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Feedback Regulation of *GA5* Expression and Metabolic Engineering of Gibberellin Levels in Arabidopsis

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The gibberellin (GA) 20-oxidase encoded by the *GA5* gene of Arabidopsis directs GA biosynthesis to active GAs, whereas that encoded by the *P16* gene of pumpkin endosperm leads to biosynthesis of inactive GAs. Negative feedback regulation of *GA5* expression was demonstrated in stems of Arabidopsis by bioactive GAs but not by inactive GA. In transgenic Arabidopsis plants overexpressing *P16*, there was a severe reduction in the amounts of C_{20}–GA intermediates, accumulation of large amounts of inactive GA_{25} and GA_{17}, a reduction in GA_4 content, and a small increase in GA_1. However, due to feedback regulation, expression of *GA5* and *GA4*, the gene coding for the subsequent 3β-hydroxylase, was greatly increased to compensate for the effects of the *P16* transgene. Consequently, stem height was only slightly reduced in the transgenic plants.

INTRODUCTION

Gibberellins (GAs) are tetracyclic diterpenoid compounds that play an important role in many aspects of plant growth and development, such as promotion of cell division and extension, seed germination, stem growth, flowering, and fruit set (Crozier, 1983). The pathway of GA biosynthesis has been elucidated in extensive biochemical studies, and the enzymes involved have been characterized.

The GA biosynthetic pathway can be divided into three stages according to the type of reactions and enzymes involved (Graebe, 1987; Hedden and Kamiya, 1997; Kende and Zeevaart, 1997; Phillips, 1998). Stage 1 involves the cyclization of geranylgeranyl diphosphate to *ent*-copalyl diphosphate, which in turn is converted to *ent*-kaurene. The enzymes that catalyze these reactions are called *ent*-copalyl diphosphate synthase and *ent*-kaurene synthase, which in Arabidopsis are encoded by the *GA1* (Sun and Kamiya, 1994) and *GA2* (Yamaguchi et al., 1998b) genes, respectively.

In the second stage, the P450 monooxygenase *ent*-kaurene oxidase, encoded by *GA3* of Arabidopsis (Helliwell et al., 1998), sequentially oxidizes C-19 of *ent*-kaurene via *ent*-kaurenol and *ent*-kaurenal to *ent*-kaurenoic acid, which is further oxidized to *ent*-7α-hydroxy kaurenoic acid. This is followed by contraction of the B ring with extrusion of C-7 to give GA_{12}–aldehyde.

The first step of stage 3 involves oxidation of GA_{12}–aldehyde to GA_{12}. Further metabolism of GA_{12} differs among species with respect to the position and sequence of oxidative steps. Common in higher plants is the early 13-hydroxylation pathway, which involves hydroxylation at C-13 to give GA_{53}. Both GA_{12} and GA_{53} are substrates for the multifunctional enzyme GA 20-oxidase in parallel pathways ($GA_{12} \rightarrow GA_{15} \rightarrow GA_{24} \rightarrow GA_9$ and $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$) in which C-20 is successively oxidized and eliminated. In addition, small amounts of the 20-carboxylic acids GA_{25} and GA_{17} are produced (Figure 1).

Three GA 20-oxidases have been isolated from Arabidopsis (Phillips et al., 1995), of which one, *GA5*, is highly expressed in elongating stems (Phillips et al., 1995; Xu et al., 1997). Next, GA_9 and GA_{20} are converted by 3β-hydroxylase (encoded by *GA4* in Arabidopsis) to the biologically active forms GA_4 and GA_1, respectively. Finally, these bioactive GAs are deactivated by 2β-hydroxylation: GA_1 to GA_8 and GA_4 to GA_{34} (Figure 1). Oxidations in stage 3 of the pathway are catalyzed by dioxygenases that use 2-oxoglutarate and molecular oxygen as cosubstrates.

With the elucidation of the GA biosynthetic pathway comes the question: how is GA biosynthesis regulated? Graebe (1987) has suggested that GA biosynthesis is activated and deactivated at different developmental stages and in different organs of higher plants. With the cloning of several GA biosynthetic genes (see Hedden and Kamiya, 1997), it is now possible to investigate the regulation of GA

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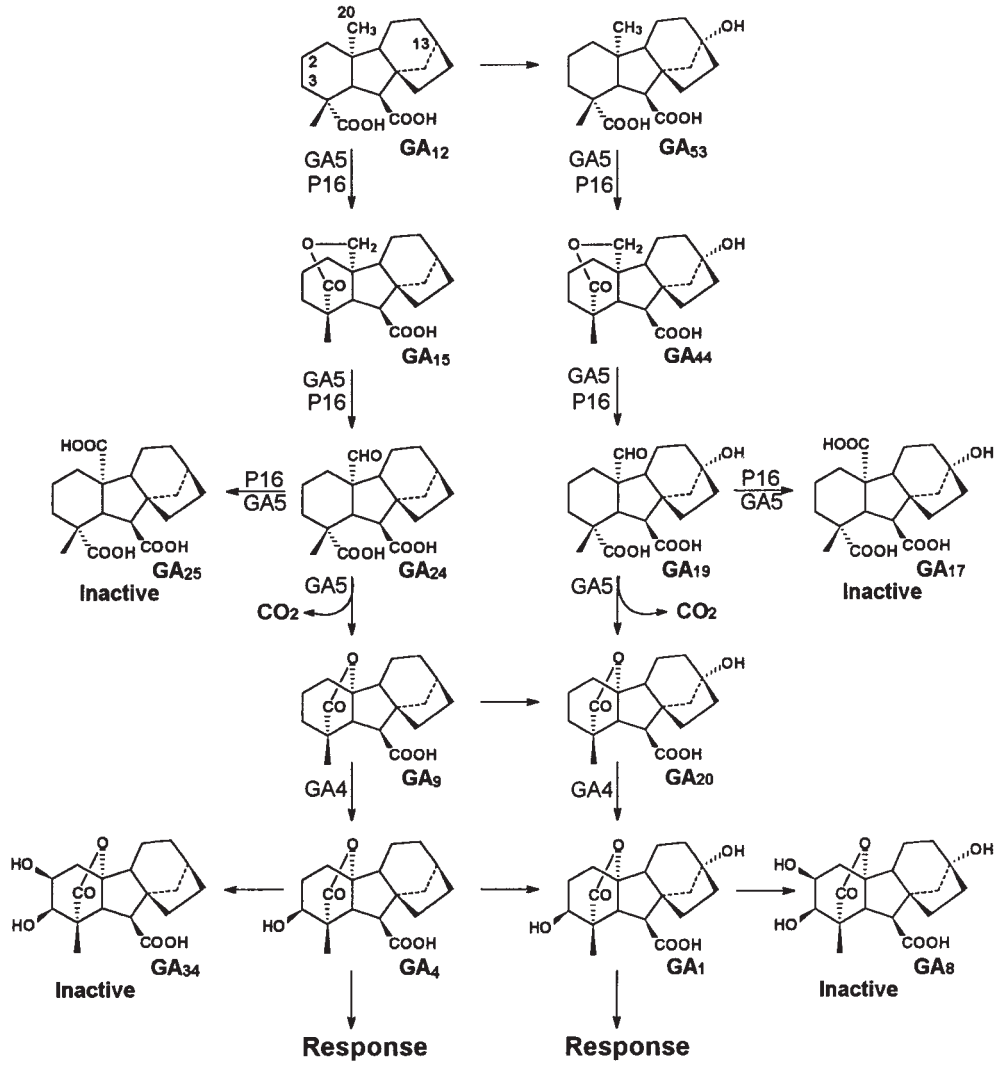

Figure 1. Stage 3 of the GA Biosynthetic Pathway in Arabidopsis.

The multifunctional enzyme GA 20-oxidase (GA5) oxidizes and eliminates C-20 of GA_{12} and GA_{53} in two parallel pathways to the C_{19}–GAs GA_9 and GA_{20}, respectively. The products of these reactions are converted by 3β-hydroxylase (GA4) to bioactive GAs: GA_9 to GA_4 and GA_{20} to GA_1. These active GAs are inactivated by 2β-hydroxylase to GA_{34} and GA_8, respectively. A GA 20-oxidase of pumpkin endosperm, P16, oxidizes C-20 exclusively to 20-carboxylic acids: GA_{12} to GA_{25} and GA_{53} to GA_{17}. All GAs with C-20 as a carboxyl group are biologically inactive.

biosynthesis at the molecular level in response to both endogenous and environmental signals. In Arabidopsis, it has been found that the amount of *GA5* transcript is increased by long days, but *GA4* expression is not under photoperiodic control (Xu et al., 1997). Expression of both the *GA4* and *GA5* genes is sensitive to feedback regulation in Arabidopsis. Treatment with GA reduced the amount of both transcripts in hypocotyls, leaves, and floral shoots (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995; Cowling et al., 1998). However, expression of *GA3* is not subject to feedback regulation by GA (Helliwell et al., 1998). Here, we report on the expression of *GA5* in the elongating stems of GA-deficient and GA-insensitive mutants after the application of active and inactive GAs.

To obtain further insight into the feedback regulation of GA biosynthesis, we also expressed in Arabidopsis a gene coding for an enzyme that only oxidizes C-20 of GAs. GA 20-oxidases normally oxidize and eliminate C-20 of C_{20}–GAs, thus producing C_{19}–GAs (Figure 1). However, a GA 20-oxidase encoded by a cDNA isolated from pumpkin endosperm, here called *P16*, oxidizes C-20 to a carboxyl group, thus giving rise to the biologically inactive com-

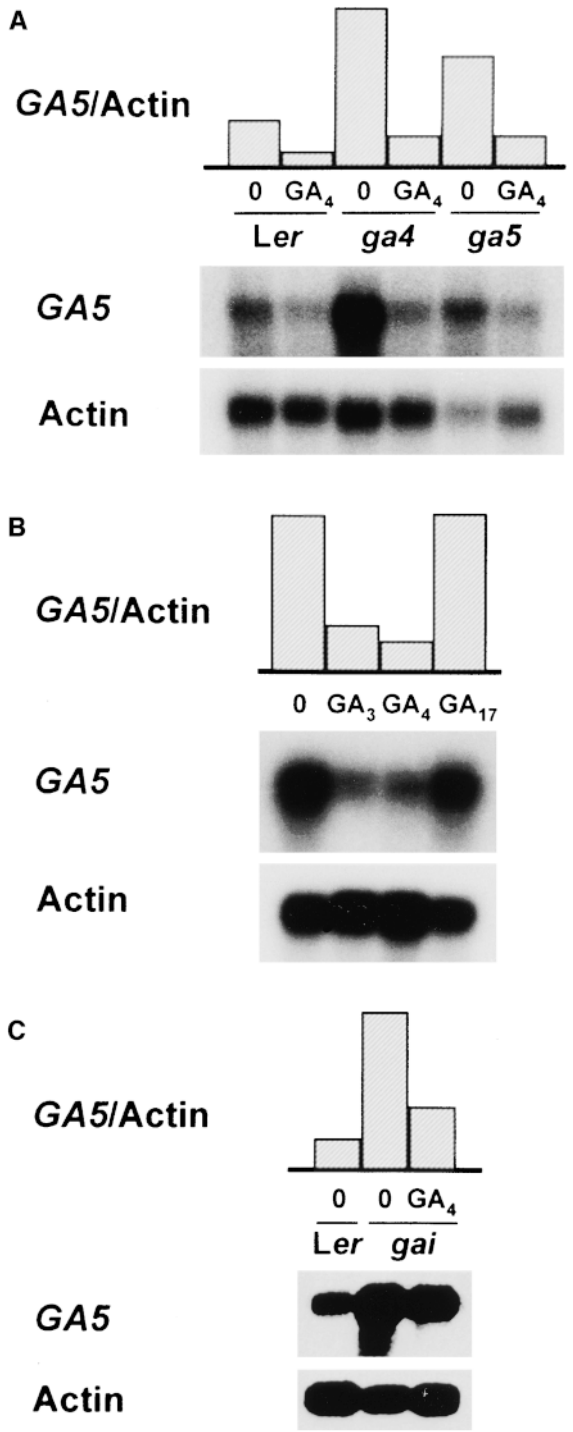

Figure 2. Feedback Regulation by GA of *GA5* Expression in Stems of Wild-Type Arabidopsis and GA-Deficient Mutants.

(A) Effects of GA_4 on the accumulation of the *GA5* transcript in *ga4* and *ga5* mutants. Wild-type Landsberg *erecta* plants and the mutants *ga4* and *ga5* were sprayed with a solution of 100 μM GA_4. The upper 2-cm sections of the stems were harvested 24 hr later. Poly(A)$^+$-enriched RNA (2 to 3 μg) isolated from stems was separated by electrophoresis. The blot was hybridized successively with antisense *GA5* and actin mRNA. The ratio of *GA5*/actin mRNA in the different treatments is indicated at the top.

(B) Effects of GA_3, GA_4, and GA_{17} on the accumulation of the *GA5* transcript in the *ga4* mutant. *ga4* plants were sprayed with a solution of 100 μM GA_3, GA_4, or GA_{17}. The upper 2-cm sections of the stems were harvested 24 hr later. Poly(A)$^+$-enriched RNA (2 to 3 μg) isolated from stems was separated by electrophoresis. The blot was hybridized successively with antisense *GA5* and actin mRNA. The ratio of *GA5*/actin mRNA in the different treatments is indicated at top.

(C) Effects of GA_4 on the accumulation of the *GA5* transcript in the *gai* mutant. *gai* plants were sprayed with a solution of 100 μM GA_4. The upper 2-cm sections of the stems were harvested 24 hr later. Poly(A)$^+$-enriched RNA (2 to 3 μg) isolated from stems was separated by electrophoresis. The blot was hybridized successively with antisense *GA5* and actin mRNA. The ratio of *GA5*/actin is indicated at the top.

pounds GA_{25} and GA_{17} (Figure 1; Xu et al., 1995). It was of interest, therefore, to determine whether the constitutive expression of the pumpkin 20-oxidase, *P16*, would lower the level of active GAs and thus reduce stem growth. Our results demonstrate that expression of the transgene *P16* stimulates production of GA_{25} and GA_{17}, but this is accompanied by increased transcript levels of *GA4* and *GA5* so that an adequate level of active GAs is maintained for stem growth to occur at close to its normal rate.

RESULTS

Negative Feedback Regulation of *GA5* Expression in the Stems of Arabidopsis Plants

The *ga4* and *ga5* mutants are impaired in the final steps of the GA biosynthetic pathway (Figure 1; Koornneef and van der Veen, 1980) so that these mutants have reduced levels of the 3β-hydroxy-GAs GA_1 and GA_4 (Talon et al., 1990a). We found that the level of *GA5* transcript in elongating stems of these mutants was considerably higher than in stems of Landsberg *erecta* plants. However, when GA_4 was applied, the level of *GA5* transcript was reduced in the mutants as well as in stems of wild-type plants (Figure 2A). These results demonstrate that expression of *GA5* in the stems of Arabidopsis is under negative feedback regulation by GA. Thus, the reduced stature of *ga4* and *ga5* plants is due to lower levels of active GAs and is accompanied by a higher level of *GA5* mRNA. This negative feedback regulation of *GA5* transcript level is exerted by active GAs, such as GA_3 and GA_4, but not by inactive GA_{17} (Figure 2B).

The GA-insensitive mutant, *gai*, has a semidwarf stature (Koornneef et al., 1985) and increased levels of active GAs

compared with wild-type plants (Talon et al., 1990b). It was found in these experiments that *GA5* mRNA accumulated to a higher level in stems of the *gai* mutant than in stems of wild-type plants (Figure 2C). Thus, in contrast to the GA-deficient *ga4* and *ga5* mutants, a higher level of active GAs in the *gai* mutant is accompanied by a higher level of *GA5* mRNA. However, application of GA_4 reduced accumulation of *GA5* mRNA in stems of the *gai* mutant (Figure 2C).

Generation and Characterization of Transgenic Arabidopsis Plants Overexpressing *P16*

In the GA biosynthetic pathway, successive oxidation of C-20 of C_{20}–GAs leads to the loss of this carbon atom as CO_2 and to the formation of C_{19}–GAs, which subsequently are converted to biologically active GAs (Figure 1). In addition, C_{20}–GAs can also be converted to inactive C-20 carboxylic acids. The GA 20-oxidase encoded by *P16* from pumpkin endosperm is functionally different from other GA 20-oxidases in that it converts C_{20}–GAs predominantly to the inactive C-20 carboxylic acids GA_{17} and GA_{25} (Lange et al., 1994; Xu et al., 1995). We introduced the *P16* gene under control of the cauliflower mosaic virus 35S promoter (Figure 3) into Arabidopsis to determine whether this would result in redirecting the flow in the GA biosynthetic pathway to inactive forms (Figure 1), in the expectation that this would result in plants with reduced stem height.

Molecular Analysis of Transgenic Plants

To confirm that the transgenic plants did carry the *P16* gene, we performed DNA gel blot analysis with genomic DNA that had been digested with BamHI. Upon electrophoresis and hybridization of the blot with ^{32}P-labeled *P16*, the digests of all three transgenic lines gave a single band (Figure 4), indicating that each transgenic line carried a single copy of the *P16* gene. The fact that there is a BamHI site at the junction of the cauliflower mosaic virus 35S promoter and the transgene excludes the possibility that the transgenic plants carried tandem repeats. Hybridized bands among the three transgenic lines differed in size, indicating that *P16* was integrated at different sites of the genome.

To determine whether the transgene was transcribed, we analyzed mRNA from stems by RNA gel blotting. As shown in Figure 5A, the expression level of *P16* in plants of lines 5A and 5H was very high; in plants of line 5D, *P16* mRNA was barely detectable. Protein gel blot analysis was also performed to check for the formation of P16 protein in the transgenic plants. Figure 5B shows that plants of lines 5A and 5H accumulated the P16 protein, whereas 5D had no detectable amount of P16 protein. This indicates that *P16* in line 5D is not well expressed in its integrated chromosomal matrix.

Upregulation of GA5 *and* GA4 *Expression in Transgenic Plants*

To determine expression of the *GA5* and *GA4* loci in the transgenic plants, we conducted RNA gel blotting with mRNA from stems. The results presented in Figure 5A show that *GA5* and *GA4* transcript levels had increased markedly in lines 5A and 5H but not in line 5D. This demonstrates that the expression of the native genes for both GA 20-oxidase (*GA5*) and 3β-hydroxylase (*GA4*) was significantly upregulated in two of the three lines of transgenic plants.

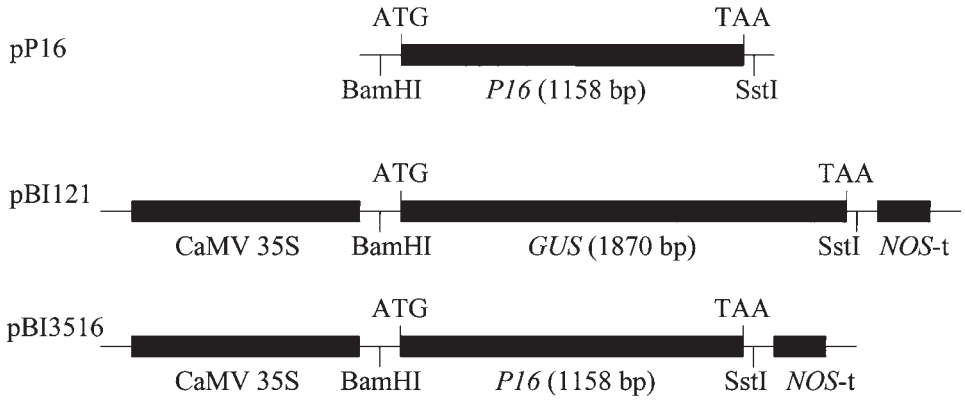

Figure 3. Structure of the pBI3516 Plasmid Used for Transformation of Arabidopsis.

Schematic presentation of the construct p*35S–P16* used for expression of GA 20-oxidase from pumpkin endosperm in Arabidopsis. The β-glucuronidase (*GUS*) gene was removed from pBI121 by digestion with BamHI and SstI, and the *P16* gene was inserted in the same sites. The cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator signal (*NOS*-t) are also indicated. The resulting plasmid was designated pBI3516.

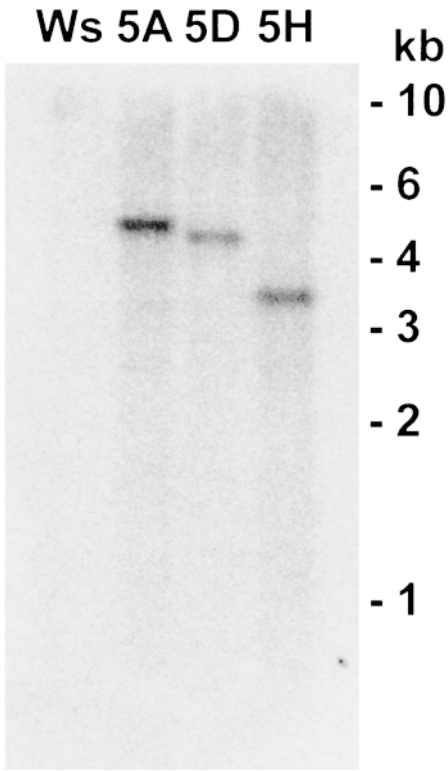

Figure 4. Genomic DNA Gel Blot Analysis of Wassilewskija and Three Transgenic Lines.

Genomic DNA from wild-type Wassilewskija (Ws) plants and the transgenic lines 5A, 5D, and 5H was digested with BamHI and hybridized with ^{32}P-labeled *P16* cDNA. All three transgenic lines show a single band that hybridizes with *P16*, indicating that each line contains a single copy of the transgene, because *P16* does not contain a BamHI restriction site. Numbers at right indicate the positions of the markers in kilobases.

Phenotype of Transgenic Plants

Stem height of plants of lines 5A and 5H was only slightly reduced, whereas that of plants of lines 5D was not reduced at all compared with that of wild-type plants (Figure 6). No other morphological differences were observed between wild-type and transgenic plants.

Analysis of GAs in Transgenic Plants

A quantitative analysis of the endogenous GAs showed that there were large decreases in the 20-methyl (GA_{53}), 20-hydroxy lactone (GA_{44}), and 20-aldehyde (GA_{24} and GA_{19}) GAs (Table 1) and large relative increases in the 20-carboxylic acids GA_{25} and GA_{17} (Table 2) in all three transgenic lines but especially in lines 5A and 5H. These results demonstrate that the *P16* transgene is not only expressed at the mRNA level but that an active enzyme is produced with the same catalytic properties in Arabidopsis as reported for the recombinant protein (Lange et al., 1994; Xu et al., 1995).

The non–13-hydroxylated C_{19}–GAs GA_9 and GA_4 were considerably reduced in the transgenic plants, especially in lines 5A and 5H. By contrast, the 13-hydroxylated C_{19}–GAs GA_{20}, GA_1, and GA_8 showed slight increases. This trend was observed in three separate experiments. Much less *P16*

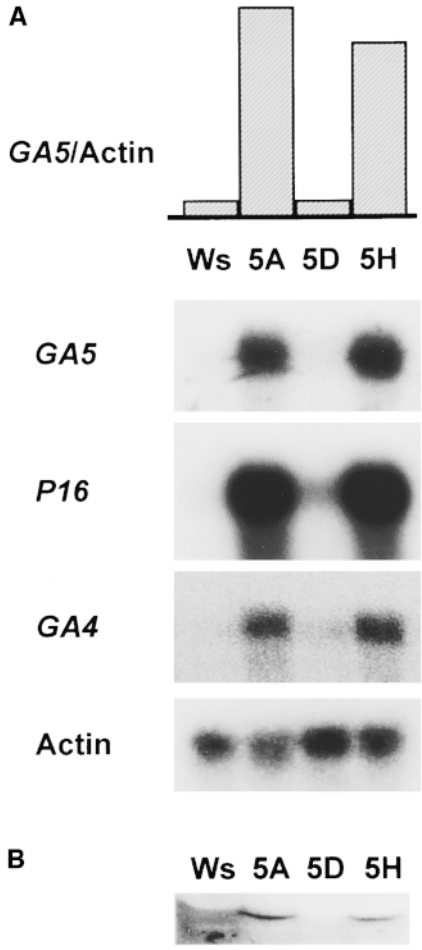

Figure 5. Expression of *GA5*, *P16*, *GA4*, and Actin mRNA in Stems of Wassilewskija and Three Transgenic Lines.

(A) RNA gel blot analysis. Poly(A)$^+$-enriched RNA (2 to 3 μg) isolated from stems was separated by electrophoresis. The blot was hybridized successively with Arabidopsis *GA5* and *GA4* antisense RNA, antisense *P16* of pumpkin, and Arabidopsis actin. The ratio of *GA5*/actin mRNA in plants of wild-type Wassilewskija (Ws) and in three transgenic lines is indicated at top.

(B) Protein gel blot analysis. Approximately 30 μg of protein isolated from leaves was loaded per lane and analyzed by electrophoresis. The blot was cross-reacted with an antiserum raised against the P16 protein purified from pumpkin endosperm.

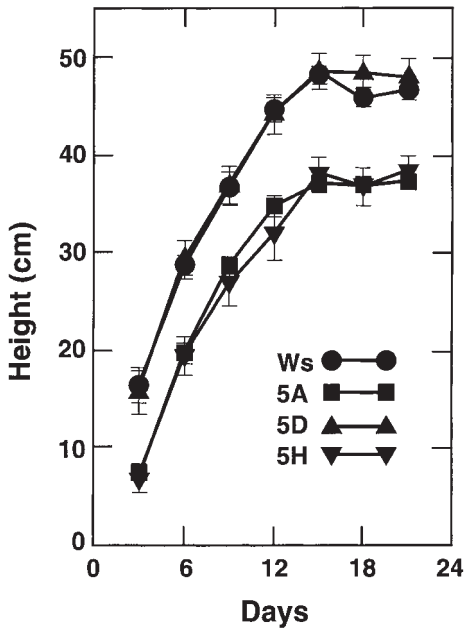

Figure 6. Stem Growth of Wassilewskija and Three Transgenic Lines Expressing the *P16* Transgene.

The plants were grown initially under SD conditions and later transferred to LD conditions consisting of a 9-hr photoperiod of high photon flux density, which was extended with light from incandescent lamps for 15 hr, for a total of 24 hr a day. Days are numbered from the time of transfer to LD conditions. Twenty plants of ecotype Wassilewskija (Ws) and 20 plants of each transgenic line were measured. Error bars indicate the standard error of the mean.

mRNA accumulated in line 5D than in lines 5A and 5H (Figure 5A), and these differences in *P16* transcript levels were correlated with the observed changes in GA levels (Tables 1 and 2).

As shown in Table 2, there were very large relative increases in the levels of GA_{17} and GA_{25} in transgenic plants expressing *P16* compared with the levels found in wild-type plants. The greater increases in GA_{17} than in GA_{25} should not be interpreted to mean that more GA_{17} than GA_{25} is produced in the transgenic plants. In fact, the opposite is most likely the case, because the non–13-hydroxylated pathway is the main GA biosynthetic pathway in Arabidopsis (Talon et al., 1990a). Consequently, more GA_{25} than GA_{17} is found in wild-type Arabidopsis; thus, normalization of the data (see footnotes in Table 2) to those of the wild type starts with a much higher base value for GA_{25} than it does for GA_{17}.

DISCUSSION

In these experiments, we have demonstrated that negative feedback regulation of *GA5* expression operates at the level of transcription in elongating stems of Arabidopsis and presumably plays an important role in maintaining the plant's height within a certain range. Expression of *GA5* increased when the endogenous levels of active GAs were reduced by either genetic (GA-deficient mutants; Figures 2A and 2B) or chemical (GA biosynthesis inhibitors; data not shown) methods. Application of GA_4 or GA_3 reduced the accumulation of *GA5* mRNA (Figure 2B). However, neither application of GA_{17} (Figure 2B) nor increased endogenous levels of GA_{17} or GA_{25} (Table 2) reduced accumulation of *GA5* mRNA, clearly demonstrating that active GAs are required not only for growth but also for feedback regulation of *GA5* expression. Similarly, it has been shown in Arabidopsis that GAs that promote hypocotyl elongation can downregulate *GA4* transcript abundance, whereas inactive GAs and GA analogs cannot (Cowling et al., 1998).

Both GA_4 and GA_3 promoted stem elongation and reduced the accumulation of *GA5* mRNA (Figure 2B). Yet GA_3 has not been identified in Arabidopsis (Talon et al., 1990a). This indicates that the receptor for transduction of the signal that regulates *GA5* expression and growth is not highly specific. In this respect, the signal transduction pathway appears quite similar, if not the same, for GAs regulating stem elongation and *GA5* expression. This raises the question of whether *GA5* expression is regulated by changes in stem growth itself or more directly by GA signal transduction. In GA-deficient mutants impaired in the genes for enzymes that catalyze the final steps in the pathway, a correlation exists between increased expression of *GA5*, a decrease in the levels of active GAs, and reduced stem height (Talon et al., 1990a; Phillips et al., 1995; Xu et al., 1995). However, this correlation does not hold in the GA-insensitive mutant *gai*, which has a semidwarf phenotype. More *GA5* transcript accumulated in this mutant than in wild-type plants (Figure 2C;

Table 1. GA Content of Arabidopsis Ecotype Wassilewskija and of Three Transgenic Lines Transformed with *P16* of Pumpkin Endosperm

GAs	Ws[a]	5A[a]	5D[a]	5H[a]
Non–13-OH-GAs				
GA_{24}	59.0	0.1	4.8	0.0
GA_9	2.0	0.5	2.4	0.5
GA_4	6.2	1.7	2.1	1.3
13-OH-GAs				
GA_{53}	13.0	0.2	0.9	0.2
GA_{44}	1.8	0.4	1.8	0.3
GA_{19}	11.8	2.1	7.6	1.7
GA_{20}	0.2	0.3	0.4	0.3
GA_1	0.2	0.3	0.3	0.3
GA_8	19.6	25.0	21.2	24.3

[a] Nanograms of different GAs per gram dry weight of Wassilewskija (Ws) and three transgenic lines.

Table 2. Increase in Levels of GA_{25} and GA_{17} in Three Transgenic Lines Transformed with *P16* of Pumpkin Endosperm Compared with That of Ecotype Wassilewskija

GAs	Ws[a]	5A[a]	5D[a]	5H[a]
Non–13-OH-GA				
GA_{25}: *m/z* 312/316[b]	1	65	58	75
13-OH-GA				
GA_{17}: *m/z* 492/436[c]	1	378	244	643

[a] Fold increase in transgenic plants compared with Wassilewskija (Ws).
[b] *m/z* 312 of GA_{25}/*m/z* 316 of ^{2}H-labeled GA_{24}, normalized to 1 for Ws.
[c] *m/z* 492 of GA_{17}/*m/z* 436 of ^{2}H-labeled GA_{19}, normalized to 1 for Ws.

Peng et al., 1997), despite the higher levels of active GAs in the mutant, indicating that GAI plays a role in the regulation of *GA5* expression. In previous work, treatment of *gai* plants with GA_4 caused a slight increase in GA5 mRNA in leaves (Xu et al., 1995). However, here we observed that application of a high concentration of GA_4 decreased *GA5* RNA in stems of *gai* plants (Figure 2C). This difference in *GA5* expression may be due to differential regulation of *GA5* in stems and leaves of *gai* plants. It is also important to note that the *gai* mutant is not completely insensitive to GA. In the double mutant *gai ga1*, in which the GA level is reduced, the phenotype becomes more extreme dwarf (Koornneef et al., 1985). A similar extreme phenotype is seen after treatment with the GA biosynthesis inhibitor paclobutrazol (Peng et al., 1997).

Using GA biosynthesis inhibitors to control plant stature is a common practice in agriculture. Molecular cloning of genes for GA biosynthetic enzymes (Hedden and Kamiya, 1997) makes it possible to target plant stature for genetic engineering. Because several studies have shown that steps catalyzed by GA 20-oxidase may be limiting GA biosynthesis (Coles et al., 1996; Hedden and Kamiya, 1997; Xu et al., 1997), GA 20-oxidase was chosen as an engineering target. The P16 protein, a fusion GA 20-oxidase encoded by a cDNA from pumpkin endosperm, produces inactive tricarboxylic acid GAs as its major products in vitro (Lange et al., 1994; Xu et al., 1995). It was expected that overexpression of *P16* would redirect the GA biosynthetic flow to inactive GA_{17} and GA_{25}, leading to a reduction in active GA_1 and GA_4 (Figure 1). Accumulation of GA_{17} and GA_{25} was indeed found in transgenic plants overexpressing *P16* (Table 2), but only small changes in stem height were observed; *GA5* and *GA4* mRNA accumulated to very high levels in these plants (Figure 5A). Thus, GA homeostasis was maintained by upregulating expression of the native genes in the transgenic plants.

Analysis of the endogenous GA content in *P16*-overexpressing plants showed a severe reduction in the amounts of C_{20}–GA intermediates with a large accumulation of inactive tricarboxylic acid GAs (Table 2). Although there was a substantial decrease in the amount of active GA_4, only a small effect on stem growth was observed in lines 5A and 5H, and no effect at all was observed in line 5D (Table 1 and Figure 6). The small increases in GA_1 and substantial decreases in GA_4 in the transgenic plants further support the conclusion that GA_4 is the major bioactive GA for stem elongation in Arabidopsis (Xu et al., 1997). Apparently, endogenous GA_4 is saturating for stem growth in wild-type plants, and a considerable reduction in its content is required before it becomes limiting for growth. Another possibility is that sensitivity to GA increases as the levels of active GAs are reduced. This would mean that in the *P16*-overexpressing plants, GA sensitivity is increased so that their stature is maintained relatively unchanged, despite reduced levels of GA_4.

P16 was expressed much less at the mRNA and protein levels in line 5D than in lines 5A and 5H (cf. Figures 5A and 5B). Nevertheless, the C_{20}–GAs GA_{24} and GA_{53} were much reduced in plants of line 5D compared with wild-type plants, whereas GA_{19} showed only a slight decrease and GA_{44} remained unchanged (Table 1). Considering the very low levels of C_{20}–GAs and the very high levels of GA_{17} and GA_{25} in plants of lines 5A and 5H (Table 2), it appears that the P16 enzyme had reached saturating levels in these two transgenic lines, although this was clearly not the case in line 5D. Although no P16 protein was detectable by immunoblotting in plants of line 5D, an adequate amount of enzyme must have been produced to cause the large increases in GA_{17} and GA_{25} (Table 2).

Reduced plant height is a desirable trait in modern agriculture. In principle, two strategies can be envisioned by which stem height in plants can be reduced by metabolic engineering of GA levels: (1) by expressing GA biosynthetic genes in the antisense orientation and (2) by overexpressing genes that deactivate GAs. In Arabidopsis, we overexpressed *P16* from pumpkin endosperm, which catalyzes the production of inactive 20-carboxylic acids, and observed only a slight decrease in stem height due to feedback regulation that resulted in much increased transcript levels of the native *GA4* and *GA5* genes. Thus, upregulation of *GA4* and *GA5* expression, and presumably of the corresponding enzymes as well, compensated for the increased deactivation of GAs by P16. Other target enzymes for genetic engineering of reduced GA levels and plant stature include 2β-hydroxylase and GA-conjugating enzymes. Aside from feedback regulation, the success or failure of this approach depends on the affinity of two competing enzymes, with one activating and the other deactivating a common substrate, such as GA_9, to either GA_4, an active GA, or GA_{51}, an inactive GA.

METHODS

Plant Material and Gibberellin Treatment

Plants (*Arabidopsis thaliana*) were grown as described previously (Talon et al., 1990a). All mutants used were in ecotype Landsberg

erecta. Short-day (SD) conditions consisted of 9 hr of light from fluorescent tubes and incandescent lamps (150 to 200 μmol m^{-2} sec^{-1}) at 23°C, followed by 15 hr of darkness at 20°C. The plants were grown under SD conditions for 4 weeks and then transferred to long-day (LD) conditions. LD conditions consisted of the same photoperiod as did SD conditions but were extended with 15 hr of low-intensity light from incandescent lamps (10 μmol m^{-2} sec^{-1}) at 20°C. When required, plants were sprayed with an aqueous solution of 100 μM of the gibberellins GA_4, GA_3, or GA_{17} containing 5% (v/v) ethanol and 0.05% Tween 20.

For DNA and protein extraction, rosette leaves were harvested before stem elongation. For mRNA extraction, the upper 2-cm portion of the stem was harvested when the stems were 2 to 5 cm long. All material was harvested at the end of the main light period.

Plants to be used for GA measurements were initially grown in SD conditions for 6 weeks from sowing and then exposed to 3 LD. At the time of harvest, all plants had stems with flower buds. The entire plant (rosette leaves and stems with leaves and flower buds) was collected, frozen in liquid N_2, and lyophilized.

Construction of Plasmid and Transformation of Arabidopsis

Plasmid pP16, which contains the GA 20-oxidase cDNA from pumpkin endosperm (Xu et al., 1995), was digested with BamHI and SstI, and *P16* was inserted into the same sites of pBI121 (Jefferson et al., 1987) (Clontech, Palo Alto, CA). The resulting plasmid was designated pBI3516 (Figure 3). This plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 (Clontech).

For transformation of Arabidopsis ecotype Wassilewskija, cotyledons were removed from 7-day-old seedlings and cocultured with LBA4404 harboring pBI3516. Transgenic plants (T_0) were regenerated from these cotyledon explants, as described previously (Barghchi et al., 1994). All analyses were performed on T_2 plants, which had been tested for homozygosity in the T_3 generation.

DNA Isolation and Gel Blot Analysis

Genomic DNA was extracted as described previously (see Focus, Volume 12, published by Bethesda Research Laboratories). Digested DNA samples were separated by electrophoresis in a 0.8% agarose gel and transferred to nylon membranes. The blots were prehybridized and hybridized in 0.5 M Na_2HPO_4, pH 7.2, 7% SDS, and 1 mM EDTA at 68°C and washed in 40 mM Na_2HPO_4, pH 7.2, 5% SDS, and 1 mM EDTA at 68°C. They were then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

mRNA Isolation and RNA Gel Blot Analysis

Total RNA was extracted by using an Extract-A-Plant RNA isolation kit (Clontech). mRNA was then purified by using a PolyATract mRNA isolation system (Promega). RNA gel blots were prepared by electrophoresis of 2 to 3 μg of poly(A)$^+$ RNA in agarose gels in the presence of formaldehyde (Sambrook et al., 1989), followed by transfer to nylon membranes. The blots were probed with ^{32}P-labeled antisense RNA. Hybridization was performed as given for DNA gel blotting except that the temperature was 70°C. The blots were washed in 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 70°C and exposed to a PhosphorImager screen. The relative amounts of mRNA were determined with a PhosphorImager. Each blot was sequentially probed with *GA5*, *GA4* (Chiang et al., 1995), *P16*, or actin (clone 40F11 from Dr. T. Newman, Michigan State University) antisense RNA probes after the blots were stripped. The ratio of *GA5*/actin mRNA was used to compare *GA5* mRNA levels in different treatments. Each experiment was repeated at least twice with similar results.

The hybridization conditions used did not cause cross-hybridization with transcripts of homologs of *GA5* (Phillips et al., 1995; Y.-L. Xu, unpublished results). A homolog of *GA4*, *GA4H*, has been isolated that was predominantly expressed during Arabidopsis seed germination. Cross-hybridization between the mRNAs of these two genes was below the level of detection (Yamaguchi et al., 1998a).

Protein Extraction and Protein Gel Blot Analysis

Protein from pumpkin (*Cucurbita maxima*) endosperm was precipitated with ammonium sulfate, and the 40 to 70% fraction with GA 20-oxidase activity was purified by anion exchange, gel filtration, and hydrophobic chromatography. The partially purified enzyme preparation was subjected to semipreparative native PAGE, and gel sections were assayed for enzyme activity. Proteins in the zone with the highest enzyme activity were eluted and used for immunization of rabbits. Polyclonal antibodies were obtained that immunoprecipitated the GA 20-oxidase activity. These antibodies were used to isolate the *P16* cDNA clone, which encodes a GA 20-oxidase, by immunoscreening a cDNA library made from pumpkin endosperm (Xu et al., 1995).

Proteins were extracted from Arabidopsis rosette leaves by using 62.5 mM Tris-HCl buffer at pH 6.8. Protein concentration was estimated by the Bradford (1976) method. Cold (−20°C) acetone was then added to a final concentration of 80% (v/v) to precipitate proteins. Precipitated proteins were dissolved in loading buffer (62.5 mM Tris-HCl, pH 6.8, 5% SDS, 10 mM DTT, and 10% glycerol).

Extracted proteins were electrophoresed on 10% SDS–polyacrylamide gels. After electrophoresis, the polypeptides were electrotransferred to a nitrocellulose filter. The filter was developed with the ProtoBlot II AP system (Promega).

Quantification of GAs

The GAs were extracted, purified, and quantified by the use of deuterated GAs as internal standards (Talon et al., 1990a), except for the tricarboxylic acids GA_{25} and GA_{17}, which were not available in deuterated form. Because these GAs were collected in the same fractions as their corresponding 20-aldehydes, GA_{24} and GA_{19}, they were quantitatively related to the internal standards ^{2}H-labeled GA_{24} and ^{2}H-labeled GA_{19} by gas chromatography–selected ion monitoring. The ions used for calculating the increases in GA_{25} and GA_{17} in transgenic plants in comparison with Wassilewskija represent major fragments in the mass spectra of GA_{25}, GA_{17}, and ^{2}H-labeled GA_{24} and GA_{19} (see footnotes in Table 2).

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