The Plant Journal (1999) **17**(5), 547–556

Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes

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Summary

Gibberellin (GA) 20-oxidase catalyses consecutive steps late in GA biosynthesis in plants. In *Arabidopsis*, the enzyme is encoded by a gene family of at least three members (*AtGA20ox1*, *AtGA20ox2* and *AtGA20ox3*) with differential patterns of expression. The genes are regulated by feedback from bioactive GAs, suggesting that the enzymes may be involved in regulating GA biosynthesis. To investigate this, we produced transgenic *Arabidopsis* expressing sense or antisense copies of each of the GA 20-oxidase cDNAs. Over-expression of any of the cDNAs gave rise to seedlings with elongated hypocotyls; the plants flowered earlier than controls in both long and short days and were 25% taller at maturity. GA analysis of the vegetative rosettes showed a two- to threefold increase in the level of GA_4, indicating that GA 20-oxidase normally limits bioactive GA levels. Plants expressing antisense copies of *AtGA20ox1* had short hypocotyls and reduced rates of stem elongation. This was reflected in reduced levels of GA_4 in both rosettes and shoot tips. In short days, flowering was delayed and the reduction in the rate of stem elongation was greater. Antisense expression of A*tGA20ox2* had no apparent effects in long days, but stem growth in one transgenic line grown in short days was reduced by 20%. Expression of antisense copies of *AtGA20ox3* had no visible effect, except for one transgenic line that had short hypocotyls. These results demonstrate that GA levels and, hence, plant growth and development can be modified by manipulation of GA 20-oxidase expression in transgenic plants.

Introduction

The gibberellin (GA) plant hormones are involved in many developmental processes, including germination, stem extension, flowering and fruit development. Modification of these processes by application of chemicals that alter GA content is common agronomic practice. For example, GA_3 is used to stimulate berry growth in seedless grape production (Christadoulou *et al*., 1968), and inhibitors of GA biosynthesis are used as growth retardants to control the stature of cereals and ornamental pot plants (Hedden and Hoad, 1994). An alternative approach to the application of such chemicals would be to modify the content of GAs by genetic manipulation of their biosynthesis. The recent cloning of several genes involved in GA biosynthesis (reviewed by Hedden and Kamiya, 1997) has provided the means to test the feasibility of this approach.

Mutants of *Arabidopsis thaliana* with reduced production of GAs have demonstrated the role of these hormones in stem elongation and flowering in this species. Six loci, *GA1–GA6*, that encode GA-biosynthetic enzymes have been identified (Koornneef and van der Veen, 1980; Sponsel *et al*., 1997). Null mutations in *GA1*, which encodes copalyl-diphospate synthase (CPS; Sun and Kamiya, 1994), prevent bolting, although flower production without stem elongation is initiated in long days. In short days, both stem elongation and flower formation are completely inhibited in such mutants (Wilson *et al*., 1992). In contrast, null mutations in *GA4*, which encodes GA 3β-hydroxylase (Chiang *et al*., 1995; Williams *et al*., 1998) and *GA5*, encoding a GA 20-oxidase (Xu *et al*., 1995), result in semi-dwarfs with normal flower production. Mutations in *GA6*, which is also thought to encode a GA 20-oxidase, result in short inflorescences, reduced flower fertility and short siliques (Sponsel *et al*., 1997). GA 20-oxidase and 3β-hydroxylase catalyze the final steps in the biosynthesis of the active hormones, as outlined in Figure 1. The former is a multi-functional enzyme that removes carbon-20 in the formation of the C_{19}-GA skeleton.

The capacity of the *ga4* and *ga5* mutants to accumulate reduced levels of bioactive GAs (Talon *et al*., 1990) that support some stem elongation, despite the apparent absence of functional enzymes encoded by these genes, indicates that there are multiple genes encoding GA 3β-hydroxylase and 20-oxidase in *Arabidopsis*. This has been confirmed for GA 20-oxidase, for which three cDNAs encoding functionally identical enzymes were cloned (Phillips *et al*., 1995). The three 20-oxidase genes are differentially expressed; one, designated *AtGA20ox1* and corresponding

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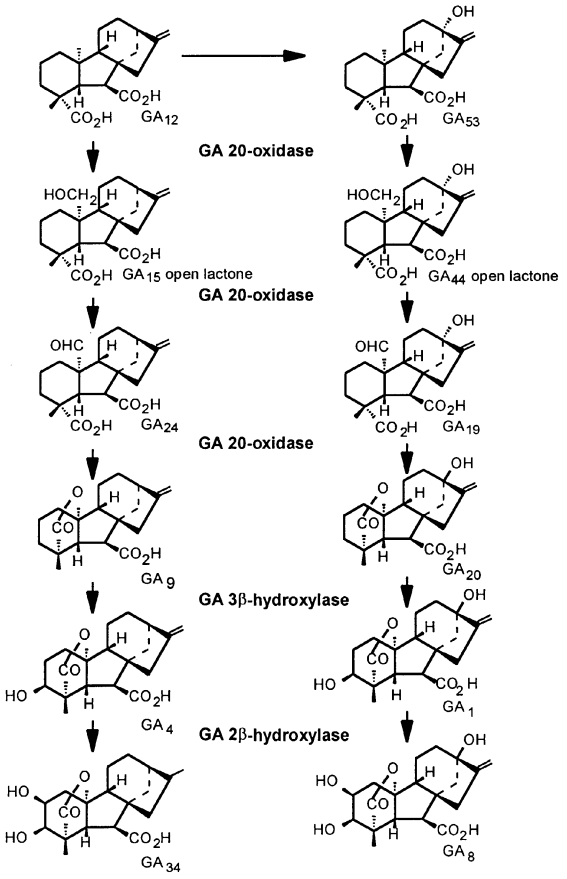

Figure 1. Biosynthetic pathways from GA_{12} and GA_{53} to the bioactive products GA_4 and GA_1, and their respective inactive 2β-hydroxylated catabolites GA_{34} and GA_8.
GA 20-oxidases catalyse successive oxidation reactions on C-20, resulting in its removal and the formation of the C_{19}-GA skeleton.

to the *GA5* gene*, is expressed mainly in the stem, but also in the inflorescence. A second (*AtGA20ox2*) is expressed in the inflorescence and developing silique, while the third (*AtGA20ox3*) is expressed only in the silique. These genes are thus apparently involved in different developmental processes that are controlled by GA.

In the present work, we investigate the function of the GA 20-oxidase genes in *Arabidopsis* by suppressing each gene through expression of antisense mRNA. These experiments also test the feasibility of reducing GA biosynthesis and, hence, plant stature, by genetic manipulation of GA 20-oxidase gene expression. In addition, we describe the effects of over-expressing the 20-oxidase genes constitutively in order to determine the influence of GA 20-oxidase activity on GA biosynthesis.

*Gene nomenclature: In consultation with the gibberellin community, we propose a rational system of nomenclature for GA biosynthetic genes. The three Arabidopsis GA 20-oxidase genes, *At2301* (*GA5*), *At2353* and *YAP169* have thus been renamed as *AtGA20ox1, AtGA20ox2* and *AtGA20ox3*, while the GA 3β-hydroxylase gene, *GA4*, becomes *AtGA3ox1*.

Results

Over-expression of Arabidopsis *GA 20-oxidase genes*

At least 15 lines over-expressing each of the *Arabidopsis* GA 20-oxidase cDNAs were obtained; all lines were phenotypically very similar irrespective of the particular 20-oxidase cDNA being over-expressed. The seedlings showed differences from the control line 96 h after imbibition (Figure 2a and Table 1). Compared with those of controls, hypocotyls and petioles of the 20-oxidase seedlings were approximately 50% longer and resembled controls treated with GA_3. Treatment of the 20-oxidase seedlings with GA_3 resulted in no further increase in hypocotyl or petiole length. The over-expressing lines flowered earlier than the control, a trait that was apparent in the T2 generation, in which early flowering was apparently dose-dependent, homozygous lines bolting earlier than heterozygotes (data not shown). In long days, bolting occurred in the homozygous (T4) lines at six leaves as opposed to nine leaves for the control, a difference of about 4 days (Figure 3a). In this respect also, the over-expressing lines mimicked control plants that had been treated with GA (Figure 2b). The difference in stem height in the 20-oxidase lines compared with the control persisted to maturity, when the former were approximately 25% taller (Figures 2b and 3a), although growth rates were only slightly higher (Figure 3a). In short days, the over-expressing lines bolted at 24 leaves, 10 days earlier than the control, which bolted at 29 leaves (Figures 2c and 3b). At maturity, the 20-oxidase lines were approximately 33% taller than controls.

Northern blots of mRNA from shoot tips of the 20-oxidase over-expressing and control lines were probed with cDNA for each GA 20-oxidase gene (Figure 4a). High levels of expression of the transgenes were indicated for each line by the presence of two intense bands of 1.3 kb and 1.5 kb in lines over-expressing *GA20ox1*, and 1.4 kb and 1.6 kb in lines over-expressing *GA20ox2* and *GA20ox3*. The smaller transcript in each case has the expected size based on the cDNA sequence that was inserted into the expression cassette, while the larger band appears to be due to a secondary transcription initiation site within the double CaMV 35S promoter used.

Expression of antisense GA 20-oxidase mRNA

For each antisense construct, at least 13 transgenic lines were identified and at least five homozygous (T4) lines were investigated. At the seedling stage, hypocotyl and petiole lengths in two lines transformed with antisense *AtGA20ox1* cDNA (lines A1–1 and A1–2) and one line transformed with antisense *AtGA20ox3* (line A3–1) were reduced compared with the control line (Figure 2a and

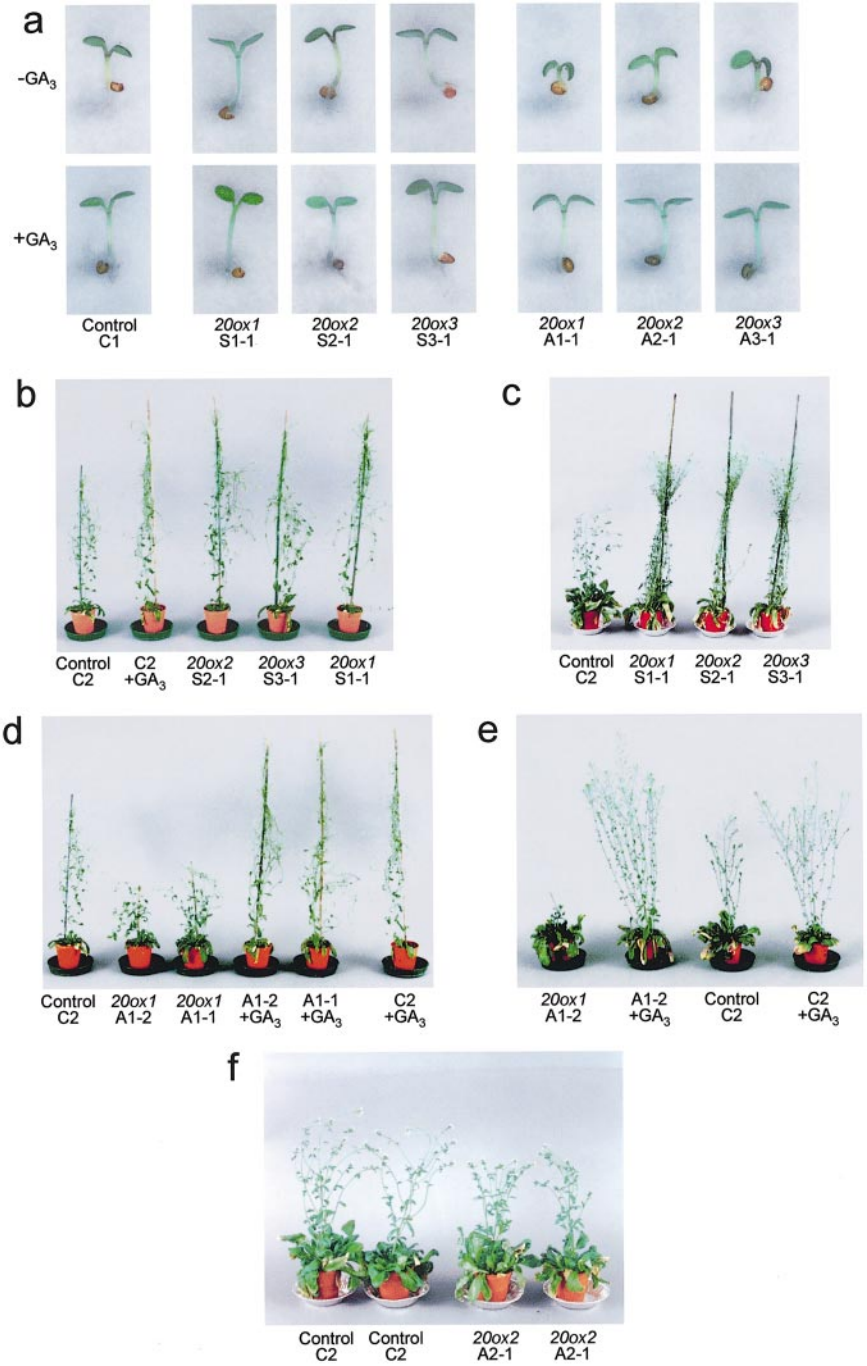

Figure 2. Expression of sense and antisense copies of GA 20-oxidase genes in *Arabidopsis*: effects on plant development.
(a) Seedlings of homozygous transgenic lines 96 h after imbibition and stratification, grown in long days on media ± GA_3; (b) over-expression: effects on growth in long days, 56 days after planting; (c) over-expression: effects on growth in short days, 63 days after planting; (d) antisense expression of *AtGA20ox1*: effects on growth in long days, 56 days after planting, showing restoration of growth by GA_3 application; (e) antisense expression of *AtGA20ox1*: effects on growth in short days, 63 days after planting, showing restoration of growth by GA_3 application; (f) antisense expression of *AtGA20ox2*: effects on growth in short days, 63 days after planting.

Table 1). Cotyledons of these antisense GA 20-oxidase lines were darker green than those of the control and were epinastic. Treatment of the seedlings with GA_3 restored their growth to that of the GA_3-treated control. None of the plants transformed with antisense *AtGA20ox2* were distinguishable from the controls at this stage. Growth of the antisense *AtGA20ox3* line A3–1 increased to that of the control from about 96 h after sowing and, thereafter, this line was phenotypically identical to control plants (Figure 3c,d). In long days, the antisense *AtGA20ox1* plants bolted at the same time as the control (at nine leaves), but the rate of stem extension of the antisense plants was reduced such that, at maturity, they were about 40% shorter (Figures 2d and 3c). Stem length in the *AtGA20ox1* antisense lines was similar to that of the controls when the plants were treated with GA_3 (Figure 2d).

Bolting of the antisense *AtGA20ox1* lines was delayed in short days, beginning at 33 leaves as opposed to

Table 1. Hypocotyl lengths (mm) of homozygous transgenic seedlings, 96 days after imbibition on media ± 10 μM GA_3; means of 20 seedlings per line with standard error

	Over-expressing lines				Antisense lines		
	Control C1	S1–1	S2–1	S3–2	A1–1	A2–1	A3–1
− GA_3	0.80 ± 0.01	1.33 ± 0.01	1.25 ± 0.02	1.25 ± 0.02	0.56 ± 0.01	0.82 ± 0.01	nd
+ GA_3	1.17 ± 0.02	1.34 ± 0.01	1.25 ± 0.02	1.27 ± 0.02	1.16 ± 0.01	1.28 ± 0.02	nd

nd, not determined.

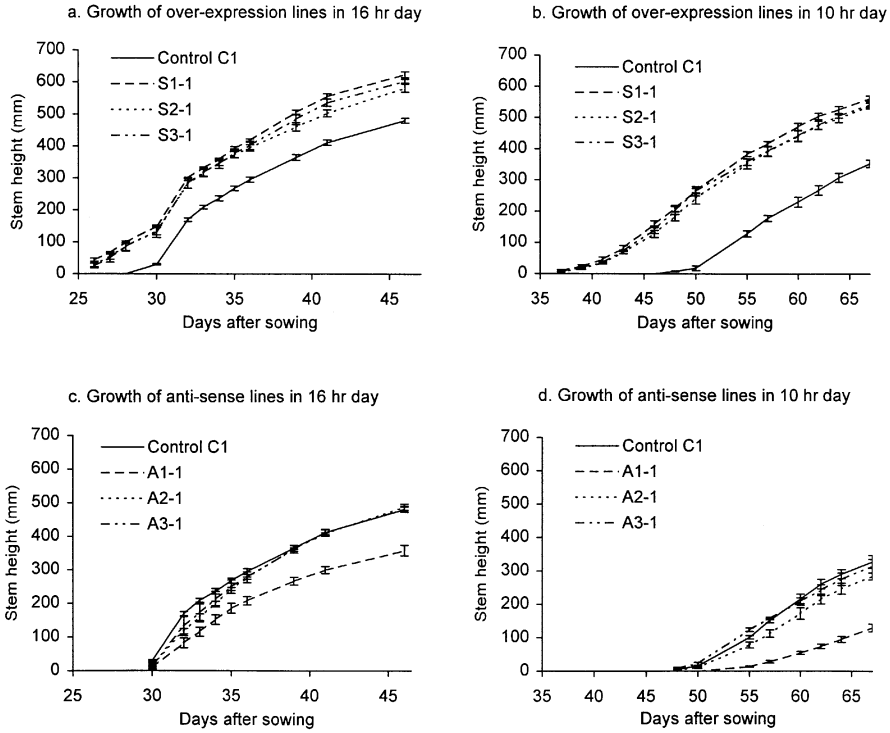

Figure 3. Stem heights of *Arabidopsis* expressing sense and antisense copies of GA 20-oxidase genes.

29 leaves for the control (Figure 3d). Furthermore, stem extension was reduced substantially, such that the final stem heights of the two lines were 37% and 50% of the control (Figures 2e and 3d). Flower numbers and morphology, as well as silique size and numbers in these antisense lines were similar to those of the control. Stem growth of one *AtGA20ox2* antisense line (A2–1) was slightly reduced in short days, with a final height of about 80% that of the control (Figures 2f and 3d). Flowering time in long or short days was not affected in this line, which grew normally in long days (Figure 3c). Flower fertility appeared to be reduced in this line, which set fewer and smaller siliques than control plants (data not shown). No antisense *AtGA20ox3* lines had a detectable phenotype after the seedling stage.

mRNA from the shoot tips of the antisense lines, grown in long days, was analysed by Northern hybridization with cDNA probes (Figure 4b). A major band of antisense message was observed at 1.5 kb for the two *AtGA20ox1* antisense lines A1–1 and A1–2 that gave an altered phenotype, with a minor, unexplained band at 2.2 kb. Bands at 1.4 and 1.6 kb were present in the *AtGA20ox2* antisense line A2–1 (Figure 4b). Only one other antisense transformant that was analysed, expressing antisense to *AtGA20ox1*, contained antisense transcript (data not shown). All other antisense lines had incorporated the transgene (data not shown), but did not show an altered phenotype. By probing with antisense RNA, the abundance of native *AtGA20ox1* transcript in the antisense *AtGA20ox1* lines A1–1 and A1–2 was shown to be very markedly reduced compared with the control and lines expressing antisense mRNA for the other GA 20-oxidase genes (Figure 4b).

Effect of altered expression of GA 20-oxidase genes on GA content

In order to determine effects of altered GA 20-oxidase gene expression on GA content, plants over-expressing the *Arabidopsis* GA 20-oxidase genes, as well as control plants,

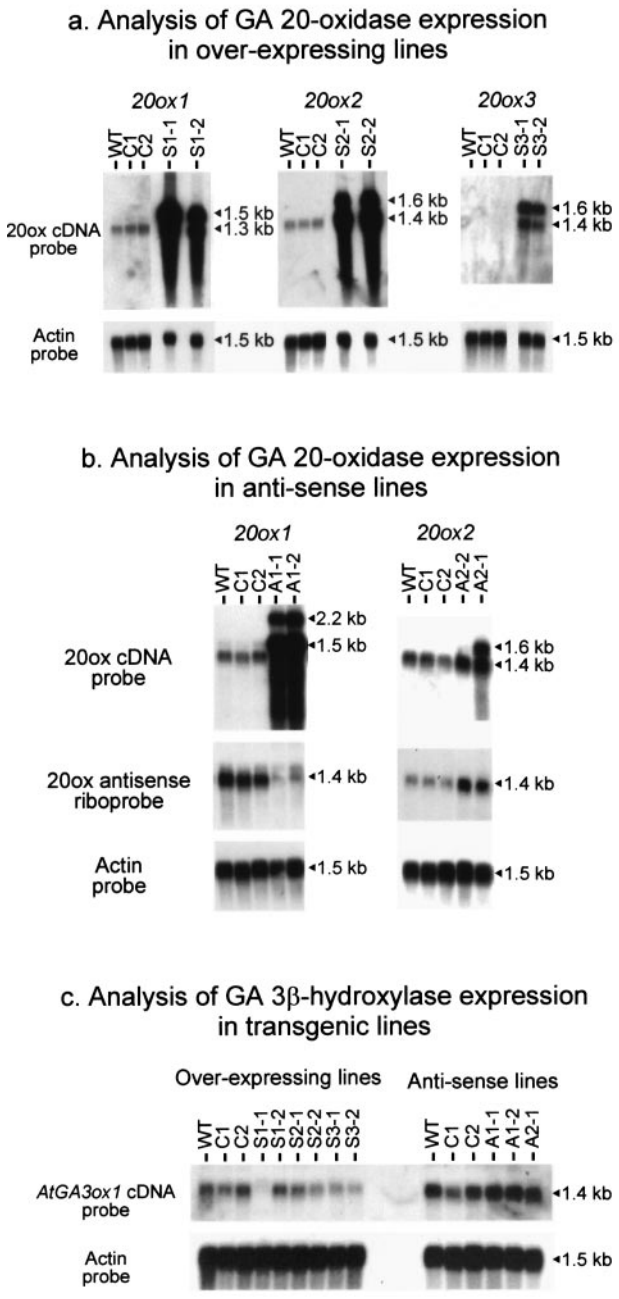

Figure 4. Hybridization of Northern blots showing expression of transgenes and endogenous genes in shoot tips of transgenic *Arabidopsis*.
All over-expressing lines and their controls were grown under long days in glasshouse conditions, while antisense lines and their controls were grown under long days in controlled environment. The actin probe (*Act-2* gene, bp 1447–1832; An *et al.*, 1996) was used as a loading control.
(a) Analysis of GA 20-oxidase gene expression in shoot tips of over-expressing and control lines using GA 20-oxidase cDNA probes.
(b) Analysis of GA 20-oxidase expression in shoot tips of antisense and control lines using GA 20-oxidase cDNA probes and antisense riboprobes.
(c) Analysis of GA 3β-hydroxylase (*AtGA3ox1*) expression in GA 20-oxidase over-expressing and antisense lines.

Table 2. Concentration (ng g^{-1} fresh weight) of GAs in shoot tips of controls and plants over-expressing GA 20-oxidase transgenes grown in the glasshouse under long days

Genotype	GA_4	GA_{34}	GA_1	GA_8	GA_{20}	GA_{19}
Control line C1	2.9	8.7	0.5	0.1	0.3	1.1
Control line C2	2.3	4.9	0.1	0.3	0.4	0.5
AtGA20ox1 line S1–1	3.7	7.1	0.1	1.5	0.7	0.2
AtGA20ox2 line S2–1	2.9	10.4	0.4	1.0	0.7	0.4
AtGA20ox3 line S3–2	3.9	6.4	0.2	1.4	0.6	0.8

Table 3. Concentration (ng g^{-1} fresh weight) of GAs in shoot tips of a control and of plants expressing antisense GA 20-oxidase genes grown under controlled environment conditions in long days

Genotype	GA_4	GA_{34}	GA_1	GA_8	GA_{20}	GA_{19}
Control line C2	12.4	2.2	1.0	0.1	0.6	1.6
AtGA20ox1 line A1–1	3.0	0.8	0.4	0.1	0.2	0.6
AtGA20ox1 line A1–2	8.9	1.6	1.0	0.1	0.2	0.6
AtGA20ox2 line A2–1	5.5	4.4	0.9	0.1	0.3	0.6

were grown in long days in the glasshouse. The levels of several C_{19}-GAs and the C_{20}-GA GA_{19} in the shoot tips were determined by combined gas chromatography–mass spectrometry (GC–MS) (Table 2). The GAs analysed included the biologically active compounds, GA_1 and GA_4, and their respective inactive 2β-hydroxylated metabolites, GA_8 and GA_{34}. In *Arabidopsis*, the major biologically active GA is GA_4 (Talon *et al.*, 1990); this was also evident from our analysis, which showed that the level of GA_4 was much higher than that of GA_1 in the shoot tips. With the exception of GA_8, there was no consistent difference between controls and GA 20-oxidase over-expressing lines S1–1, S2–1 and S3–2 in the levels of the GAs analysed. However, the GA_8 content was elevated approximately 10-fold in the over-expressing lines, which bolted early and produced longer stems. A similar analysis was undertaken of the shoot tips of the two *AtGA20ox1* (A1–1 and A1–2) and single *AtGA20ox2* (A2–1) antisense lines and a control (C2) grown in long days under controlled environment conditions (Table 3). In this case, there was some reduction in the amount of GA_4, GA_{20} and GA_{19} in the antisense lines. It should be noted that the shoot tips of control line C2 contained approximately fivefold more GA_4 when grown in the controlled environment than when grown in the glasshouse, presumably due to differences in light intensity and quality.

The elongating shoot tip was selected for analysis because it is the most rapidly growing tissue and was expected to be the most active in GA production. However, this tissue is quite heterogeneous in terms of tissue types, containing flower buds and flowers at different developmental stages. Thus, variation in GA content between

Table 4. Concentration (ng g^{-1} fresh weight) of GAs in rosettes of controls and plants expressing sense or antisense GA 20-oxidase genes grown under controlled environment conditions in long days

Genotype	GA_4	GA_{34}	GA_{20}	GA_{19}
Control line C1	0.3	0.5	0.2	0.2
AtGA20ox1 line S1–1	0.9	2.0	0.4	nd
AtGA20ox2 line S2–1	0.9	2.5	0.4	0.2
AtGA20ox3 line S3–2	0.7	1.4	0.3	0.3
AtGA20ox1 line A1–1	0.1	0.7	0.2	0.4
AtGA20ox1 line A1–2	0.1	0.4	0.2	0.4
AtGA20ox2 line A2–1	0.1	0.4	0.2	0.4

nd, not determined.

samples, due to differences in tissue distribution, could mask the relatively small differences expected in plants over-expressing the GA 20-oxidase genes. Therefore, in a separate experiment, each line, grown in long days, was harvested before flower initiation and the total rosette analysed for GA content (Table 4). The amounts of GA_4 and GA_{34} were much lower than in the shoot tip, but were clearly elevated in the lines in which the *Arabidopsis* 20-oxidase cDNAs were over-expressed. Gibberellins A_1 and A_8 were present at too low levels to be quantified accurately in this tissue.

Effect of transgenes on GA 3β-hydroxylase gene expression

It has been shown that the GA 20-oxidase genes and the GA 3β-hydroxylase gene *AtGA3ox1* (*GA4*) respond to increased GA concentrations by a reduction in transcript levels that, it is suggested, constitute a biostatic mechanism that limits changes in the levels of the bioactive GAs (Chiang *et al.*, 1995; Phillips *et al.*, 1995). Hence, the effect of increased or decreased levels of GA 20-oxidase on bioactive GA levels in the transgenic plants might be limited by feedback regulation operating through the final enzyme in the biosynthetic pathway, GA 3β-hydroxylase. To investigate this possibility, we examined the transcript levels for *AtGA3ox1* in all our transgenic lines (Figure 4c). In several of the lines over-expressing GA 20-oxidases there was a substantial reduction in *AtGA3ox1* transcripts, particularly in line S1–1. However, some lines, such as S1–2, showed no decrease in *AtGA3ox1* expression. There is no evidence that *AtGA3ox1* is up-regulated by GA deficiency in either of the 20-oxidase antisense lines (A1–1 and A1–2) that show a semi-dwarf habit, suggesting that the *AtGA3ox1* gene may be less sensitive than stem elongation to changes in GA levels.

Discussion

Over-expression of the Arabidopsis *GA 20-oxidase genes increases C_{19}-GA formation resulting in a GA-overproduction phenotype*

Plants that constitutively express the *Arabidopsis* GA 20-oxidase genes behaved like control plants that have been treated with GA. As expected, since they encode functionally similar enzymes (Phillips *et al.*, 1995), over-expression of each of the three genes gave the same result. The GA-overproduction phenotype is apparent throughout development; relative to controls and the Columbia wild-type, the 20-oxidase-over-expressing lines have longer hypocotyls and petioles, larger rosette leaves, accelerated flowering and bolting, and longer stems (Figure 2). This phenotype is consistent with the higher C_{19}-GA content detected in the rosette leaves (Table 4). The major biologically active GA in *Arabidopsis* is GA_4 (Talon *et al.*, 1990), which is elevated two- to threefold in rosettes of the over-expressing lines. However, no consistent differences in the amounts of GA_4 and GA_1 between the lines that over-expressed the *Arabidopsis* GA 20-oxidase cDNAs and controls were apparent in shoot tips of bolting plants. The amounts of these GAs were much higher than those in the rosettes, which may be due partly to the contribution from floral tissues. Variation in the distribution of these tissues between samples could result in large differences in GA content and thereby mask the influence of the introduced enzymes. It is of interest that the level of GA_8 correlated positively with accelerated bolting, being 10-fold higher in shoot tips of these lines than in controls and lines expressing antisense mRNA (Tables 2 and 3). Gibberellin A_8, the inactive metabolite of GA_1, may be turned over more slowly than GA_1 and may, therefore, provide a record of the flux through the pathway to this GA (the early 13-hydroxylation pathway) at a previous stage. There was no equivalent increase in the amount of GA_{34} formed by the non-13-hydroxylation pathway, suggesting a more rapid turnover of GA_{34} than GA_8 or compartmentation in the shoot tip of the pathways to GA_8 and GA_{34}, with the latter unaffected by the action of the transgene.

Stimulation of GA production in vegetative tissues and of growth resulting from increased GA 20-oxidase gene expression suggests that this enzyme plays an important role in both processes. The results obtained indicate that GA concentration is normally limiting for many aspects of *Arabidopsis* development and, therefore, regulation of GA biosynthesis will be an important factor in developmental control. The accumulation of GA_{24} and GA_{19} in wild-type *Arabidopsis* stems (Talon *et al.*, 1990) is consistent with the conversion of these intermediates to GA_9 and GA_{20}, respectively, by GA 20-oxidase being slow, possibly rate-

determining steps in GA biosynthesis. This is supported by the present results which indicate that GA 20-oxidase activity has a relatively large influence on the GA-biosynthetic pathway. Evidence that the enzyme is regulated at the transcript level in *Arabidopsis* by GA action in a type of feedback control (Phillips *et al.*, 1995) and by photoperiod (Xu *et al.*, 1997) also points to an important role for 20-oxidase in determining GA concentrations.

A similar regulatory role has also been proposed for the final enzyme in the biosynthetic pathway, encoded by *AtGA3ox1* (*GA4*), whose transcript level is also feedback-regulated by bioactive GAs (Chiang *et al.*, 1995; Cowling *et al.*, 1998). Some of the transgenic lines over-expressing GA 20-oxidases showed a decrease in *AtGA3ox1* expression, presumably due to feedback from an increase in bioactive GAs, while most lines showed little or no feedback effect. Thus, although all over-expressing lines show an identical advance of flowering time and increase in stem height, feedback control of the *AtGA3ox1* gene is variable. This may indicate that these processes are differentially responsive to changes in bioactive GA levels. Interestingly, the GA 20-oxidase over-expressing lines which have reduced expression of *AtGA3ox1* nevertheless have increased levels of the bioactive GAs and their catabolites, implying that GA 3β-hydroxylase never becomes seriously limiting, a suggestion supported by the lack of a dramatic rise in the GA 3β-hydroxylase substrate GA_{20}. It is clear that claims for feedback mechanisms operating through transcript levels must be supported by evidence of changes in protein levels and enzyme activity.

Expression of antisense GA 20-oxidase mRNA results in semi-dwarfism

In contrast to plants transformed with sense GA 20-oxidase DNA, most of which expressed the transgenes and had an altered phenotype, few plants transformed with antisense DNA expressed the antisense mRNA, despite incorporation of the transgenes. Only those plants in which a high abundance of antisense transcript could be detected were phenotypically different from the wild-type or controls. The reason for the failure to detect expression of the antisense genes is unclear but may be due to premature termination of transcription.

Two lines were obtained in which large amounts of antisense mRNA for the stem-specific GA 20-oxidase gene *AtGA20ox1* were present with a resulting decrease in the content of endogenous transcript to very low levels (Figure 4b). These plants grew as GA-responsive semi-dwarfs in long days (Figure 2d). Compared with controls, hypocotyl and petiole lengths were reduced in the seedlings, rosette leaves were smaller and there was a marked reduction in stem height. Stem height reduction was much more pronounced and flowering was delayed when plants were grown in short days (Figure 2e), possibly as a result of reduced responsiveness to GA in short days (Xu *et al.*, 1997). Flowering in *Arabidopsis* is absolutely dependent on GA in short days, but can occur in long days even with highly attenuated GA concentrations (Wilson *et al.*, 1992). The antisense plants resemble the semi-dwarf mutants *ga4* and *ga5* (Koornneef and van der Veen, 1980), although height reduction in the mutants is more severe. This may be due, at least partly, to the different genetic backgrounds of the mutants, which are in Landsberg *erecta*, and the antisense GA 20-oxidase plants, which are in Columbia.

The *AtGA20ox1* antisense plants contained substantial amounts of C_{19}-GAs, particularly in the shoot tip, despite the very severe reduction in gene expression. Similarly, the *ga5* mutant, in which the GA 20-oxidase protein encoded by *AtGA20ox1* is predicted to be truncated and therefore inactive (Xu *et al.*, 1995), produces C_{19}-GAs, albeit at reduced levels (Talon *et al.*, 1990). Production of C_{19}-GAs in the mutant must therefore result from the action of other GA 20-oxidases, such as that encoded by *AtGA20ox2*, which is expressed in the inflorescence (Phillips *et al.*, 1995). Since flower initiation precedes stem elongation in *Arabidopsis*, C_{19}-GAs produced by *AtGA20ox2* activity may contribute to stem growth throughout bolting. Therefore, stem elongation in *Arabidopsis* is determined by at least two GA 20-oxidase genes.

It proved more difficult to obtain plants with altered phenotype from expression of antisense mRNA for the other two GA 20-oxidase genes (*AtGA20ox2* and *AtGA20ox3*). A primary transformant after introduction of antisense *AtGA20ox3*, the silique-specific gene, was dwarfed only at the young seedling stage, indicating a role for this gene in germination and early seedling development. However, no dwarf seedlings were recovered in the next generation and further work on this line has not been possible. Transgenic line A2–1 expressing antisense *AtGA20ox2* mRNA grew normally in long days, but was slightly reduced in height relative to controls when grown in short days; the difference in height was due to shorter internodes within the inflorescence. The plant resembles the recently described *ga6* mutant which, on the basis of its response to applied GAs, is thought to have defective GA 20-oxidase activity (Sponsel *et al.*, 1997). The possibility that *ga6* is due to a mutation in the *AtGA20ox2* gene is currently being investigated.

The results of our experiments, particularly with antisense *AtGA20ox1*, indicate that it is feasible to reduce GA content and, therefore, modify plant stature by down-regulating GA 20-oxidase gene expression. By expressing very high levels of antisense mRNA, it was possible to over-ride the feedback mechanism by which plants up-regulate GA 20-oxidase transcript levels in response to reduced content of biologically active GAs (Phillips *et al.*, 1995; Xu *et al.*, 1995).

Experimental procedures

Plant materials

All the work described was carried out using *Arabidopsis thaliana* ecotype Columbia. Seeds were sown onto the surface of pre-wetted Fisons F2 potting compost supplemented with Osmocote at 2 g l^{-1} and vermiculite at 20% (w/v). The imbibed seeds were stratified at 4°C for 3 days before transfer to a glasshouse under 16 h supplemented natural light (yielding approximately 200 μmol m^{-2} s^{-1}), 8 h dark. Plants for *Agrobacterium*-mediated transformation were transplanted into 15 cm pots of compost, 10–12 per pot, and grown as described above. For day length studies, stratified seeds in compost were transferred to growth chambers containing a mixture of fluorescent and tungsten lamps yielding 400 μmol m^{-2} s^{-1} light under a 16 h light (23°C), 8 h dark period (18°C; 'long days'), or 10 h light, 14 h dark ('short days'); where appropriate, plants were sprayed weekly with 10 μM GA_3. For analysis of seedling growth, seeds were sown onto 3 mM paper soaked in 0.5 g l^{-1} MES pH 5.8 and 1× Murashige-Skoog media with Gamborg's B5 vitamins (MS medium) ± 10 μM GA_3, stratified as above and grown in a growth room at 25°C under 16 h light, 8 h dark. Plants for GA analysis were grown in long days in seed trays (22 cm × 36 cm) at a density of 40 plants per tray. For analysis of rosettes, plants were grown in controlled environment and harvested 22 days after sowing, when the whole plants above soil level were frozen immediately in liquid nitrogen. For analysis of stems, plants were grown either in controlled environment (control and antisense lines) or in the glasshouse (controls and over-expressing lines) until they had bolted to a height of about 10 cm (approximately 30 days after sowing). The shoot tips (the upper 2 cm of the bolting stem, including flowers and flower buds) and the 8 cm of stem beneath were harvested and frozen immediately in liquid nitrogen.

Plasmid constructs

The three *Arabidopsis* GA 20-oxidase cDNA clones, *AtGA20ox1*, *AtGA20ox2* and *AtGA20ox3* (Phillips *et al.*, 1995), cloned in pBSII/SK-(Stratagene) in the antisense orientation with respect to *Lac*α, were amplified by PCR using specific 5′ primers (*AtGA20ox1*: GAGAATTCAAA*A*TGGCCGTAAAGTTTCG; *AtGA20ox2*: GAGAATTCAGAA*ATG*GCGATACTATGC; *AtGA20ox3*: GAGAATTCAAAA*ATG*GCAACGGAATGC; the *Eco*RI site and start ATG are underlined) together with a 3′ vector primer (SKP: CGCTCTAGAACTAGTGGATC). The amplified fragments were digested with *Eco*RI and inserted into the *Eco*RI site of pCGN1761 (Calgene), between the double CaMV 35S promoter and the tml 3′-end. After identification of constructs with the cDNAs in both sense and antisense orientations by restriction endonuclease digestion, the inserts were sequenced using the T7 dideoxynucleotide chain termination kit (Amersham) with specific primers to confirm the absence of introduced mutations. The sense and antisense expression cassettes of each cDNA were excised from pCGN1761 as *Xba*I fragments and inserted into the *Xba*I site of the binary vector pCIB200 (Ciba Agricultural Biotechnology), which carries the neomycin phosphotransferase gene of Tn5 under the Nos promoter as a selectable marker in plants.

Plant transformation

The binary expression constructs and, as a control, the binary vector pCIB200 itself were introduced into *Agrobacterium tumefaciens* strain GV3101 carrying the pAD1289 plasmid conferring over-expression of *VirG* (Hansen *et al.*, 1994) by electroporation (Shen and Forde, 1989). These were introduced into *Arabidopsis* by the vacuum infiltration method (Bechtold *et al.*, 1993). To identify transgenic plants, seeds from infiltrated plants were grown on MS agar supplemented with kanamycin (50 μg ml^{-1}) for approximately 14 days and resistant plants were transferred to compost. Transgenic plants containing the T-DNA inserted at a single locus were identified by scoring T2 seeds for kanamycin resistance and these plants were propagated by self-fertilization to the T3 generation to isolate homozygous lines for each transgene. T4 plants derived from the homozygous lines were used for phenotypic, biochemical and molecular characterization. The homozygous lines discussed in this paper are designated S*X-Y* or A*X-Y* for the sense or antisense lines, respectively, where *X* is 1, 2 or 3 corresponding to the 20-oxidase cDNA used and *Y* is the isolate number. Transgenic plants containing the binary vector without insert were also generated and used as controls.

Transcript analysis

Poly-A^+ RNA was isolated by oligo-dT cellulose chromatography of total RNA, prepared by tissue extraction in buffer containing SDS and proteinase K followed by serial phenol-chloroform extractions, as described by Bartels and Thompson (1983). RNA size markers were obtained from Promega. Northern blots were pre-

Table 5. Parameters used for quantification of GAs by selected reaction monitoring using [17–$^2H_2$]GAs as internal standards

GA	Collision Energy	Parent ion (*m/z*)	Product ion scan (*m/z*)	Product ion scan (*m/z*) for quantification
GA_1	0.8	506	350–506	448
[17–$^2H_2$]GA_1	0.8	508	350–508	450
GA_4	0.7	284	150–284	224
[17–$^2H_2$]GA_4	0.7	286	150–286	226
GA_8	0.9	594	430–594	448
[17–$^2H_2$]GA_8	0.9	596	430–596	450
GA_{19}	0.8	434	250–434	374
[17–$^2H_2$]GA_{19}	0.8	436	250–436	376
GA_{20}	0.8	418	300–418	375
[17–$^2H_2$]GA_{20}	0.8	420	300–420	377
GA_{34}	0.8	506	350–506	416
[17–$^2H_2$]GA_{34}	0.8	508	350–508	418

pared by electrophoresis of 5 μg poly-A^+ RNA through agarose gels in the presence of formaldehyde (Sambrook *et al.*, 1989), followed by transfer to nitrocellulose as described by Thomas (1980). DNA fragments were labelled with ^{32}P-dCTP by random-primed labelling with Ready-to-Go DNA labelling beads (Pharmacia). Pre-hybridization and hybridization were carried out at 42°C under the conditions described by Phillips and Huttly (1994). Blots were hybridized for 16 h and then washed for 3 × 15 min in 2 × SSC, 0.1% SDS at 42°C and for 3 × 15 min in 0.1 × SSC, 0.1% SDS at 60°C. Antisense RNA probes to GA 20-oxidases were generated from the T3 promoter of pBSII/SK-plasmids using the Riboprobe System (Promega). RNA probes were hybridized as described above at 60°C, and washed for 3 × 10 min in 2 × SSC, 0.1% SDS at 60°C and for 3 × 10 min in 0.1 × SSC, 0.1% SDS at 60°C. The membranes were sealed into polythene bags and exposed to Kodak XAR film at –75°C with intensifying screens.

Gibberellin analysis

Samples (5 g fresh weight) were homogenized in 80% methanol-water (50 ml) containing [17–$^2H_2$]GAs (from Professor L.N. Mander, Australian National University, Canberra, Australia) (3–10 ng, depending on anticipated content of endogenous GAs), and 833 Bq each of the following tritiated GA standards, [1,2–$^3H_2$]GA_1, [1,2–$^3H_2$]-GA_4 (Amersham International plc), [2,3–$^3H_2$]GA_9 (gift from Dr A. Crozier, University of Glasgow, Glasgow, UK), 16,17-dihydro[15,16,17–$^3H_4$]GA_{19} (prepared by catalytic exchange with tritium gas by Amersham International plc) and [1,2,3–$^3H_3$]GA_{20} (1.41 TBq/mmol, from Professor J. MacMillan, University of Bristol, Bristol, UK). The homogenate was stirred overnight at 4°C. After filtration, the residue was re-extracted with methanol (50 ml) for 2 h and re-filtered. The combined methanol extracts were evaporated almost to dryness under reduced pressure at 35°C. The residue was resuspended in water (5 ml), adjusted to pH 8.0 (1 M KOH) and loaded onto a QAE Sephadex A-25 (Pharmacia) anion-exchange column (5 ml bed volume) pre-equilibrated with sodium formate (0.5 M), then washed with formic acid (0.2 M) and water at pH 8. After loading, the column was washed with water at pH 8 (15 ml) and GAs were eluted with 0.2 M formic acid (20 ml) and applied directly to a pre-equilibrated C_{18} Extract-Clean cartridge (500 mg) (Altech Associates). GAs were eluted with methanol (5 ml), which was then evaporated to dryness *in vacuo*. GAs were resolved by reverse phase HPLC and pooled fractions prepared for combined gas chromatography–mass spectrometry as described previously (Croker *et al.*, 1990). Samples were analysed as methyl ester trimethylsilyl ethers using an external source ion trap mass spectrometer (Finnigan GCQ). Samples in *N*-methyl-*N*-trimethylsilyltrifluoracetamide (3 μl) were diluted with 20 μl of CH_2Cl_2 and injected (1 μl) into a fused silica WCOT BPX5 capillary column (25 m × 0.22 mm × 0.25 μm film thickness) (Scientific Glass Engineering) at an oven temperature of 60°C. After 1 min, the splitter (50:1) was opened and the temperature increased at 20°C min^{-1} to 220°C and then at 4°C min^{-1} to 300°C. The He flow was controlled at a constant linear velocity of 40 cms^{-1}. The injector, interface and MS source temperatures were 220, 270 and 200°C, respectively. The mass spectrometer was operated in dual selected reaction monitoring mode. Characteristic parent ions were selected and monitored with an isolation wave form notch of 1 atomic mass unit. Full product ion scans at 0.5 sec $scan^{-1}$ were obtained for all GAs analysed. Details of ions monitored and collision energies are given in Table 5. The concentrations of GAs in the extracts were determined from previously established calibration curves of peak area ratios of the product ion for unlabelled and deuterated GAs plotted against varying molar ratios of the two compounds. The same stock solution of labelled GAs were used for production of the calibration curves.

Acknowledgements

This work was supported by grants from Ciba Agricultural Biotechnology and the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom; IACR also receives grant-aided support from BBSRC. We thank Calgene Inc. for plasmid pCGN1761 containing the CaMV35S promoter cassette.

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