plants. Bio/Technology **9,** 957–962.

Cowling, R.J., Kamiya, Y., Seto, H., and Harberd, N.P. (1998). Gibberellin dose-response regulation of *GA4* gene transcript levels in Arabidopsis. Plant Physiol. **117,** 1195–1203.

Edwards, L., Johnston, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **98,** 1349.

Fu, X., Kohli, A., Twyman, R.M., and Christou, P. (2000). Alternative silencing effects involve distinct types of non-spreading cytosine methylation at a three-gene single-copy transgenic locus in rice. Mol. Gen. Genet. **263,** 106–118.

Gale, M.D., and Marshall, G.A. (1973). Insensitivity to gibberellin in dwarf wheats. Ann. Bot. **37,** 729–735.

Gilroy, S., and Jones, R.L. (1994). Perception of gibberellin and abscisic acid at the external face of the plasma membrane of barley (*Hordeum vulgare*) aleurone protoplasts. Plant Physiol. **104,** 1185–1192.

Gómez-Cadenas, A., Zentella, R., Walker-Simmons, M.K., and Ho, T.-H.D. (2001). Gibberellin/abscisic acid antagonism in barley aleurone cells site of action of the protein kinase PKABA1 in relation to gibberellin signaling molecules. Plant Cell **13,** 667–679.

Gubler, F., Kalla, R., Roberts, J., and Jacobsen, J.V. (1995). Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: Evidence for Myb transactivation of a high-pI α-amylase gene promoter. Plant Cell **7,** 1879–1891.

Harberd, N.P., King, K.E., Carol, P., Cowling, R.J., Peng, J., and Richards, D.E. (1998). Gibberellin: Inhibitor of an inhibitor of . . . ? BioEssays **20,** 1001–1008.

Hedden, P., and Croker, S.J. (1992). Regulation of gibberellin biosynthesis in maize seedlings. In Progress in Plant Growth Regulation, C.M. Karssen, L.C. van Loon, and D. Vreugdenhil, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 534–544.

Hedden, P., and Kamiya, Y. (1997). Gibberellin biosynthesis: Enzymes, genes and their regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 431–460.

Hooley, R. (1994). Gibberellins: Perception, transduction and responses. Plant Mol. Biol. **26,** 1529–1555.

Hooley, R., Beale, M.H., and Smith, S.J. (1991). Gibberellin perception at the plasma membrane of *Avena fatua* aleurone protoplasts. Planta **183,** 274–280.

King, K.E., Carol, P., Cowling, R.J., Peng, J., Richards, D.E., and Harberd, N.P. (2001). Genetic approaches to the understanding of gibberellin-mediated plant growth regulation. In Mechanism of Action of Plant Hormones, K. Palme and J. Schell, eds (New York: Springer-Verlag). (in press).

Kohli, A., Leech, M., Vain, P., Laurie, D.A., and Christou, P. (1998). Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot-spots. Proc. Natl. Acad. Sci. USA **95,** 7203–7208.

Koornneef, M., and van der Veen, J.H. (1980). Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. **58,** 257–263.

Koornneef, M., Elgersma, A., Hanhart, C.J., van Loenen-Martinet, E.P., van Rign, L., and Zeevaart, J.A.D. (1985). A gibberellin insensitive mutant of *Arabidopsis thaliana*. Physiol. Plant. **65,** 33–39.

Maqbool, S.B., and Christou, P. (1999). Multiple traits of agronomic importance in transgenic indica rice plants: Analysis of transgene integration patterns, expression levels and stability. Mol. Breed. **5,** 471–480.

Ogawa, M., Kusano, T., Katsumi, M., and Sano, H. (2000). Rice gibberellin-insensitive gene homolog, *OsGAI*, encodes a nuclear-localized protein capable of gene activation at transcriptional level. Gene **245,** 21–29.

Peng, J., and Harberd, N.P. (1993). Derivative alleles of the *Arabidopsis* gibberellin-insensitive (*gai*) mutation confer a wild-type phenotype. Plant Cell **5,** 351–360.

Peng, J., and Harberd, N.P. (1997a). Gibberellin deficiency and response mutations suppress the stem elongation phenotype of phytochrome-deficient mutants of Arabidopsis. Plant Physiol. **113,** 1051–1058.

Peng, J., and Harberd, N.P. (1997b). Transposon-associated somatic *gai*-loss sectors in *Arabidopsis*. Plant Sci. **130,** 181–188.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The *Arabidopsis GAI* gene defines a signalling pathway that negatively regulates gibberellin responses. Genes Dev. **11,** 3194–3205.

Peng, J., Richards, D.E., Moritz, T., Caño-Delgado, A., and Harberd, N.P. (1999a). Extragenic suppressors of the Arabidopsis *gai* mutation alter the dose–response relationship of diverse gibberellin responses. Plant Physiol. **119,** 1199–1207.

Peng, J., et al. (1999b). 'Green revolution' genes encode mutant gibberellin response modulators. Nature **400,** 256–261.

Phillips, A.L., Ward, D.A., Uknes, S., Appleford, N.E.J., Lange, T., Huttly, A.K., Gaskin, P., Graebe, J.E., and Hedden, P. (1995). Isolation and expression of three gibberellin-20-oxidase cDNA clones from *Arabidopsis*. Plant Physiol. **108,** 1049–1057.

Pysh, L.D., Wysocka-Diller, J.W., Camilleri, C., Bouchez, D., and Benfey, P.N. (1999). The GRAS gene family in Arabidopsis: Sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes. Plant J. **18,** 111–119.

Richards, D.E., Peng, J., and Harberd, N.P. (2000). Plant GRAS and metazoan STATs: One family? BioEssays **22,** 573–577.

Richards, D.E., King, K.E., Ait-ali, T., and Harberd, N.P. (2001). How gibberellin regulates plant growth and development: A

molecular genetic analysis of gibberellin signalling. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52,** 67–88.

Robertson, M., Swain, S.M., Chandler, P.M., and Olszewski, N.E. (1998). Identification of a negative regulator of gibberellin action, HvSPY, in barley. Plant Cell **10,** 995–1008.

Silverstone, A.L., Mak, P.Y., Martínez, E.C., and Sun T.-p. (1997). The new *RGA* locus encodes a negative regulator of gibberellin response in *Arabidopsis thaliana*. Genetics **146,** 1087–1099.

Silverstone, A.L., Ciampaglio, C.N., and Sun T.-p. (1998). The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal-transduction pathway. Plant Cell **10,** 155–169.

Sudhakar, D., Duc, L.T., Bong, B.B., Tinjuangjun, P., Maqbool, S.B., Valdez, M., Jefferson, R., and Christou, P. (1998). An efficient rice transformation system utilizing mature seed-derived explants and a portable, inexpensive particle bombardment device. Transgenic Res. **7,** 289–294.

Talon, M., Koornneef, M., and Zeevaart, J.A.D. (1990). Accumulation of C19- gibberellins in the gibberellin-insensitive dwarf mutant *gai* of *Arabidopsis thaliana* (L.) Heynh. Planta **182,** 501–505.

Wilson, R.N., and Somerville, C.R. (1995). Phenotypic suppression of the gibberellin-insensitive mutant (*gai*) of Arabidopsis. Plant Physiol. **108,** 495–502.

Xu, Y.-L., Li, L., Wu, K., Peeters, A.J.M., Gage, D.A., and Zeevaart, J.A.D. (1995). The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: Molecular cloning and functional expression. Proc. Natl. Acad. Sci. USA **92,** 6640–6644.