

Cloning and Overproduction of Gibberellin 3-Oxidase in Hybrid Aspen Trees. Effects on Gibberellin Homeostasis and Development¹

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To broaden our understanding of gibberellin (GA) biosynthesis and the mechanism whereby GA homeostasis is maintained in plants, we have investigated the degree to which the enzyme GA 3-oxidase (GA3ox) limits the formation of bioactive GAs in elongating shoots of hybrid aspen (*Populus tremula* × *Populus tremuloides*). We describe the cloning of a hybrid aspen GA3ox and its functional characterization, which confirmed that it has 3 β -hydroxylation activity and more efficiently converts GA₉ to GA₄ than GA₂₀ to GA₁. To complement previous studies, in which transgenic GA 20-oxidase (GA20ox) overexpressers were found to produce 20-fold higher bioactive GA levels and subsequently grew faster than wild-type plants, we overexpressed an Arabidopsis GA3ox in hybrid aspen. The generated GA3ox overexpresser lines had increased 3 β -hydroxylation activity but exhibited no major changes in morphology. The nearly unaltered growth pattern was associated with relatively small changes in GA₁ and GA₄ levels, although tissue-dependent differences were observed. The absence of increases in bioactive GA levels did not appear to be due to feedback or feed-forward regulation of dioxygenase transcripts, according to semiquantitative reverse transcription polymerase chain reaction analysis of *PttGA20ox1*, *PttGA3ox1*, and two putative *PttGA2ox* genes. We conclude that 20-oxidation is the limiting step, rather than 3 β -hydroxylation, in the formation of GA₁ and GA₄ in elongating shoots of hybrid aspen, and that ectopic GA3ox expression alone cannot increase the flux toward bioactive GAs. Finally, several lines of evidence now suggest that GA₄ has a more pivotal role in the tree hybrid aspen than previously believed.

Gibberellins (GAs) form a group of more than 130 tetracyclic diterpenes, some of which are biologically active and act as growth regulators in higher plants. Work on GA-deficient mutants has established that bioactive GAs play an important role in controlling diverse developmental processes such as seed germination, stem elongation, flowering, and fruit ripening (Davies, 1995). The GA biosynthetic pathway has been elucidated and its key components identified (for review, see Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Olszewski et al., 2002). The final steps in the pathway are catalyzed by the soluble 2-oxoglutarate-dependent dioxygenases GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox), and GA 2-oxidase (GA2ox). The pathway branches at GA₁₂, which can be 13-hydroxylated into GA₅₃, marking the starting points for two parallel routes catalyzed by the above dioxygenases: the early nonhydroxylated and the 13-hydroxylated pathways forming the bioactive GAs,

GA₄ and GA₁, respectively. The multifunctional GA20ox removes a carbon by successive oxidation of GA₁₂ to GA₉ and GA₅₃ to GA₂₀. However, the final interconversion into the bioactive GA₄ or GA₁ requires the action of the enzyme GA3ox. The deactivation of the bioactive species is catalyzed by GA2ox, which can also divert GA₉ and GA₂₀ away from the route toward active GAs by forming GA₅₁ and GA₂₉, respectively. The genes encoding the three described enzymes have been cloned in several species and have been found to belong to small multigene families. In Arabidopsis there are at least four genes coding for each enzyme (Hedden et al., 2001).

Maintaining the levels of the bioactive GAs in appropriate temporal and spatial patterns, while controlling the responsiveness of each tissue to GAs, are vital processes for the plant during its growth and development. Recent studies in Arabidopsis, rice (*Oryza sativa*), and barley (*Hordeum vulgare*) have identified several positive and negative regulators of GA signaling pathways, all involved in regulating GA responsiveness during development (for review, see Olszewski et al., 2002; Gomi and Matsuoka, 2003). In addition, a large number of studies have found evidence that GA biosynthesis is finely modulated via regulation of the GA dioxygenases. Work on GA mutants and plants treated with chemical inhibitors of GA biosynthesis has shown that transcript levels of GA20ox and GA3ox increase, while GA2ox transcripts decrease, in response to lowered amounts of GA (Chiang et al., 1995; Phillips et al., 1995; Thomas et al.,

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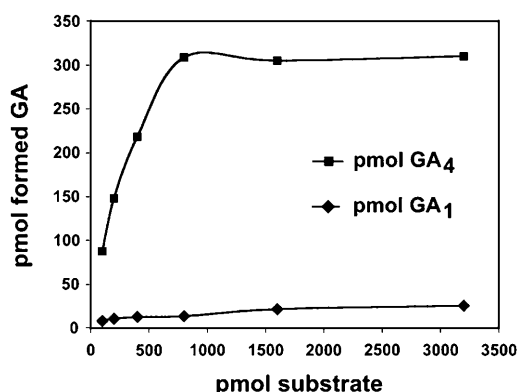


Figure 1. Competitive assay for GA 3 β -hydroxylation with cell lysates from recombinant *E. coli* expressing *PttGA3ox1* in pGEX-4T-2. Cell lysates were incubated with cofactors and a mixture of equal amounts of the substrates GA₉ and GA₂₀ at varying concentrations for 1 h at 20°C, and the corresponding formation of GA₄ and GA₁ was monitored by GC/MS-SRM using deuterated GAs as internal standards.

1999). In contrast, plants treated with bioactive GAs depress *GA20ox* and *GA3ox* transcription, and increase the transcription of *GA2ox* (Phillips et al., 1995; Yamaguchi et al., 1998; Thomas et al., 1999). Furthermore, evidence for interactions between GA signaling and biosynthesis has come from studies of GA signaling mutants. For instance, the *gai* mutant has increased levels of bioactive GAs and *GA20ox* transcript (Peng et al., 1999). As a result of the feedback and feed-forward regulation, governed by the endogenous levels of GA₁ and GA₄, the plant can maintain appropriate levels of GAs throughout its growth and development.

In hybrid aspen (*Populus tremula* $\times$ *Populus tremuloides*), expression of a *GA20ox* gene under the cauliflower mosaic virus (CaMV) 35S promoter led to a 20-fold increase in bioactive GA levels and subsequently increased growth and taller trees (Eriksson et al., 2000). Accelerated growth as a response to ectopic *GA20ox* expression and increased levels of GA growth regulator have also been observed in *Arabidopsis* (Huang et al., 1998; Coles et al., 1999), potato (*Solanum tuberosum*; Carrera et al., 2000), and tobacco (*Nicotiana tabacum*; Vidal et al., 2001), implying that this enzyme has a limiting role in regulating the production of bioactive GAs. In contrast, when the genes coding for *ent*-copalyl diphosphate synthase and *ent*-kaurene synthase, which are involved in early stages of GA biosynthesis, are overexpressed in *Arabidopsis*, no significantly increased levels of bioactive GA are observed (Fleet et al., 2003). This implies that key regulation of the flux through the GA biosynthesis pathway may take place downstream of *ent*-kaurene formation.

To complement the previous transgenic *GA20ox* overexpresser (OE) findings, we wanted to elucidate the role of *GA3ox* in controlling GA homeostasis in *Populus*. In this report, we describe the cloning and

characterization of a functional *GA3ox* isolated from *P. tremula* $\times$ *P. tremuloides* and results from a study in which we ectopically expressed an *Arabidopsis* *GA3ox* in hybrid aspen. Increased levels of *GA3ox* transcript had relatively minor effects on GA₁ and GA₄ homeostasis, although tissue-dependent differences were observed. Our results suggest that GA 20-oxidation is much more important as a rate-limiting step than GA 3 β -hydroxylation in GA-controlled shoot elongation. Finally, several lines of evidence now suggest that GA₄ may have a more central role in the tree hybrid aspen than previously believed.

RESULTS

Isolation and Functional Characterization of a GA 3-Oxidase from Hybrid Aspen

We screened a hybrid aspen cambial cDNA library (Hertzberg and Olsson, 1998) with a *PttGA3ox* PCR probe generated by reverse transcription (RT)-PCR. Out of 1,000,000 plaque-forming units, two cDNA clones were identified in a low stringency screening. Both clones were identified as partial *PttGA3ox* fragments, with identical, overlapping sections. The missing 5' end was subsequently obtained in a RACE experiment. Finally, a full-length cDNA lacking 5'- and 3'-untranslated regions was reconstructed by RT-PCR using primers based on the 5' information and the isolated cambial library clones. This last step provided confirmation that the fragments obtained originated from the same gene. The resulting full-length cDNA was named *PttGA3ox1* (GenBank accession no. AY433958). The cDNA contained an open reading frame of 1,122 bp encoding a putative protein of 374 amino acids. This deduced amino acid sequence showed the highest similarity to a *GA3ox* of *Nicotiana glauca*, *NsGA3ox2*, with which it shared 71% identity (AF494090). The binding sites of Fe²⁺ and 2-oxoglutarate in one member of the 2-oxoglutarate-dependent dioxygenase family have been determined by x-ray crystallography (Valegard et al., 1998), and these conserved sites were also found in the deduced amino acid sequence of *PttGA3ox1*. To

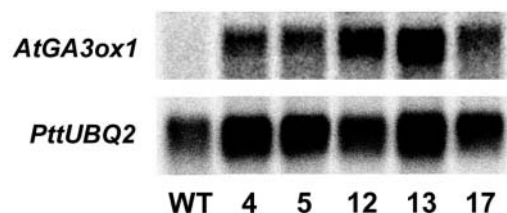


Figure 2. Northern analysis of *AtGA3ox1* expression in young expanding leaf tissue of WT and 35S-*AtGA3ox1* transgenic lines (4, 5, 12, 13, and 17). Twenty micrograms of total RNA was loaded per lane and hybridized under stringent conditions at 65°C to a full-length *AtGA3ox1* probe. A ubiquitin-like EST, *PttUBQ2*, was used as loading control.

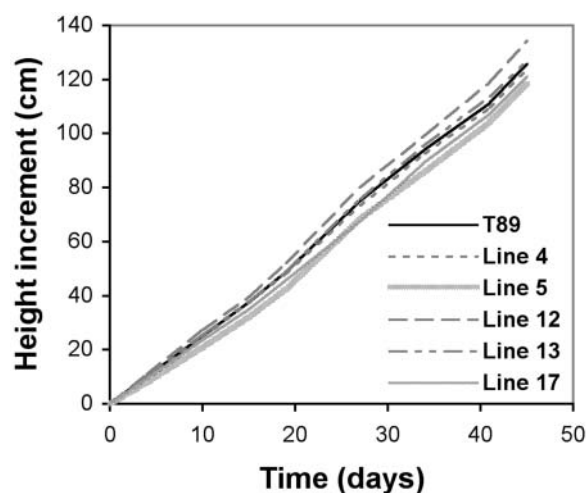


Figure 3. Relatively minor changes observed in height growth increment of 35S-AtGA3ox1 transgenic plants. Actively growing hybrid aspen plants cultivated in the greenhouse under LD conditions were monitored during a 45-d period. The depicted growth pattern is based on averages per line, $n = 9$ except for line 17 ($n = 5$). Student's t test analysis of the significance of differences between each line and WT based on the last data point per genotype showed that line 5 ($P < 0.01$) and line 17 ($P < 0.05$) were shorter and line 12 taller ($P < 0.01$) than WT.

test whether *PttGA3ox1* encoded a functional GA3ox we subcloned the coding region in the expression vector pGEX-4T-2 to produce an inframe fusion protein and expressed this (and an empty vector construct serving as a control) in the *Escherichia coli* strain XL1 Blue. Lysates of bacteria, in which recombinant GA3ox protein production had been induced, and controls were used for functional assays. The recombinant PttGA3ox1 protein converted ^{14}C -labeled GA₂₀ and GA₉ to GA₁ and GA₄, respectively, as identified by gas chromatography-mass spectrometry (GC-MS; data not shown), demonstrating that the clone encoded a functional enzyme. No conversion of substrate took place when the vector control was incubated with substrate. We then used unlabeled substrates at various concentrations in a competitive assay and analyzed products by GC/MS-SRM. The recombinant PttGA3ox1 protein was shown to have a higher affinity for GA₉ than GA₂₀, and formed GA₄ more efficiently than GA₁ (Fig. 1).

Southern blot analysis using the full-length *PttGA3ox1* sequence as a probe suggested the occurrence of at least three GA3ox genes in *Populus* (data not shown). The existence of four *Populus* GA3ox genes was confirmed by BLAST searches of *PttGA3ox1* in the ongoing *Populus trichocarpa* genome sequencing program (<http://genome.jgi-psf.org/poplar0/poplar0.home.html>; data not shown). In comparison, GA3ox in *Arabidopsis* is encoded by four genes, and their functions have been confirmed by heterologous expression in *E. coli* (Hedden et al., 2001).

Generation of Overexpression Lines

Transgenic hybrid aspen plants expressing the *Arabidopsis* GA4 gene (*AtGA3ox1*) under control of the CaMV 35S promoter were obtained by *Agrobacterium tumefaciens*-mediated transformation. Growth traits of 12 of the resulting transgenic lines and wild type (WT) were characterized. The results were used to select five representative lines (lines 4, 5, 12, 13, and 17) for further studies, after confirming that the transgenic plants were OEs by RNA blot analysis. The selected lines showed high levels of *AtGA3ox1* expression, with line 12 having the highest and line 5 the lowest levels (Fig. 2). Southern blot analysis of genomic DNA from the selected lines confirmed that they were independent of each other (data not shown). It also revealed that lines 12 and 13 contained one insert and the other lines two or more inserts.

No Dramatic Alterations in *AtGA3ox* OE Morphology

Anticipating that they would display changes in GA-related traits, we characterized the transgenic lines in terms of height increment, final internode lengths, and number of internodes formed. The total growth increment of the transgenic GA3ox OE lines was similar to WT during the period plant growth was monitored (Fig. 3). Line 12 had the highest overall total height growth, line 5 the lowest, while WT and the other lines were intermediate. Thus, ectopic expression of GA3ox resulted in lines that were slightly taller or shorter than WT. All transgenic lines formed fewer internodes than WT (Table I), with line 12 forming the least. The increase in total height increment in line 12, despite forming fewer internodes, was due to its internodes being significantly (approximately 30%) longer than WT (Table I). The results of the growth and developmental studies were consistent with data from the initial growth characterization of the 12 lines (data not shown).

Table I. Comparison of internode formation between WT and *AtGA3ox1* OEs

	Internode Length	Number of Internodes Formed
	cm	
WT	2.8 ± 0.036	45 ± 0.75
Line 4	3.0 ± 0.033*	41 ± 0.47*
Line 5	2.9 ± 0.040	41 ± 0.65*
Line 12	3.7 ± 0.045*	36 ± 0.84*
Line 13	2.9 ± 0.048	43 ± 0.58
Line 17	2.9 ± 0.018	42 ± 0.49*

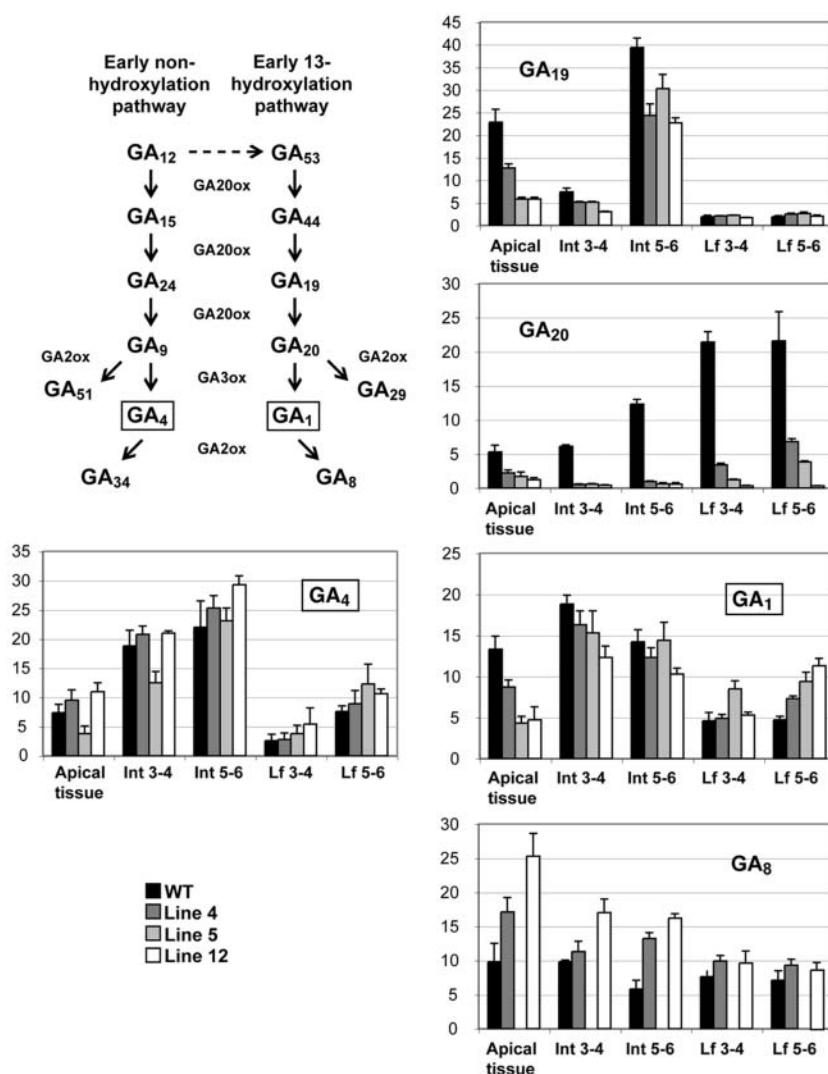
Effects of Altered GA Biosynthesis on GA Levels and Expression of *PttGA20ox1*, *PttGA3ox1*, and Two Putative *PttGA2ox* Genes

The substrate of GA3ox, GA₂₀, was dramatically reduced in all lines and tissues analyzed, providing strong evidence of successful overexpression at the protein level (Fig. 4). In accordance with this finding, in vivo metabolic studies of ¹⁴C-labeled GA₂₀ confirmed that GA3ox enzyme activity was increased in transgenic plants (data not shown). Unexpectedly, however, no marked increase in bioactive GAs was observed. The bioactive GA₁ and GA₄ levels remained unchanged or increased in leaf tissue compared to WT, and in apical and internode tissues the GA₁ levels generally decreased while the GA₄ levels slightly increased in lines 4 and 12. In contrast, the levels of the deactivated GA₈ increased in apical and internode tissues, while remaining largely unaffected in leaves.

To elucidate whether the transcriptional regulation of GA biosynthesis had been altered in OEs, thus limiting the formation of bioactive GAs, we investi-

gated the expression of genes encoding three key enzymes in GA biosynthesis (*PttGA20ox1*, the endogenous *PttGA3ox1*, and two putative *PttGA2ox* genes) by semiquantitative RT-PCR. The expression of the Arabidopsis homologs has been demonstrated to be feedback and feed-forward regulated by bioactive GAs, respectively (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995; Thomas et al., 1999) and previously both *PttGA20ox1* and *PttGA3ox1* have been shown to be feedback regulated in hybrid aspen (Eriksson and Moritz, 2002; M. Chono, unpublished data). We found that *PttGA20ox1* and *PttGA3ox1* had not been down-regulated in the OE lines; instead, they had remained unchanged or even become up-regulated as in young elongating stem tissue (Fig. 5). Moreover, the putative GA2ox exhibited distinct expression patterns; *PttGA2ox1* was mainly expressed in apical tissue, while *PttGA2ox2* was mainly expressed in internode tissue, but neither of the genes was up-regulated due to the ectopic GA3ox expression (Fig. 5; data not shown). Therefore the lack of a large

Figure 4. GA content of apical, internode, and leaf tissue in 35S-AtGA3ox1 transgenic plants and WT. Tissue from nine individuals was pooled per genotype and tissue type. GAs from 200 mg fresh weight of pooled tissue were purified and analyzed by GC/MS-SRM using ²H₂-GAs as internal standards. Data presented are the means of three technical replicates of pooled sample ± SD. GA₈ levels in line 5 were not measured. Boxed GAs depict bioactive GAs.



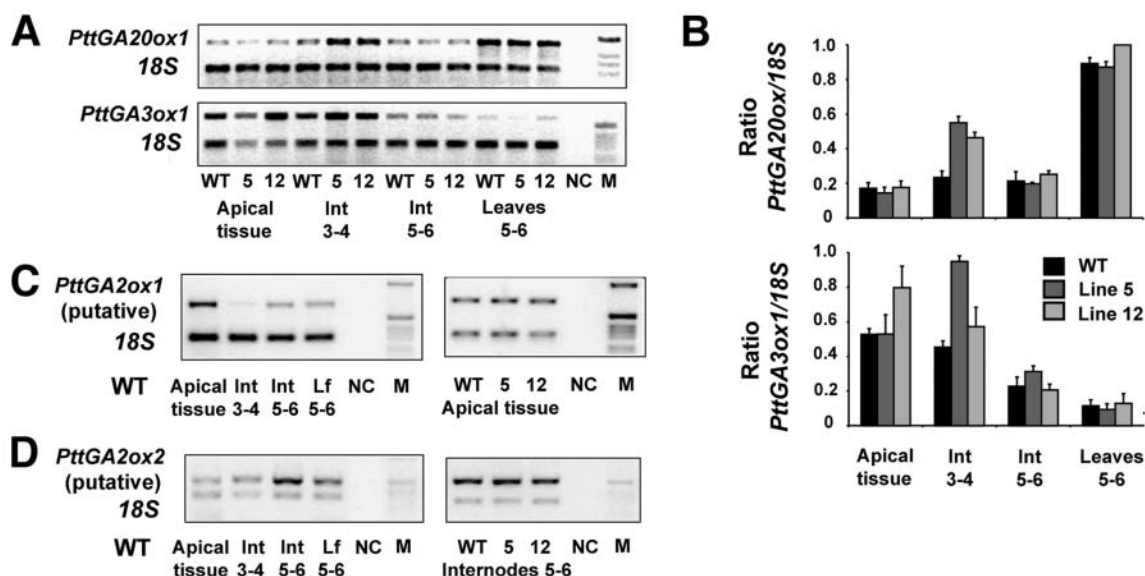


Figure 5. GA dioxygenase expression levels, as determined by semiquantitative RT-PCR in various tissues of WT and the 35S-AtGA3ox lines 5 and 12. Different gene-specific and intron-spanning primer pairs were used together with a primer pair for the internal standard 18S. One cDNA source was used per sample that originated from pooled plant tissues (nine plants per genotype). A, The expression of *PttGA20ox1* and *PttGA3ox1* in various tissues of WT and line 5 and 12. B, Quantification results of two GA genes were normalized to the internal standard 18S. The average of 3 to 4 independent RT-PCRs were calculated and results plotted; bars depict se. C, The *PttGA2ox1* expression was analyzed first in WT and then compared to line 5 and 12 in apical tissue. D, *PttGA2ox2* transcript levels were determined in WT and compared to line 5 and 12 in young expanding internodes. Int, internodes; NC, negative control—no cDNA added to the PCR mixture; M, DNA size marker.

increase in bioactive GAs in the transgenic lines was not caused by any major changes in the expression of the examined GA dioxygenases. More likely, there is a limiting activity of GA20ox in supplying GA₉ and GA₂₀ as substrates for GA3ox. As a consequence, ectopic GA3ox will quickly deplete the limiting substrate pool without largely affecting the bioactive GA levels.

GA₄ Levels, But Not GA₁ Levels, in Expanding Internodes Are Correlated with Final Internode Lengths

The morphological data from our growth characterization had shown that line 12 developed 30% longer internodes than WT, and the GA analysis had revealed slightly elevated levels of GA₄ in line 12 internodes (Table I; Fig. 4). Therefore, an additional experiment was conducted in which line 12 was grown together with WT to investigate the influence of GA₁ and GA₄ on final internode length. For this purpose, actively elongating internodes representing stages at which they were 0% to 20%, 20% to 40%, and 40% to 80% of their final length were harvested from five plants per genotype (Table III), and treated as replicates in a further analysis of GAs. Compared to the previous experiment (Fig. 4), the GA levels were much higher, especially for GA₄ (Table II). This could be explained by changes in the growth conditions, which increased growth rates considerably (data not shown), in addition to the use of a new, improved GA extraction

method, which extracted some GAs, such as GA₄, more efficiently from different tissues of *Populus* (Fig. 4; Table II). Thus it seems that the initial GA analysis (Fig. 4) underestimated the levels of GA₄.

A number of different multivariate statistical tools can be used for displaying data that contain a large number of variables. In Principal Component Analysis (PCA; Wold et al., 1987) the aim is to extract one or more principal components or latent variables to describe the majority of variance in a data set. Although the dataset acquired from the GA analysis does not fulfill the general multivariate requirement that the number of variables should be much larger than the number of samples, PCA can still provide useful visual displays of the data. The PCA-score plot using all GA values as X-matrix shows that the different classes of samples, i.e. the three developmental stages of internode elongation and the two genotypes, are clearly distinct (Fig. 6A). This is because the GA levels differ both between lines and between internodes in various stages of elongation. Partial Least Square to Latent Structures (PLS) is a supervised method in which any underlying covariation between an X-matrix, here GA-levels, and a Y-matrix, here internode length, can be found (Wold et al., 2001). We found a correlation between GA₄ levels and internode length for both WT (Fig. 6B) and line 12 (data not shown). The absence of a similar correlation for the bioactive GA₁ leads us to suggest that the absolute levels of GA₄ in the earlier stages of internode elongation determine the final internode length. The longer internodes of line 12

Table II. GA contents in elongating internodes of WT hybrid aspen and transgenic 35S-AtGA3ox1 in a complementary experiment

GA contents of internodes in developmental stages at which they had reached (a) 20% to 40%, (b) 40% to 60%, and (c) 60% to 80% of their final length are shown (pg mg⁻¹ fresh weight).

Genotype	GA ₅₃	GA ₁₉	GA ₂₀	GA ₁	GA ₈	GA ₉	GA ₄	GA ₃₄
WT-a	24.3 ± 7.0 ^a	29.8 ± 7.0	26.8 ± 7.3	22.7 ± 4.3	138.0 ± 14.9	18.0 ± 3.6	199.8 ± 40.7	119.7 ± 24.8
WT-b	1.2 ± 0.2	6.1 ± 0.8	31.5 ± 3.1	14.6 ± 2.7	76.0 ± 11.1	11.7 ± 0.9	14.0 ± 3.6	39.3 ± 1.5
WT-c	3.1 ± 1.2	15.1 ± 4.0	34.0 ± 2.4	30.0 ± 5.2	40.3 ± 4.4	11.2 ± 1.1	7.7 ± 3.4	15.4 ± 1.1
Line 12-a	31.3 ± 19.0	15.7 ± 7.7	nd ^b	19.8 ± 10.7	212.7 ± 98.5	nd	571.5 ± 237.6	65.3 ± 33.6
Line 12-b	0.7 ± 0.1	1.3 ± 0.1	nd	3.4 ± 0.7	49.6 ± 5.2	nd	62.9 ± 6.2	33.9 ± 1.6
Line 12-c	0.9 ± 0.2	4.2 ± 0.8	nd	21.1 ± 2.6	35.0 ± 2.2	nd	36.0 ± 7.4	18.7 ± 2.3

^aValues represent the average of five biological replicates ± SE. ^bnd, Not detectable.

compared to WT can thus be explained by its consistently higher GA₄ levels (Table II).

As our results suggested that GA₄ levels determine the final length of internodes (Fig. 6B), we performed a sensitivity assay to determine which GA has the strongest effect on *P. tremula* hypocotyl elongation. There was a significant difference in the response to GA₄ as compared to GA₁ in our assay. However, the difference was only subtle, providing relatively weak support for a higher responsiveness to GA₄ (Fig. 7). As a consequence, it is more likely that the increased internode elongation observed in line 12 is due to the higher concentration of GA₄ (Table II).

DISCUSSION

It is well established that GA 3-oxidase catalyzes the conversion of inactive GA species into GAs with biological activity and that it is subject to strict developmental controls in the life cycle of a plant (Lester et al., 1997; Martin et al., 1997; Williams et al., 1998; Itoh et al., 1999; Yamaguchi et al., 2001). As well as cloning a *P. tremula* × *P. tremuloides* GA3ox, in this study we investigated transgenic hybrid aspen expressing the *AtGA3ox1* under the CaMV 35S promoter, in order to broaden our understanding of the regulation of GA biosynthesis. Because GA 3-oxidase catalyzes the last step in the formation of bioactive GAs, it was tempting to believe that overexpression of the enzyme may increase the flux of GA precursors to bioactive GAs.

We identified a GA3ox from hybrid aspen that possessed the ability to convert GA₉ and GA₂₀ into the bioactive GA₄ and GA₁, respectively, demonstrating

that we had cloned a functional GA3ox. Furthermore, the GA₄-producing activity of PttGA3ox1 was higher than its GA₁-producing activity, suggesting that it may make a more important contribution to the early nonhydroxylated pathway than the early 13-hydroxylation pathway. Data on the expression of *PttGA3ox1* during early seedling development in *P. tremula* shows that its expression is 40% inhibited, via feedback regulation, by the bioactive GA₄ at 36 h following germination, according to semiquantitative RT-PCR analysis (M. Chono, unpublished data). The transcript profile of *PttGA3ox1* has now been extended to apical tissue and young expanding internodes, sites in which GA3ox expression and GA-induced stem elongation are known to occur (Itoh et al., 1999).

To elucidate the way in which GA homeostasis is maintained and the consequences of altering GA levels in planta, we overexpressed the Arabidopsis GA4 gene *AtGA3ox1* in hybrid aspen. The total growth increment remained relatively stable in relation to WT, but the GA3ox OEs developed fewer internodes and the internode length remained the same or increased. The substrate for GA3ox GA₂₀ was dramatically reduced in all the transgenes and tissues (Fig. 5). Nevertheless, the levels of the bioactive GA₁ and GA₄ did not increase correspondingly, although some tissue-dependent changes occurred.

The transcriptional regulation of the GA biosynthesis is well documented: treatment with bioactive GAs triggers feedback regulation of *GA20ox* and *GA3ox*, and feed-forward regulation of *GA20ox* transcription, enabling GA homeostasis to be maintained (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995; Thomas et al., 1999). It could be argued that the absence of an increase in bioactive GA formation may be due to the

Table III. Internode lengths of WT and two selected transgenic 35S-AtGA3ox1 lines in three developmental stages of elongation

Data are means from five biological replicates, in mm, ± SE.

Plant Line	Internode A 20%–40%	Internode B 40%–60%	Internode C 60%–80%	Final Internode Length 100%
WT	2.0 ± 0	11.2 ± 1.1	18.4 ± 0.5	32 ± 1.2
Line 5	2.0 ± 0	8.4 ± 0.6	18.6 ± 1.4	29 ± 0.9
Line 12	2.5 ± 0.2	12.4 ± 0.5	22.2 ± 0.7	39 ± 0.9

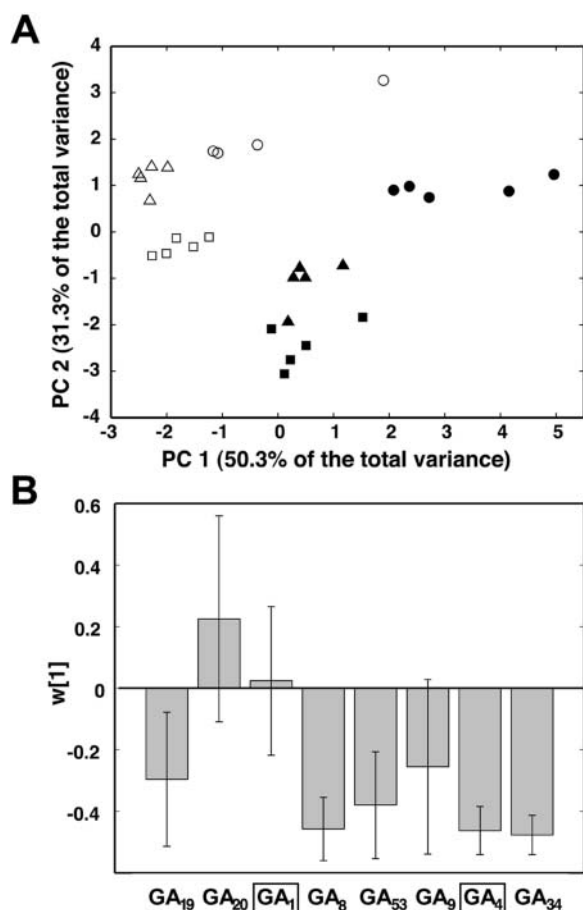


Figure 6. A, PCA-score plot from the analysis of GAs in five WT and five line 12 plants. All variables were log-transformed, centered, and scaled to unit variance. Each point in the plot corresponds to a separate sample type, i.e. internode tissue of different developmental stages. Internode A, circles; internode B, triangles; and internode C, boxes. Line 12 is represented by white symbols, and WT is depicted in black. A clear separation can be observed between all samples, suggesting developmental regulation of GA levels. B, PLS-loading plots from the first loading vector (w) in the analysis of GA levels and internode length from internodes at different developmental stages of five WT plants. The height of the bars shows the relative correlation between GAs and internode length. If the confidence interval (calculated with jack-knifing; Efron, 1986; Martens and Martens, 2000) does not include 0, a variable is considered significant. Bioactive GAs are boxed, and the plot shows that GA₄ levels, and not GA₁ levels, are correlated with internode length.

shortage of GA3ox substrate and/or increased deactivation activity. Scarcity of the substrates GA₂₀ and GA₉ may, in turn, be due to inherent limitations on the activity of GA20ox and/or to down-regulation of GA20ox in the transgenics. To seek evidence for alterations in dioxygenase gene regulation, we first determined the transcript levels of *PttGA20ox1* and the endogenous *PttGA3ox1* in lines 5, 12, and WT. The regulation of both genes was similar in that the transcript levels remained unchanged or increased compared to WT (Fig. 5). In *Arabidopsis*, GA20ox and

GA3ox are both encoded by at least four genes (Hedden et al., 2001), so the possibility that the expression of other gene family members is negatively affected in the transgenic lines cannot be excluded. Because both *PttGA20ox1* and *PttGA3ox1* have been shown to be feedback regulated by bioactive GAs (Eriksson and Moritz, 2002; M. Chono, unpublished data), it is unlikely that only other family members would respond to altered GA levels. Instead, we conclude that GA20ox and GA3ox are not down-regulated. Similarly, semiquantitative RT-PCR of two putative *PttGA20ox* genes revealed unaltered transcript profiles in the OEs suggesting that the deactivation of GA₁ and GA₄ had not been changed. It must be emphasized, however, that changes at the protein or activity levels of the gene family members of GA20ox or GA2ox may also contribute to an altered GA biosynthesis. Without conclusive evidence of increased feedback effects of GA20ox and GA3ox, or feed-forward regulation of GA2ox in the OEs, an alternative explanation for the lack of elevated bioactive GAs must be sought. Unlike the relatively minor phenotype of 35S-GA3ox plants, ectopic GA20ox expression in hybrid aspen elevates the bioactive GA levels 20-fold, leading to highly increased internode elongation (Eriksson et al., 2000). Therefore, we suggest that GA 20-oxidation, relative to GA 3 β -hydroxylation, acts as a limiting step in GA biosynthesis controlling shoot elongation. As a consequence, ectopic GA3ox will quickly deplete the limiting substrate pool, explaining the observed GA profile in 35S-GA3ox plants. In accordance with our findings, a recent study has shown that overexpression of *ent-copalyl diphosphate synthase* and *ent-kaurene synthase*, which catalyze the first two GA-committed steps, does not result in increased levels of bioactive GAs either (Fleet et al., 2003).

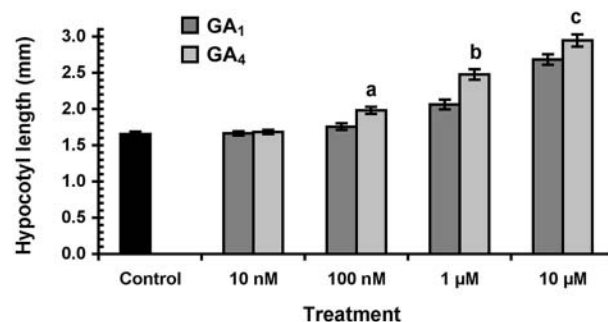


Figure 7. Sensitivity of *P. tremula* seedlings to GA₄ and GA₁. Hypocotyl length of 2.5-d-old seedlings are presented as an average of 40 seedlings $\pm$ SE, except for the 10- μ M treatment ($30 \pm$ SE). Letters indicate statistically significant differences, according to Student's *t* test, in sensitivity to GA₄ as compared to GA₁; a, $P < 0.001$; b, $P < 0.0001$; and c, $P < 0.01$. At GA₄ concentrations greater than 100 nM and GA₁ concentrations higher than 1 μ M the seedlings grew significantly taller than controls, $P < 0.00001$. The experiment was repeated once with *P. tremuloides* seedlings with very similar results.

An interesting observation was that line 12 developed 30% longer internodes, causing this line to grow more quickly than WT, although it formed fewer internodes. In order to study if GAs (and if so, which ones) determine final internode length, we sampled internodes from line 12 and WT in three representative stages of internode elongation and determined the levels of GAs in the individual internodes. The PCA-score plot (Fig. 6A) showed that GA levels of each class of internode developmental stage was clearly separated from the others, suggesting that the GA levels are not only different between WT and line 12, but they are also developmentally regulated along the elongating shoot. Furthermore, the results from the PLS analysis showed that GA₄ levels, but not GA₁ levels, are correlated with internode length (i.e. developmental stage; Fig. 6B). As GAs are considered to act in the subapical parts of the elongating shoot (Olsen et al., 1995; Vogler et al., 2003) it is possible that GA₄ is the main bioactive GA involved in *Populus* internode elongation and ultimately determines their final length, explaining both the 30% longer internodes in line 12 and the correlation between GA₄ and developmental stage.

The early nonhydroxylation pathway, in which GA₄ is produced, has previously been suggested to be less active in hybrid aspen based on feeding studies to shoot cuttings using radioactively labeled GA₁₂ (Eriksson and Moritz, 2002). However, recombinant PttGA20ox1 was shown to be capable of metabolizing both GA₁₂ and GA₅₃ and therefore able to accept substrates of both pathways. In our study, GA₄ levels were found to be higher than GA₁ levels, why the focus turned to GA₄ as the main bioactive GA in hybrid aspen. We determined the relative sensitivity of *P. tremula* seedlings to these GAs and found that they were slightly more responsive to GA₄ (Fig. 7). It is widely accepted that GA₄ is the bioactive GA in *Arabidopsis*, since it is more sensitive to, and has higher concentrations of, GA₄ than GA₁ (Xu et al., 1997; Cowling et al., 1998). Although the response to GA₄ in *Populus* is only marginally higher, the results from the functional PttGA3ox1 assay, which revealed a higher formation rate of GA₄ than GA₁, the higher concentration of GA₄, and the correlation between GA₄ and final internode length suggest that GA₄ plays a pivotal role in hybrid aspen growth and development.

Finally, one of the phenotypic changes observed in the transgenic lines was the lower number of leaves formed, especially in the most strongly divergent transgenic line. The process that governs the rate of leaf formation is initiated in the peripheral zone of the shoot apical meristem and is measured in plastochrons. It is separate from the subsequent cell divisions and cell elongation in the subapical region, which dictate final internode length (Steeves and Sussex, 1989). Further plastochron investigations are now required which can detect regional GA differences in various apical regions, for instance the

peripheral zone of the apex where the leaf primordia are established, but at present we are unable to achieve such analytical resolution.

In conclusion, our study has shown that GA 3 β -hydroxylation is much less important as a rate-limiting step than GA 20-oxidation in the formation of the GAs that control shoot elongation in hybrid aspen. In addition, by using the most strongly divergent transgenic line and WT we showed that the GA₄ levels are correlated with final internode lengths, explaining the increased stem elongation of this OE line. The role of GA₄ as the predominant bioactive GA was further supported by its higher concentrations as compared to GA₁.

MATERIAL AND METHODS

Cloning of *PttGA3ox1*

Total RNA was isolated from growing apical buds of hybrid aspen, using the cetyl-trimethyl-ammonium bromide (CTAB) method (Chang et al., 1993). Poly(A⁺)RNA was then isolated using Dynabeads Oligo(dT)₂₅ resin as described by the manufacturer (Dynal, Oslo, Norway), and first-strand cDNA was synthesized using the first strand cDNA synthesis kit according to the manufacturer's protocol (Amersham-Pharmacia Biotech, Uppsala). A PCR probe was generated using this cDNA as template with degenerate primers based on conserved regions of GA3ox sequences. The 5' primer was 5'-ATGTGGTMMNGARGGNTTYAC-3' and the 3' primer was 5'-GTRTGIG-SIGCNARNCCCAT-3'. A band of expected size was purified by gel electrophoresis, subcloned, and sequenced. The 0.3-kb insert was radiolabeled and used to probe a hybrid aspen λ GT11 cambial cDNA library (Hertzberg and Olsson, 1998) using standard techniques (Sambrook et al., 1989). From the 1,000,000 plaque-forming units that were screened at low stringency, two partial cDNA clones were identified, subcloned, and sequenced. The missing 5' end was obtained by RACE on a Marathon (CLONTECH Laboratories, Palo Alto, CA) double-stranded cDNA library originating from the cDNA described above, using the gene specific primer 5'-TGGTGAATCCCTCT-GACCACATGAGC-3' and adapter primer 1 supplied by the manufacturers, using their protocol. Finally, the complete cDNA was reconstructed in a PCR experiment with the 5' primer including an *Eco*RI site, 5'-CCCTCCAGTAA-GAATTCCTCATGCCTTCAAG-3' and the 3' primer including an *Xho*I site, 5'-GTGATCACTCGAGTCCTAACCAACTTTTACAC-3'. A Marathon (CLONTECH) cDNA library of growing apical buds was used as a template in the PCR, using the Advantage PCR system (CLONTECH). The product of the expected size was purified by gel electrophoresis, subcloned, sequenced, and the full-length clone was named *PttGA3ox1* (GenBank accession no. AY433958).

Heterologous Expression and Enzyme Assays

The entire coding region of *PttGA3ox1* was subcloned in frame into the expression vector pGEX-4T-2 (Amersham-Pharmacia Biotech), and both this construct and empty vector controls were subsequently transformed into the *Escherichia coli* strain XL1 Blue. Heterologous expression was induced, and lysates were prepared as described in Martin et al. (1997). The presence of induced protein in the crude extract was confirmed by SDS-PAGE analysis. The enzyme assay mixtures, consisting of 0.1 M Tris-HCl, 4 mM 2-oxoglutarate, 4 mM ascorbate, 4 mM dithiothreitol, and 0.5 mM FeSO₄ added to the lysate (10 μ L) in a final volume of 100 μ L, were incubated for 1 h with gibberellin substrate at 20°C. The following GAs (purchased from Professor L. Mander, Australian National University, Canberra, Australia) were used as substrates: ¹⁴C₂-17,17-GA₉ (1,900 Bq mol⁻¹), ¹⁴C₂-17,17-GA₂₀ (1,900 Bq mol⁻¹), ¹⁴C₂-17,17-GA₁₉ (1,900 Bq mol⁻¹), ¹⁴C₂-17,17-GA₁ (1,370 Bq mol⁻¹), ¹⁴C₂-17,17-GA₄ (1,370 Bq mol⁻¹), GA₉, and GA₂₀.

Plant Transformation and Growth Conditions

cDNA corresponding to the *Arabidopsis* GA4 locus, *AtGA3ox1* (supplied by Professor Howard Goodman, Harvard Medical School, Boston), was

ligated in sense orientation into the *Bam*HI cloning site of the binary vector pPCV702.kana (Walden et al., 1990) under the control of the CaMV 35S promoter. The construct was transformed into hybrid aspen, *Populus tremula* × *Populus tremuloides* Michx. clone T89, and plants were regenerated essentially as previously described (Nilsson et al., 1992). Following an initial characterization of 12 transgenic lines, five representative lines were chosen for further studies. WT control plants did not receive any plant vector but were subjected to simultaneous vegetative propagation in tissue culture as transgenes. Once the plantlets had developed sufficient roots, they were potted in a fertilized peat:perlite mixture (5:1) and grown in a growth chamber under long day (LD) conditions described elsewhere (Eriksson et al., 2000) for 34 d, when the growth characterization began. More detailed analysis of three different stages of internode elongation in WT and line 12 was carried out under greenhouse LD conditions, with an 18/6-h photoperiod, 60% humidity, 20°C, during January and February with supplementary lighting (Osram Powerstar HQI-BT 400 W/D, Osram, Germany) switched on and off when incoming light fell below and increased above 20 W/m², respectively, during the photoperiod. In both growth studies, plants were watered daily and fertilized with a complete nutrient solution (SuperbaS, Supra Hydro, Landskrona, Sweden) once a week.

RNA Blot Analysis

Total RNA was extracted from young leaf tissues of transgenic lines and WT plants. The RNA was isolated, prepared for gel blot analysis, and hybridized. The relative RNA amounts were then quantified as previously described (Eriksson et al., 2000). The full-length *AtGA3ox1* and a hybrid aspen ubiquitin-like expressed sequence tag (EST; A1165341) were used as probes under high stringency conditions at 65°C (last wash 0.1 × sodium chloride/sodium phosphate/EDTA and 0.1% SDS) to analyze the ectopic *GA3ox* expression and reference gene expression patterns, respectively.

Growth Measurements, Sampling, and Statistical Analysis

Thirty-five days after potting, the plants were marked at an actively growing internode, approximately 10 cm above the root-stem junction. This was used as a reference point for measuring internode formation and height increment. The first internode was defined as the first one below a leaf at least 1 cm long, and the number of internodes was counted between the first internode and the reference point. Measurements were made approximately every 5 d until the plants reached the age of 80 d. At this point, apical tissues were sampled by dissecting out as many leaf primordia as possible, internodes 3 to 4, internodes 5 to 6, and the corresponding leaves of these internodes. Nine individual plants per genotype were analyzed and subsequently harvested, except for five plants of line 17. After sampling, the plant material was frozen immediately in liquid nitrogen. Tissues from the different individual plants of each line were pooled, ground in liquid nitrogen, and divided for GA analysis and RT-PCR analysis. For the complementary study of WT and line 12 internode elongation, the first 20 internodes were individually measured to establish the final internode length per genotype and plant. Actively elongating internodes representing stages at which they had reached 0% to 20%, 20% to 40%, and 40% to 80% of their final length were harvested from five plants per genotype, and internodes were sampled and processed for GA analysis as separate biological samples. The dataset was analyzed by multivariate statistical tools using SIMCA-P+ 10.0.4.0 software (Umetrics, Umeå, Sweden).

RT-PCR Analysis

First-strand cDNA was synthesized using the first strand cDNA synthesis kit according to the manufacturer's protocol (Amersham-Pharmacia Biotech) from total RNA isolated using the CTAB method (Chang et al., 1993) from the tissues described in "Growth Measurements, Sampling, and Statistical Analysis" (see above). Gene-specific and intron-spanning primer pairs, capable of amplifying a 0.47-kb 3' end fragment of *PtGA2ox1* and a 0.60-kb 3' end fragment of *PtGA3ox1*, respectively, were GA20THR Fw (5'-TTAGGCACCGTCCTCATTTGT-3'); GA20THR.Rev (5'-AATAGCAGCCCCCAAGTGCAT-3') and 3bHf11 (5'-AAGCTCATGTGGTCAGAGGG-ATTC-3'); and CN10-1ny (5'-GTGATTGGGCGATAGAGAGAGG-3'). Primers to amplify GA2ox were designed based on sequences of two putative GA2ox genes generated from a *Populus* EST sequencing effort: *PtGA2ox1*

(GenBank accession no. BU835271) and *PtGA2ox2* (GenBank accession no. BU877509). Gene-specific and intron-spanning primer pairs, capable of amplifying a 0.73-kb *PtGA2ox1* fragment and a 0.58-kb *PtGA2ox2* fragment, respectively, were T071g08For (5'-ACTTCTTTGCCAAAACATTTCGATGA-3'); T071g08Rev (5'-AGGCTTGATGGGTGAACCATTTCT-3') and V035d04For (5'-CAGCTGTGAAGAAAATGGCA-3'); and V035d04Rev (5'-ATCAGCC-AATCTGGAGCTGT-3'). The RT-PCR was performed with a gene-specific primer pair and an 18S primer pair as an internal control (QuantumRNA 18S internal standards, Ambion, Austin, TX) according to the manufacturer's instructions. PCR products were analyzed on a 1.5% (w/v) agarose gel containing ethidium bromide and the signal intensities were determined with a Gel Doc 1000 DNA Gel Analysis and Documentation System and Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). Each experiment was repeated at least three times using the same cDNA source, originating from pooled material of five to nine plants per genotype. For the *GA2ox* and *GA3ox* reactions the average mean and SE values of relative transcript abundance were calculated.

GA₁ and GA₄ Treatment of *P. tremula* Seedlings

Approximately 50 wild *P. tremula* seeds per treatment were sown on filter paper in petri dishes drenched in water with GA₁ or GA₄ at the following concentrations: 10 nM, 100 nM, 1 μM, and 10 μM. Controls received no addition of GA. The petri dishes were cold-treated for 5 d at 4°C before being placed under LD greenhouse conditions for 2 d, after which they were harvested in order to analyze the lengths of their hypocotyls. The seedlings were analyzed under a Leica MZFLIII microscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera, and the lengths were determined using the program Leica QWin 2.3. Forty seedlings for each treatment, except the 10-μM treatment (30 seedlings), were measured. The experiment was repeated once with *P. tremuloides* seedlings, and similar results were obtained.

Quantification of GAs

GAs from 200 mg (fresh weight) of tissue were purified and analyzed, essentially as described by Eriksson et al. (2000) by GC/MS-SRM (JMS-MStation 700, JEOL, Tokyo), using ²H₂-GAs (Professor L. Mander) as internal standards. For the GA analysis of the internodes in specific developmental stages, the extraction was modified as follows. Plant tissues, between 10 and 50 mg fresh weight, were extracted using an MM 301 Vibration Mill (Retsch, Haan, Germany) at a frequency of 30 Hz s⁻¹ for 3 min after adding 3 mm tungsten carbide beads (Retsch) to each tube to increase the extraction efficiency. After centrifugation in an Eppendorf centrifuge for 10 min at 14,000 rpm, the supernatant was further purified as earlier described (Eriksson et al., 2000).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY433958, BU835271, and BU877509.

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