Gibberellin Dose-Response Regulation of *GA4* Gene Transcript Levels in Arabidopsis[1]

Rachel J. Cowling[2], Yuji Kamiya, Hideharu Seto, and Nicholas P. Harberd*

Department of Molecular Genetics, John Innes Centre, Colney Lane, Norwich NR4 7UJ, United Kingdom (R.J.C., N.P.H.); and Laboratory for Plant Hormone Function, Frontier Research Program (Y.K.), and Plant Function Laboratory (H.S.), The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351–01, Japan

The gibberellins (GAs) are a complex family of diterpenoid compounds, some of which are potent endogenous regulators of plant growth. As part of a feedback control of endogenous GA levels, active GAs negatively regulate the abundance of mRNA transcripts encoding GA biosynthesis enzymes. For example, Arabidopsis *GA4* gene transcripts encode GA 3β-hydroxylase, an enzyme that catalyzes the conversion of inactive to active GAs. Here we show that active GAs regulate *GA4* transcript abundance in a dose-dependent manner, and that down-regulation of *GA4* transcript abundance is effected by GA$_4$ (the product of 3β-hydroxylation) but not by its immediate precursor GA$_9$ (the substrate). Comparison of several different GA structures showed that GAs active in promoting hypocotyl elongation were also active in regulating *GA4* transcript abundance, suggesting that similar GA:receptor and subsequent signal transduction processes control these two responses. It is interesting that these activities were not restricted to 3β-hydroxylated GAs, being also exhibited by structures that were not 3β-hydroxylated but that had another electronegative group at C-3. We also show that GA-mediated control of *GA4* transcript abundance is disrupted in the GA-response mutants *gai* and *spy-5*. These observations define a sensitive homeostatic mechanism whereby plants may regulate their endogenous GA levels.

GAs are a family of hormones that are essential for development and growth of plants (Hooley, 1994). However, the molecular mechanism for GA perception thought to involve a GA:GA-receptor interaction has yet to be elucidated. Although more than 100 different GA structures have been identified in plants, only a few possess biological activity, suggesting a high degree of specificity to the GA:GA-receptor interaction (Takahashi et al., 1990).

The study of GA action has been advanced by the use of mutants that are affected in GA biosynthesis or signal transduction (Ross, 1994). Examples of GA-biosynthesis mutants in Arabidopsis are the GA-deficient dwarfs *ga1*, *ga4*, and *ga5* (Koornneef and van der Veen, 1980). A WT phenotype can be recovered in all of these mutants by applying active GAs. GA biosynthesis is a multistep pathway involving *ent*-kaurene synthesis and oxidation, followed by further oxidations of the GA skeleton, the latter being catalyzed by 20-oxidase and 3β-hydroxylase (Graebe, 1987; Talon et al., 1990a). *GA1*, *GA4*, and *GA5* are loci that encode the copalyl diphosphate synthase (Sun and Kamiya, 1994; Hedden and Kamiya, 1997), 3β-hydroxylase (Chiang et al., 1995; Hedden and Kamiya, 1997), and 20-oxidase (Phillips et al., 1995; Xu et al., 1995) enzymes of GA biosynthesis, respectively. 3β-Hydroxylation is widely held to be a final step in the biosynthesis of active GAs, converting GA$_9$ and GA$_{20}$ (inactive) to GA$_4$ and GA$_1$ (active), respectively.

Recently, it has been shown that expression of 20-oxidase and *GA4* genes is negatively regulated by exogenous GA (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995). These observations are consistent with evidence suggesting that the activities of GA biosynthesis enzyme can be down-regulated by GA (Hedden and Croker, 1992). A negative feedback loop of this nature requires that GAs active in feedback regulation can somehow be distinguished from similar (but inactive) structures in the GA biosynthesis pathways. In the case of feedback regulation of *GA4* transcript levels, this could occur in one of two ways: Either the immediate products of the 3β-hydroxylation reaction or all biologically active GA structures negatively regulate *GA4* transcript levels. Here we describe experiments designed to determine which of these possible mechanisms is responsible for the regulation of *GA4* transcript levels.

The Arabidopsis GA signal transduction mutant *gai* has the dwarf, dark-green characteristics of a GA-deficient plant. However, the phenotype conferred by *gai* cannot be rescued by the application of GA, and the *gai* mutant therefore displays reduced responses to both endogenous and exogenous GA (Koornneef et al., 1985; Peng and Harberd, 1993, 1997; Wilson and Somerville, 1995; Peng et al., 1997). *gai* has higher than wild-type levels of endogenous active GAs (Talon et al., 1990b), suggesting that negative

Abbreviations: BX-112, prohexadione calcium BX-112; PAC, paclobutrazol; QRT-PCR, quantitative RT-PCR; RT-PCR, reverse-transcription PCR.

feedback regulation of GA levels is perturbed in this mutant. Another class of Arabidopsis GA signal transduction mutants, the *spy* mutants, display resistance to the GA biosynthesis inhibitor PAC (Jacobsen and Olszewski, 1993; Wilson and Somerville, 1995; Jacobsen et al., 1996). *spy* mutants are able to germinate and display elongation growth in concentrations of PAC that are inhibitory to wild-type plants.

In this paper we describe experiments that define the nature of the feedback regulation of *GA4* transcript levels by active GAs. Using the GA-deficient *ga1–3* mutant (Sun and Kamiya, 1994), we show that *GA4* transcript levels are negatively regulated by the product of the 3β-hydroxylation reaction, GA_4, but not by the immediate precursor, GA_9. Feedback regulation occurs in a dose-dependent manner that closely mirrors stimulation of hypocotyl elongation. We also show that the presence of a 3β-OH group does not always confer activity for feedback, and that GAs that are active in feedback do not have to be 3β-hydroxylated. Finally, we show that feedback regulation of *GA4* transcript levels is disrupted in *gai* and *spy* mutants. These results indicate that negative feedback regulation of *GA4* transcript levels occurs by perception of active GAs via a receptor/signal transduction pathway that is similar to that involved in GA-mediated elongation growth.

MATERIALS AND METHODS

Chemicals

GA_1 was a gift from Prof. Sassa (Yamagata University Yamagata, Japan), and GA_4 was obtained from Kyowa Hakko Co., Ltd. (Tokyo, Japan). GA C was prepared from GA_1 (Cross, 1960): treatment of GA_1 with Dowex-resin 50W-X2 (H^+ form) in refluxing methanol:water, 2:5 (v/v), for 7 h gave GA C in 84% yield. *epi*-GA_4 was prepared from GA_4: treatment of GA_4 with potassium *tert*-butoxide in *tert*-butyl alcohol at room temperature for 7 d afforded *epi*-GA_4 in 92% yield (Aldridge et al., 1965). 3-oxo-GA_9 was prepared from GA_4 by oxidation (Aldridge et al., 1965). All of the GAs were purified by preparative high-performance column chromatography. The purity of GAs was about 100% as checked by GC-MS.

Plant Material and Growth Procedures

An Arabidopsis Landsberg *erecta* laboratory strain (wild type) was used throughout. *ga1–3* and *gai* mutants were originally isolated from mutagenized WT (Koornneef and van der Veen, 1980; Koornneef et al., 1985). Seeds homozygous for the *spy-5* allele (also isolated from mutagenized wild type) were kindly donated by R. Wilson (Wilson and Somerville, 1995).

After sterilization (Ezura and Harberd, 1995), *ga1–3* seeds were chilled for 5 d at 4°C in sterile 10^{-6} M GA_4 solution to initiate and synchronize germination. After a thorough rinsing in sterile water they were plated individually (50/plate) on germination medium (Ezura and Harberd, 1995) containing GA or inhibitors at the required

concentration. Sterilized wild type and *spy-5* seeds were directly sown (50/plate) before chilling for 5 d at 4°C. The seeds were then grown in a standard growth room at 20°C with a 16-h light/8-h dark cycle.

GAs (previously purified by HPLC) were dissolved in methanol and then in sterile water. A small volume (no more than 1/1000 volume) was then added to 20 mL of cooled molten germination medium in Petri dishes. The inhibitors PAC (Zeneca Agrochemicals, Wilmington, DE) and BX-112 (Kumiai Chemical Research Institute, Shizuoka, Japan) were made up and added in the same way. Hypocotyls were measured directly to the nearest 0.5 mm using samples of 8 to 10 per treatment.

QRT-PCR

RNA was prepared from seedlings (entire aerial parts) harvested 2 weeks after germination (about 20 per sample). Approximately 5 μg of each RNA sample was then used in a first-strand cDNA synthesis reaction (containing RNase inhibitor) using a standard poly-dT adapter primer and Moloney murine leukemia virus reverse transcriptase, diluted 10-fold. The following oligonucleotides were made to amplify fragments of the *GA4* (Chiang et al., 1995, 1997), *APT1* (Moffat et al., 1994) and γ-*TIP* (Ludevid et al., 1992) cDNAs: OLLY23, 5′-TCCCAGAATCGCTAA-GATTGCC-3′; OLLY42, 5′-CCTTTCCCTTAAGCTCTG-3′; OLLY26, 5′-CGATTTCCGTAAACTTTGGC-3′; OLLY28, 5′-ATCCATTGGATAGGATGTGG-3′; OLLY40, 5′-CAT-CTTGAAGCTTAAATC-3′; and OLLY22, 5′-GACTCG-AGTCGACAT-CGA(T)$_{17}$-3′. OLLY23 and OLLY42 amplify a 478-bp fragment of *APT1* cDNA. OLLY26 and OLLY28 amplify a 398-bp fragment of *GA4* cDNA. Both of these products can be distinguished by size from products resulting from amplification of any contaminant genomic DNA because the primer sequences are on either side of at least one intron. For the amplification of γ-*TIP* cDNA, OLLY40 was used with a poly-T primer (OLLY22) to ensure that only cDNA would be amplified, as a fragment of approximately 1.1 kb (the γ-*TIP* genomic DNA sequence is unknown). In each case, the PCR product was cloned and sequenced, using standard techniques, so as to verify the sequence of the amplified fragments. The cloned fragments were later released from the cloning vectors via restriction endonuclease digestion and used as hybridization probes.

The above cDNA solutions (5 μL) were used as the templates in a standard 50-μL PCR reaction (with 0.25 mM dNTPs and 2 ng/μL each primer) of up to 30 to 34 cycles of 1 min each at 94°C, 55°C, and 72°C. OLLY23/OLLY42 and OLLY26/OLLY28 primer pairs were used in separate reactions to avoid primer competition (Murphy et al., 1990). After at least 10 cycles, 4-μL aliquots were removed from the reactions every 2 to 4 cycles. PCR products were separated by electrophoresis, blotted, and hybridized using standard techniques ([^{32}P]dCTP-labeled hybridization probes, as described above). QRT-PCR products were quantified by phosphor imaging, using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Curves were constructed by plotting the radioactivity of the PCR product (*y*-axis; $\log_{10}$ scale) against the number of cycles (*x*-axis)

(see Fig. 1B). This confirmed the approximately exponential nature of the PCRs (all gradients were between 0.22 and 0.3 [the theoretical maximum for PCR]). Expression of the two genes was compared at points where both reactions were progressing exponentially, which gives a ratio of *GA4/APT1* expression (Noonan et al., 1990). Within each experiment, these ratios were normalized to the sample grown on GM alone or as described in the figure legends. However, in some samples the expression of the *GA4* gene was so low that PCR products could not be detected within this range, and a ratio could not be calculated, so in the figures this is described as not detected. A product was always detected after 30 cycles, suggesting that the *GA4* gene was never completely repressed in our experiments, but at this point the control gene reactions had already saturated. It is also possible that the primers used to amplify the *GA4* product, although shown to preferentially amplify product derived from the *GA4* gene itself, might also amplify the products of any genes closely related in sequence to *GA4*.

RESULTS

GA4 Transcript Levels Are Elevated in the *ga1–3* Mutant

GA4 expression was followed relative to the expression of a control gene, *APT1*, by QRT-PCR using the kinetic method. This method permits quantification of the abundance of a specific mRNA with respect to another endogenous control mRNA (Chelly et al., 1988; Murphy et al., 1990; Noonan et al., 1990). *APT1* is expressed at a low level in all tissues of Arabidopsis (Moffat et al., 1994). These experiments were performed using the *ga1–3* mutant,

which has greatly reduced levels of endogenous active GAs due to a deletion in a gene encoding copalyl diphosphate synthase (Sun and Kamiya, 1994; Hedden and Kamiya, 1997). Transcripts were compared in *ga1–3* seedlings treated with exogenous GA_4 and untreated controls. GA_4 was chosen for these experiments because it is the most abundant 3β-hydroxylated GA and probably the main active GA in Arabidopsis (Talon et al., 1990a). As shown in Figure 1, the levels of control *APT1* transcript in *ga1–3* seedlings are not significantly affected by GA_4 treatment (Fig. 1A), and are not significantly different from that of WT (Fig. 1B).

γ-*TIP* transcript levels are up-regulated following GA treatment of the GA-deficient *ga1–2* mutant (Phillips and Huttly, 1994). We compared the effects of exogenous GA_4 on the accumulation of γ-*TIP* and *GA4* transcripts in *ga1–3* (Fig. 1A). As expected, γ-*TIP* transcripts were not detected in the untreated *ga1–3* controls, but were clearly detectable in the GA_4-treated *ga1–3* sample. The behavior of *GA4* transcripts in these experiments is the converse of that of γ-*TIP*, in that *GA4* transcripts were clearly detectable in the untreated *ga1–3* controls, but were only just detectable in the GA_4-treated *ga1–3* sample.

ga1–3 mutant seedlings contained elevated levels of *GA4* transcript compared with the wild type (Fig. 1B). These elevated transcript levels were restored to wild-type levels by the addition of exogenous GA_4 (Fig. 1B). The GA_4-treated *ga1–3* sample required at least six more PCR cycles to produce the same amount of *GA4* amplification product than did the nontreated *ga1–3* control, whereas *APT1* was expressed at the same level in both samples (Fig. 1B). The efficiency of the PCR reaction was similar for all samples
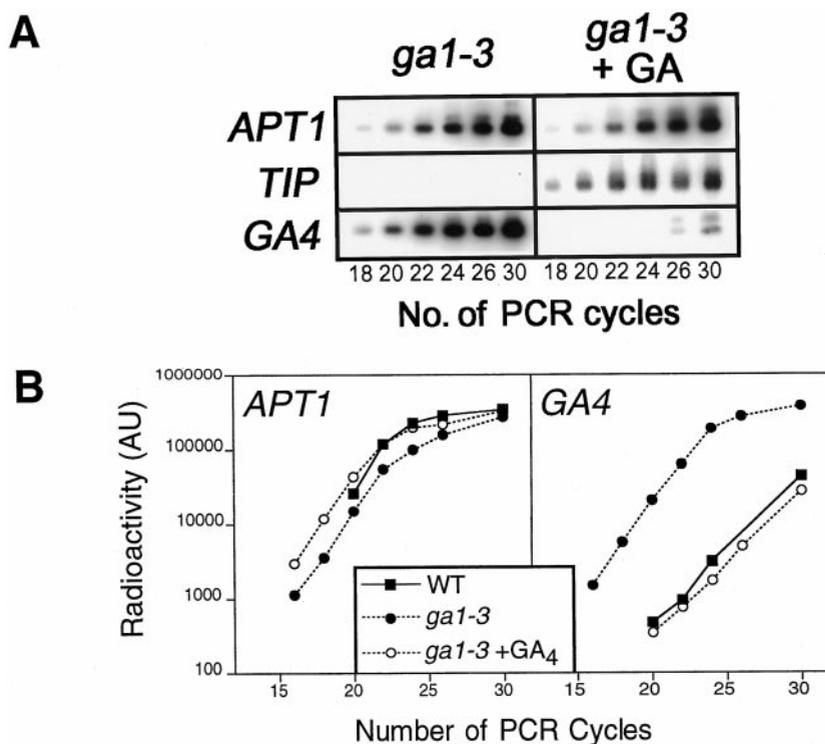

Figure 1. A, QRT-PCR analysis of *GA4* and γ-*TIP* (*TIP*) transcripts relative to those of the *APT1* control gene in GA_4-treated (10^{-7} M; *ga1–3* + GA) and untreated *ga1–3* (*ga1–3*). The relevant primer pairs (see "Materials and Methods") were used on poly-T-primed cDNA samples in separate reactions. Aliquots taken after the stated number of cycles were separated on a 1.2% agarose gel, blotted, hybridized to radioactively labeled probes of a known sequence, and visualized by phosphor imaging. B, Kinetics of RT-PCR reactions shown in A compared with others using wild-type samples. Radioactivity of hybridized filters was measured using Image-Quant software (see "Materials and Methods") and plotted on a $\log_{10}$ scale (*y* axis) (AU, arbitrary units) against the number of PCR cycles (*x* axis). In the GA_4-treated sample, the *GA4*-derived primers amplified two products, the smaller of which was of the correct size to be the *GA4* sequence and the signal intensity of which was measured. Gradients over the linear portions (exponential phases) of the curves range from 2.2 to 2.8. RT-PCR product levels were compared before saturation occured (cycle 24). WT, wild type.

and for both genes during the exponential phase (Fig. 1B). The elevated level of *GA4* transcript in the *ga1–3* mutant is thus equivalent to an induction of *GA4* gene expression of over 60-fold (64). This result is consistent with previous observations that *GA4* transcripts accumulate in a *ga4* mutant to higher levels than in the wild type, and confirms that *GA4* transcript abundance is negatively regulated by GAs (Chiang et al., 1995).

GA-Mediated Feedback Control of *GA4* Transcript Abundance Is GA Dose Dependent

For the following experiments, a steady-state estimate of *GA4* mRNA abundance was obtained by calculating the ratio of *GA4:APT1* transcripts using information from plots such as the one in Figure 1B. The phosphor imager value for the *GA4* gene was divided by that of the *APT1* gene at the value of x (no. of PCR cycles), where both reactions were approximately exponential, and prior to saturation (cycle 22 for Fig. 1B). This ratio approximates the ratio of initial templates in the PCR reaction at cycle 0, providing all of the reactions have similar efficiencies (Noonan et al., 1990). Within each experiment all samples had been prepared and processed at the same time, and ratios were calculated at the same number of cycles. Within each experiment these values were normalized to the sample grown on medium alone (i.e. in the absence of hormone or inhibitors), which was arbitrarily given the *GA4:APT1* ratio of 1. All experiments were repeated with separate RNA samples, PCRs, and hybridizations; representative data are shown.

The effects of a range of GA concentrations on *GA4* transcript abundance and hypocotyl elongation were compared (Fig. 2). At a high GA_4 dose, *ga1–3* hypocotyls were
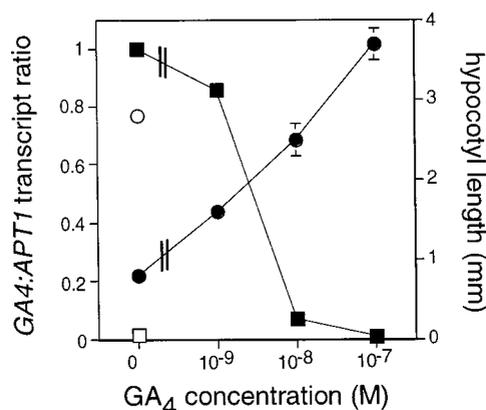
Figure 2. *ga1–3* seedlings were assayed for relative *GA4* mRNA levels and hypocotyl length 2 weeks after germination on medium containing the stated concentration of GA_4. QRT-PCR results (*GA4: APT1* ratios, calculated after 22 cycles, when the reaction had yet to saturate) were normalized with respect to *ga1–3* grown on germination medium only (=1). Results from untreated wild-type seedlings are shown for a comparison (open symbols). Error bars represent SE hypocotyl length (sometimes smaller than symbol width). ●, *ga1–3* hypocotyl elongation; ○, wild-type hypocotyl elongation; ■, *GA4/ APT1* transcript ratios in *ga1–3*; □, *GA4/APT1* transcript ratios in the wild type.

as long as those of untreated wild type, whereas *GA4* transcript levels were as low as those of untreated wild type. However, as the GA_4 dose decreased, *ga1–3* hypocotyls became progressively shorter, and *GA4* transcript levels became progressively higher.

Feedback Regulation by Active GAs?

GA_9 is 3β-hydroxylated by the 3β-hydroxylase enzyme in planta to form GA_4 (Fig. 3A). We investigated the hypothesis that regulation of expression of the *GA4* gene is an example of product inhibition by testing the effects of GA_9 (substrate) and GA_4 (product) on the accumulation of *GA4* transcripts in the *ga1–3* mutant. To prevent conversion of GA_9 to GA_4, the inhibitor BX-112 was used. BX-112 prevents both 3β- and 2β-hydroxylation of GAs (Nakayama et al., 1990a, 1990b). Figure 3B shows that GA_9 and GA_4 caused a marked increase in *ga1–3* hypocotyl length. However, if BX-112 was included in the medium, the effect of GA_9 was greatly reduced, whereas that of GA_4 was relatively unaffected. The simplest explanation for this is that GA_9 is not active in itself, but becomes active following 3β-hydroxylation to GA_4. 3β-Hydroxylation is largely, but not completely, abolished by the BX-112 treatment; thus, only a small amount of the inactive GA_9 is converted to GA_4 in the presence of BX-112.

Seedling samples were assayed for *GA4* transcript levels relative to the control gene *APT1* by comparing levels of RT-PCR products after 22 cycles (Fig. 3C). *GA4* transcript levels were high in the *ga1–3* mutant, but were repressed by both GA_9 and GA_4 in the absence of BX-112. No *GA4* RT-PCR product was detected in the GA_9 (without BX-112) or GA_4 (without BX-112) samples (after 22 cycles) even though the control gene was expressed at the same level in both samples, as it is in nontreated *ga1–3* (see "Materials and Methods"; data not shown). However, in the presence of BX-112, GA_9 reduced *GA4* transcript accumulation in *ga1–3* only very slightly, whereas GA_4 was equally effective in reducing *GA4* transcript accumulation in *ga1–3* in the presence or absence of BX-112. The simplest explanation for these observations is that GA_4 is active in the feedback control of *GA4* transcript abundance, whereas GA_9 is not. Thus, a GA endogenous to Arabidopsis (GA_4) regulates a product-inhibition pathway controlling the abundance of transcripts that encode an enzyme required for the biosynthesis of that GA.

GA Structure-Activity Relationships in the *GA4* Transcript Feedback Response

It might be predicted that only 3β-hydroxylated GAs would feedback regulate the abundance of *GA4* transcripts (which encode the 3β-hydroxylase). It has also been suggested that the 3β-OH group is the key to GA activity in some plants (Reeve and Crozier, 1974). A range of GA structures (Fig. 4A) were tested for their activity in stimulating hypocotyl elongation in *ga1–3* seedlings at a set concentration of 10^{-7} M. This is the lowest concentration at which exogenous GA_4 stimulates hypocotyl elongation sufficiently to make a *ga1–3* hypocotyl of equivalent length to
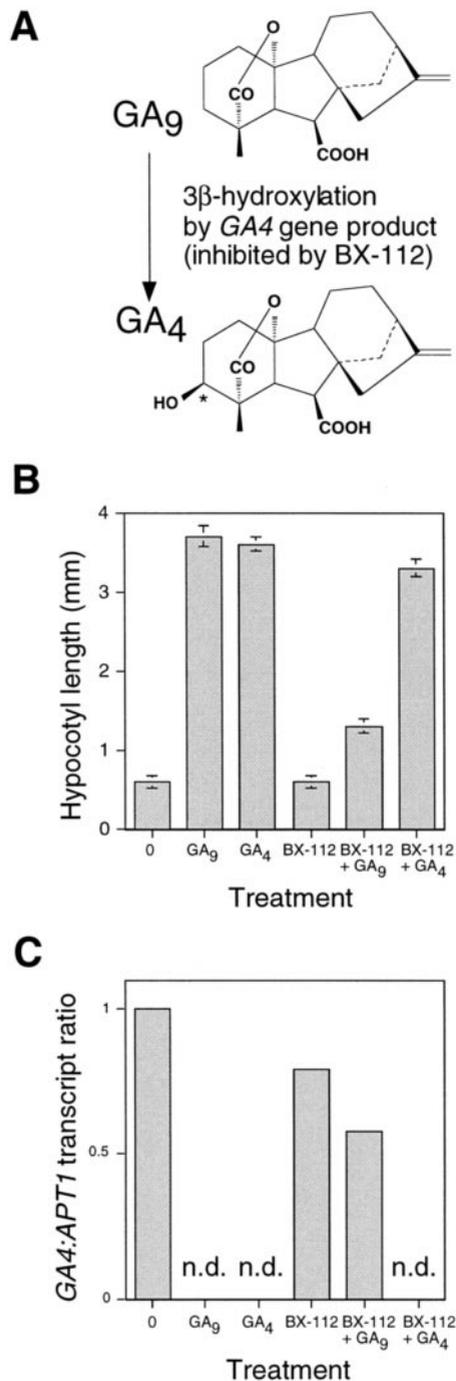
Figure 3. *GA4* transcript levels are regulated by GA_4 but not by GA_9. A, GA_9 is converted to GA_4 by the addition of a 3β-OH group on C-3 (*). This reaction is catalyzed by the *GA4* gene product and inhibited by BX-112. B, *ga1–3* seedlings were grown for 2 weeks on germination medium supplemented with 10^{-4} M BX-112, 10^{-7} M GA_9, and 10^{-7} M GA_4 as stated. Hypocotyl lengths were measured as described in Figure 2, error bars represent SE. C, *ga1–3* seedlings treated as in B were assayed for *GA4* transcript levels as in Figure 2. RT-PCR products were compared after 22 cycles. Results are presented as *GA4:APT1* product ratios, normalized with respect to *ga1–3* grown on germination medium only (=1). n.d., *GA4* transcript not detected (see "Materials and Methods").

an untreated wild-type hypocotyl (Fig. 2). The results of these experiments are shown in Table I. GA_4, 3-oxo-GA_9, and *epi*-GA_4 exhibited strong activity as stimulators of *ga1–3* hypocotyl elongation, whereas GA_1, GA C, and GA_4-methyl ester all exhibit low activity. For the purposes of the present paper the GAs used in these experiments are classified as active or inactive as stimulators of *ga1–3* hypocotyl elongation (Table I).

Levels of *GA4* and *APT1* transcripts were compared via QRT-PCR (after 22 cycles) in *ga1–3* seedlings treated with the above GA structures (Table I). *GA4* transcripts were not detected in the GA_4, 3-oxo-GA_9, or *epi*-GA_4 samples, despite detectable expression of the *APT1* gene. Thus, GA_4, 3-oxo-GA_9, and *epi*-GA_4 are all active as negative regulators of *GA4* transcript abundance. Conversely, *GA4* transcripts were detected at levels similar to that of the untreated *ga1–3* control in the GA_1, GA C, and GA_4-methyl ester samples (a small reduction in the GA_1-treated *GA4:APT1* ratio is indicative of GA_1 possessing low activity).

These experiments show that there is a correlation between the degrees of activity exhibited by each GA structure in the two assays described above. GAs that are active in the promotion of hypocotyl elongation are also active in the negative regulation of *GA4* transcript abundance, whereas those inactive in the former assay are also inactive in the latter. Of the structures that are active (GA_4, 3-oxo-GA_9, and *epi*-GA_4), only GA_4 is 3β-hydroxylated. GC-MS analysis showed that the purity of the GA_4, 3-oxo-GA_9, and *epi*-GA_4 samples is high, and that the activity displayed by
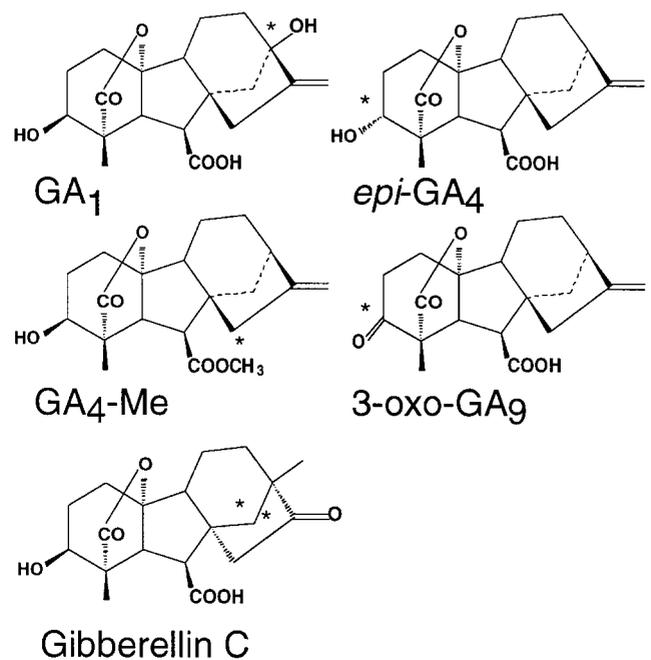
Figure 4. Structures of the GAs and GA analogs tested for biological activity. Significant differences in structure from GA_4 (see Fig. 3A) are highlighted (*). *epi*-GA_4 is 3α-hydroxylated rather than 3β-hydroxylated; GA_1 is 13-hydroxylated; GA_4-methyl ester (GA_4-Me) is esterified on the carboxyl group at carbon-7; 3-oxo-GA_9 has a ketone group at C-3 instead of a 3β-hydroxyl group; and GA C, a derivative of GA_1, has a rearrangement of the C and D rings.

Table I. *GA structure-activity relationships*

GA[a]	Hypocotyl Length[b]	GA4:APT1 Ratio[c]	Activity[d]
	mm		
3β-OH-GAs			
0	0.8 (0.1)	1.00	—
GA$_4$	3.3 (0.3)	n.d.[e]	Active
GA$_1$	1.0 (0.1)	0.41	Inactive
GA$_4$-Methyl ester	0.7 (0.1)	0.80	Inactive
GA C	0.9 (0.1)	0.98	Inactive
Non-3β-OH-GAs			
epi-GA$_4$	2.8 (0.3)	n.d.	Active
3-oxo-GA$_9$	4.1 (0.4)	n.d.	Active

[a] GA and analog structures as shown in Figure 4. [b] Seedlings were grown on germination medium containing individual GAs or analogs at 10^{-7} M. Hypocotyls were measured as described in Figure 2 with SE in parentheses. [c] Relative *GA4* transcript levels calculated after 22 cycles. *GA4* and *APT1* transcript levels were obtained initially as ImageQuant phosphor imaging values (see "Materials and Methods"), and are expressed as normalized *GA4:APT1* ratio (*gal-3* grown on germination medium only = 1). [d] Activity of each GA at 10^{-7} M. GA$_1$, although classed here as inactive, has mild activity. [e] n.d., Samples where the *GA4* transcript level was too low to be detected, preventing calculation of the *GA4:APT1* ratio. In all cases *APT1* was expressed at a similar level.

3-oxo-GA$_9$ and *epi*-GA$_4$ is not due to GA$_4$ contamination (see "Materials and Methods"). Furthermore, GA$_1$, GA C, and GA$_4$-methyl ester are all 3β-hydroxylated and yet are inactive (or have low activity in the case of GA$_1$). Thus, a specific 3β-hydroxy-GA recognition system may not be involved in the negative feedback regulation of *GA4* transcript abundance.

Feedback Control of *GA4* Transcript Abundance Is Disrupted in *gai* and *spy* Mutants

The effect of GA treatments on *GA4* transcript levels was further investigated using the wild type and the GA-response mutants *gai* and *spy-5*. These plants have different/unknown endogenous GA concentrations (Talon et al., 1990a, 1990b). For this reason, the plants were grown in media containing 10^{-7} M GA$_4$ or 10^{-7} M PAC or both. PAC inhibits the oxidation of *ent*-kaurene, an early step in GA biosynthesis, and thus reduces endogenous GA levels (Graebe, 1987). The effects of these treatments on the growth of wild-type, *gai*, and *spy-5* seedlings is shown in Figure 5A. As expected, the wild type is markedly dwarfed by PAC, and this effect is reversed by the additional presence of GA$_4$ in the medium. *gai* is dwarfed both in the presence and absence of PAC, and remains dwarfed in the GA plus PAC treatment. *spy-5* (in the absence of PAC or GA) is approximately the same size as the wild type, and is resistant to the dwarfing effects of PAC (Wilson and Somerville, 1995).

GA4 transcript levels (relative to those of *APT1*) were assayed in these mutants. As shown previously (Figs. 1 and 2), the wild type has lower levels of *GA4* transcript than does *ga1–3*. However, treatment of the wild type with 10^{-7} M PAC induces expression of the *GA4* gene over 60-fold

(see Fig. 5B). This effect can be reversed by the addition of GA$_4$ (Fig. 5B). *GA4* transcript levels were approximately 4-fold higher in *gai* than in the wild type, but were appreciably lower than in *ga1–3* (see Figs. 2 and 5C). *gai* was relatively insensitive to manipulated changes in endogenous GA levels, maintaining slightly elevated levels of *GA4* transcript (4–16 times untreated wild type), despite treatments with PAC or GA (Fig. 5C). In *gai*, *GA4* gene expression is partially, but not fully, repressed by its own high levels of endogenous GAs (Talon et al., 1990b). In addition, PAC treatment (depletion of endogenous GAs) did not induce *GA4* expression to the extent that it does in the wild type.

GA4 transcript levels were also higher in *spy-5* than in the wild type (Fig. 5C). Furthermore, these levels were relatively unaffected by treatment with PAC (compare this with the marked induction of *GA4* transcript level in the wild type by PAC). Thus, *spy-5* is a mutant that displays, on PAC, GA-independent regulation of *GA4* transcript levels and, like *gai*, blocks the *GA4* transcript abundance feedback response. This is an interesting result, because *spy* mutants show some hallmarks of constitutive GA-response mutants (Swain and Olszewski, 1996). In another such constitutive GA-response mutant, the *la cry*[s] mutant of pea, 20-oxidase transcripts accumulate to lower (rather than higher) levels than they do in wild-type plants (Martin et al., 1996).

The key conclusion from these experiments is that whereas wild-type plants display a marked increase in *GA4* transcript level following PAC treatments, *gai* and *spy-5* mutant plants do not.

DISCUSSION

We have used GAs endogenous to Arabidopsis to demonstrate that *GA4* gene expression can be controlled by the product of the 3β-hydroxylation reaction, GA$_4$, but not by the substrate, GA$_9$. Active GAs have already been shown to regulate the expression of *GA4*, *GA5*, and other 20-oxidase-encoding genes in Arabidopsis (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995) and a 20-oxidase gene in pea (Martin et al., 1996). Our experiments show that GAs that lack activity in a growth bioassay (hypocotyl-elongation response) are also inactive in the regulation of *GA4* transcript levels. Conversely, GAs that control *GA4* transcript levels also control hypocotyl elongation. These observations suggest that the control of *GA4* transcript levels and hypocotyl elongation may be regulated via common GA: GA-receptor interactions and subsequent signal transduction pathways. In addition, our results show that it is active GAs that regulate *GA4* transcript abundance, and not only GAs that are the immediate products of the 3β-hydroxylation reaction.

Negative feedback regulation of biosynthetic gene expression is potentially an important form of regulation of GA biosynthesis in plants. We have shown that control of *GA4* gene expression is sensitively GA dose dependent in the normal physiological range. Thus, incremental changes in GA level result in correlated changes in the *GA4* transcript level, providing a sensitive homeostatic mechanism
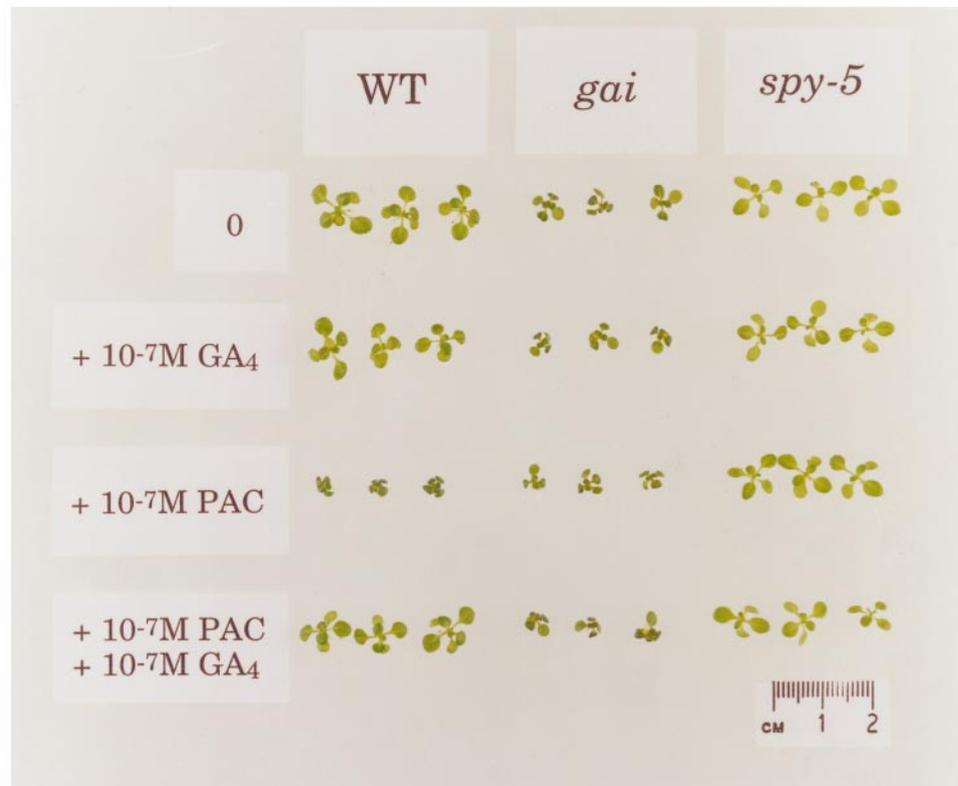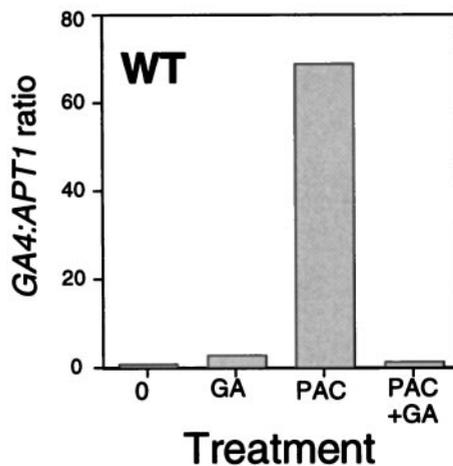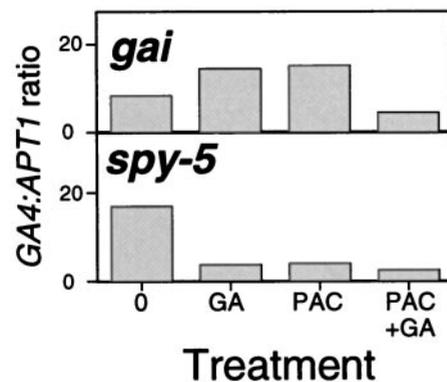
Figure 5. Effects of GA$_4$ or PAC on wild-type (WT), *gai,* and *spy-5* seedlings. A, Seedlings grown on germination medium containing 10^{-7} M GA$_4$ and/or 10^{-7} M PAC for 2 weeks. B, Effects of exogenous GA$_4$ (GA) and PAC (same concentrations as in A) on *GA4* mRNA levels in the wild type. QRT-PCR results were converted to ratios by normalizing samples to the wild-type sample grown on germination medium only. All samples were prepared at the same time and compared after 22 cycles. C, Effects of exogenous GA$_4$ (GA) and PAC (same concentrations as in A on *GA4* mRNA levels in *gai* and *spy* mutants). Samples were prepared at the same time and data were normalized as in B and compared after 22 cycles.

for the regulation of in planta GA levels. This may allow the plant to subtly monitor and alter GA production in response to developmental and environmental changes.

We tested the hypothesis that the 3β-OH group confers activity for the hypocotyl elongation and *GA4* transcript feedback responses. We found that a 3α-OH group (*epi*-GA$_4$) and a 3=O group (3-oxo-GA$_9$) could each substitute for the 3β-OH group on the GA$_4$ skeleton, creating molecules that were active in the regulation of both *GA4* transcript levels and hypocotyl elongation. Earlier experiments demonstrated that *epi*-GA$_4$ and 3-oxo-GA$_9$ are active in the regulation of cucumber hypocotyl elongation (Brian et al., 1967). In addition, recent experiments using the *d1* mutant of maize have shown that GA$_5$, which lacks a 3β-OH

group, is active in the stimulation of leaf-sheath elongation, suggesting that a 3β-OH group may not be crucial for activity (Spray et al., 1996). Furthermore, GA$_{22}$, which does not have a 3β-OH group but has a 18-OH group, promotes shoot elongation in rice in the presence of BX-112 (Kamiya et al., 1991). In this latter case it is possible that the 18-OH group compensates partially for the absence of the 3β-OH group. It is probable that the electronegative group at the C-3 position of GA$_4$ is important for the GA:GA-receptor interaction. Both 3-oxo-GA$_9$ and epi-GA$_4$ have electronegative groups at C-3 (although with a slightly different orientation than in GA$_4$), whereas GA$_9$ and the other inactive GAs do not. Furthermore, we found that GAs that possessed a 3β-OH group but were altered at other regions of the molecule (GA$_1$, GA$_4$-methyl ester, and GA C) had reduced activity. Thus, our experiments indicate that activity is retained in structures in which the -OH group at the 3β position is replaced by other groups (subject to the requirement for the electronegative group at C-3), and that activity is modified by groups at positions other than C-3. It is well known that 2β-hydroxylation of 3β-hydroxylated GAs results in a loss of activity (Takahashi et al., 1990).

Conclusions from structural studies must be tentative, as the capacity of seedlings to interconvert and transport different GA structures must be considered. However, the difference between the activities of GA$_1$ and GA$_4$ is striking (see also Sponsel et al., 1997). GA$_1$ differs from GA$_4$ by the presence in the former, and the absence in the latter, of a hydroxyl group at C-13 (see Fig. 4). Although we cannot discount the possibility that the applied GAs are converted to more active or inactive forms, it is unlikely that GA$_1$ is dehydroxylated to a more active form in vivo, because the progressive oxidation of GAs is generally thought to be an irreversible process (Graebe, 1987). Thus, the presence or absence of the 13-OH group influences the activity of the GA structure. These observations are consistent with the idea that the putative GA receptor recognizes the whole of the GA molecule and not just a particular region of it (Reeve and Crozier, 1974).

Our experiments show that the *gai* and *spy-5* mutants are both altered in the regulation of *GA4* transcript accumulation. *gai* has elevated levels of *GA4* transcript compared with the wild type. This observation is consistent with previous reports that 20-oxidase transcript levels are also elevated in *gai* (Xu et al., 1995; Peng et al., 1997), and indicates that the *gai* mutation perturbs the feedback regulation of transcripts encoding GA biosynthesis enzymes. This could explain the elevated levels of bioactive GAs in *gai* (Talon et al., 1990b): Active GAs do not down-regulate *GA4* and *GA5* transcript abundance in *gai* to the extent that they do in the wild type, resulting in higher levels of 20-oxidase and 3β-hydroxylase activities and elevated active GA levels.

The Arabidopsis *spy* mutants belong to the constitutive GA-response class of GA signal transduction mutant (Swain and Olszewski, 1996). The observation that *GA4* transcript abundance in *spy-5* is higher, rather than lower, than that of the wild type is perhaps surprising, since 20-oxidase levels in the pea *la cry^s* mutant (another constitutive GA-response mutant) are lower than in wild-type

pea (Martin et al., 1996). This apparent discrepancy may be due to the fact that different genes (*GA4*, 20-oxidase) and/or different species (Arabidopsis and pea) are involved. However, the constitutive GA-response mutants may actually represent two subclasses of mutant, one (which includes *spy*) comprising mutants that mimic wild-type plants treated with a nonsaturating GA dose, and the other (which includes *la cry^s*) comprising mutants that mimic wild-type plants treated with a saturating GA dose (Swain and Olszewski, 1996). It is possible that the above apparent discrepancy actually represents a difference between the properties of mutants from these two subclasses.

Of course, regulation of the abundance of transcripts encoding GA biosynthesis enzymes is not the only possible means of altering GA levels, and control of GA abundance may also be effected in other ways. Inactivation (by 2β-hydroxylation), conjugation, compartmentation, and transport processes may all contribute to regulating the concentration of active GAs in planta (Takahashi et al., 1990). In addition, it is possible, although untested, that feedback control may also operate via product inhibition of the enzymatic activity of GA biosynthesis enzymes. Further work will uncover the relative importance of feedback regulation of GA biosynthesis gene transcript levels in the control of the production of active GAs.

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