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Altered Growth and Wood Characteristics in Transgenic Hybrid Aspen Expressing *Agrobacterium tumefaciens* T-DNA Indoleacetic Acid-Biosynthetic Genes[1]

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A key regulator of cambial growth is the plant hormone indoleacetic acid (IAA). Here we report on altered wood characteristics and growth patterns in transgenic hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.) expressing *Agrobacterium tumefaciens* T-DNA IAA-biosynthetic *iaaM* and *iaaH* genes. Eighteen lines simultaneously expressing both genes were regenerated. Of these, four lines, verified to be transgenic by northern blot analysis, were selected and raised under controlled growth conditions. All four lines were affected in their growth patterns, including alterations in height and stem diameter growth, internode elongation, leaf enlargement, and degree of apical dominance. Two transgenic lines, showing the most distinct phenotypic deviation from the wild type, were characterized in more detail for free and conjugated IAA levels and for wood characteristics. Both lines showed an altered IAA balance, particularly in mature leaves and roots where IAA levels were elevated. They also exhibited changes in wood anatomy, most notably a reduction in vessel size, an increase in vessel density, and changes in ray development. Thus, the recent development of techniques for gene transfer to forest trees enabled us to investigate the influence of an altered IAA balance on xylem development in an intact experimental system. In addition, the results demonstrate the possibility of manipulating wood properties in a forest tree through controlled changes of IAA concentration and distribution.

IAA is an important morphogen in the differentiation of vascular tissue, as shown in experiments using both cell and tissue culture systems and intact plants (Sachs, 1981; Jacobs, 1984; Aloni, 1987; Roberts et al., 1988). In forest trees, the polar flow of IAA is crucial for maintaining the structure and activity of the vascular cambium, which gives rise to secondary growth of the stem. Experimental manipulation of the levels of IAA in the vascular cambium region by exogenous applications of auxins or auxin transport inhibitors has been demonstrated to affect almost all aspects of cambial growth, including xylem production, size and cell wall thickness of xylem cells, reaction wood, and vessel density (reviewed by Little and Savidge, 1987; Roberts et al., 1988). Although the molecular mechanisms by which IAA acts on these processes are largely unknown, it can be concluded that the IAA status in the plant is critical for the amount and quality of the produced wood. With the development of techniques for gene transfer to forest trees, modification of the endogenous hormone balance by genetic engineering is a potential tool for elucidating the hormonal regulation of cambial growth as well as a way to achieve desirable modification of wood properties.

The endogenous concentration of IAA can be altered by transforming plants with the *Agrobacterium tumefaciens* T-DNA IAA-biosynthetic genes *iaaM* and *iaaH*, encoding Trp-2-mono-oxygenase (Thomashow et al., 1986; Van Onckelen et al., 1986) and indole-3-acetamide hydrolase (Schröder et al., 1984; Thomashow et al., 1984), respectively. These two enzymes catalyze a two-step biosynthesis of IAA from Trp via indole-3-acetamide. Thus, IAA is overproduced in transgenic plants expressing the *iaaM* and *iaaH* genes. Such an approach has been used successfully to study physiology and metabolism of IAA in the herbaceous species petunia, tobacco, and Arabidopsis (Klee et al., 1987; Sitbon et al., 1991, 1992a, 1992b; Romano et al., 1993) and could also be used for studies of wood formation in forest trees. We have previously described a technique for genetic transformation and regeneration of *Populus tremula* L. × *Populus tremuloides* Michx. trees (Nilsson et al., 1992). In this paper we report use of this system for ectopic expression of the IAA-biosynthetic genes, and for the first time (to our knowledge) describe a genetically engineered forest tree with altered growth and wood characteristics as a result of the expression of the introduced genes.

MATERIALS AND METHODS

Vector Constructs

Standard DNA techniques were carried out as described by Sambrook et al. (1989). Construction of the vector pPUV72012 was based on a disarmed binary vector pPCV720 (a gift from Dr. C. Koncz, Max-Planck-Institute, Cologne, Germany), which is a derivative of the vector

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Abbreviations: *mas*, mannopine synthase; MS, Murashige and Skoog.

pPCV701 (Koncz et al., 1987), containing a selectable hygromycin marker gene and a *mas* 1′2′ promoter cassette. The bidirectional 1′2′ promoter was originally isolated from the T_R-DNA of the octopine-type Ti plasmid pTiAch5 (Velten et al., 1984), which allows simultaneous expression of two genes from the divergent 1′ and 2′ promoters (Velten and Schell, 1985). A promoter-truncated *iaaH* gene (Sitbon et al., 1992b) was linked with *Sal*I-linkers and inserted in sense orientation into the unique *Sal*I site of the pPCV720 vector on the 3′ side of the 2′ promoter and upstream of a T-DNA octopine synthase poly(A) signal. A promoter-truncated and *Bcl*I-linked *iaaM* fragment (Sitbon et al., 1992a) was then inserted into the single *Bam*HI site of the pPCV720 vector, fusing it between the 1′ promoter and the T-DNA gene 7 poly(A) signal, thus giving rise to the vector construct pPUV72012 (Fig. 1).

The cloning of the *iaaM* coding region into the pPCV720 binary vector behind the *mas* 1′ promoter to give the vector construct pPUV7201 (Fig. 1) has been described elsewhere (Sitbon et al., 1992b).

The *iaaH* coding region was cloned into the pPCV702 binary vector (Koncz et al., 1989) behind the cauliflower mosaic virus 35S promoter as described by Sitbon et al. (1992b) to give the vector construct pPUV7022 (Fig. 1).

The constructed vectors were introduced into the *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) (Koncz and Schell, 1986) by electroporation (Nilsson et al., 1992).

Transformation of Hybrid Aspen and Regeneration of Transgenic Plants

Wild-type hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.) was transformed with the vector constructs pPUV72012 and pPUV7201 and regenerated under the selection of 25 $\mu g\ mL^{-1}$ hygromycin. The regenerated, hygromycin-resistant 1′:*iaaM* plants were transformed a second time with the pPUV7022 construct and regenerated under the selection of 25 $\mu g\ mL^{-1}$ hygromycin and 80 $\mu g\ mL^{-1}$ kanamycin.

The transformation and regeneration procedures have been described in detail elsewhere (Nilsson et al., 1992). Briefly, internodal segments (5–10 mm) from sterile culture were co-cultivated with the *Agrobacterium* donor strain in liquid MS medium (Sigma) for 2 h and thereafter transferred to solid MS medium without antibiotics. After 2 d under dim-light conditions, the stem segments were washed twice with 500 $\mu g\ mL^{-1}$ aqueous Claforan (cefotaxime; Hoechst AG, Frankfurt am Mein, Germany) solution and transferred into solid MS medium supplemented with 0.1 $\mu g\ mL^{-1}$ indole-3-butyric acid, 0.2 $\mu g\ mL^{-1}$ 6-benzylaminopurine, 0.01 $\mu g\ mL^{-1}$ thidiazuron [1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea; Sigma], 250 $\mu g\ mL^{-1}$ Claforan, and the appropriate antibiotics. Proliferating shoot cultures were transferred to 300-mL glass jars containing MS medium supplemented with 6-benzylaminopurine, Claforan, and the appropriate antibiotics. Rooting was then accomplished by transferring the elongated shoots to 1-L glass jars containing half-strength solid MS medium without Suc, hormones, or antibiotics.

Growth Conditions

Rooted shoots from sterile culture, approximately 10 cm long, were potted in mineral wool and transferred to a growth chamber having a photoperiod of 18 h, a photon flux density of 300 $\mu E\ m^{-2}\ s^{-1}$ PAR, a RH of approximately 75%, and a temperature of 18/10°C (day/night). The plants were watered with a complete nutrient solution (Ingestad, 1970) containing 100 mg of nitrogen per L. The use of mineral wool as a support allowed us to replace the nutrient solution daily by ample watering, thereby maintaining stable and optimal availability of nutrients.

Documentation of Plant Growth

The plant material from sterile culture was allowed to acclimatize in the growth chamber for 15 d, at which time shoot growth had commenced. To indicate a reference point for growth measurements, the uppermost internode was marked with a thread. The growth pattern of 10 plants

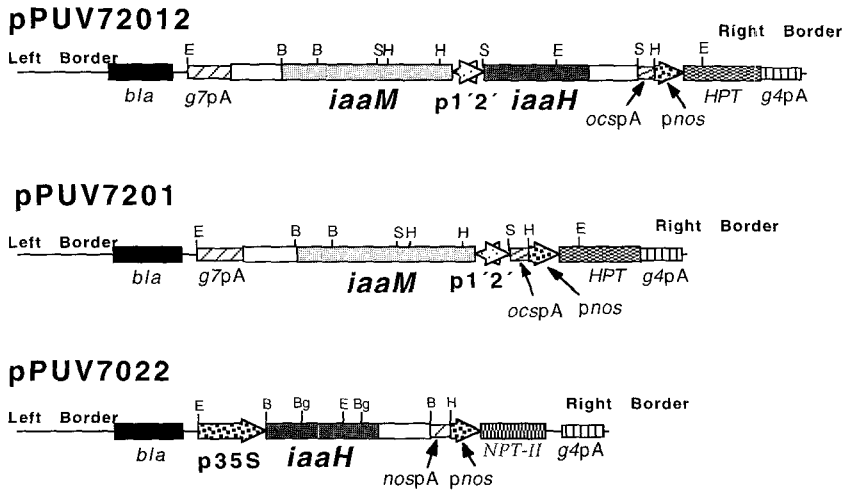

Figure 1. Schematic drawing of the binary vectors used in this study. p1′2′, Bidirectional *mas* promoter; p35S, cauliflower mosaic virus 35S promoter; *iaaM*, coding region for Trp-2-monooxygenase gene; *iaaH*, coding region for indole-3-acetamide hydrolase gene; p*nos*, promoter of the nopaline synthetase gene; *HPT*, coding region for the hygromycin phosphotransferase gene; *NPT-II*, coding region for the neomycin phosphotransferase gene; *bla*, coding region for the ampicillin resistance gene; *nos*pA, *ocs*pA, *g7*pA, and *g4*pA, polyadenylation signals from *A. tumefaciens* T-DNA nopaline and octopine synthetase genes and genes *7* and *4*; restriction enzyme sites for *Eco*RI (E), *Bam*HI (B), *Sal*I (S), *Hin*dIII (H), and *Bgl*II (Bg).

of each line was followed for 24 d by recording the number and length of leaves longer than 10 mm every 2nd or 3rd d. For each recorded occasion, plastochron age, based on a 30-mm reference leaf length, was calculated according to Erickson and Michelini (1957). At the end of the 24-d growth period the plants were measured for the following growth characteristics: plant height, length of internodes subjacent to fully expanded leaves, the length and width of the largest leaf, and stem diameter at the basal position marked with a thread. The plants were then harvested for IAA measurements and microscopic examination, and inspected for root morphology.

Quantification of IAA and IAA Conjugates

IAA was measured in the apical shoot, mature leaves, mature stem, and roots. The apical shoot was defined as all tissues above the leaf that had attained one-half the size of the fully expanded leaves. The mature leaves sampled were the two most basal full-sized leaves. Mature stem was collected between the basal reference thread and the uppermost full-sized leaf. The mature stem sample consisted of extraxylary tissues obtained by peeling the bark and scraping the surface of the exposed xylem with a scalpel. The root sample consisted of 3- to 5-cm-long root tips. The harvested plant material was immediately frozen in liquid N_2.

The procedure for measuring IAA has been described in detail by Sundberg (1990). Briefly, after homogenizing in liquid N_2 with a mortar and pestle, the plant material was extracted for 1 h in 0.05 M phosphate buffer, pH 7.0, containing 0.02% sodiumdiethyldithiocarbamate as an antioxidant and [$^{13}C_6$]IAA (Cambridge Isotope Laboratories, Woburn, MA) as an internal standard. The extract was divided into two aliquots. One of them was purified by neutral and acidic ether partitioning, followed by methylation and reversed-phase HPLC. The IAA-methyl ester fraction was collected, silylated, and quantified by GC-selected ion monitoring-MS. The m/z ratio of 202:208 was used to calculate the endogenous IAA concentration by the isotope dilution equation (Cohen et al., 1986), and the m/z ratio of 261:267 was used to check for interference. The other aliquot was hydrolyzed in 7 M NaOH at 100°C for 3 h in an N_2 atmosphere for measurement of the total amount of IAA (free IAA + hydrolyzable IAA). After neutralization with HCl, the aliquot was purified and quantified as described above. The amount of IAA conjugates (hydrolyzable IAA) was calculated as the difference between the total and free IAA concentrations.

Anatomical Analysis

Stem samples for microscopy were obtained from the basal position marked with the thread. The samples were fixed in formaldehyde:ethanol:glacial acetic acid (10:85:5, v/v), dehydrated in an ascending alcohol series, and embedded in Historesin (Reichert-Jung, Heidelberg, Germany). Transverse and longitudinal sections were sectioned at 10 μm with a rotary microtome. The sections were stained with toluidine blue, mounted in Entellan (Merck, Darmstadt, Germany), and investigated under a Zeiss Axioplan light microscope.

Xylem width, vessel size, vessel density, xylem ray density, and xylem ray cell width were estimated from the transverse sections. Xylem width was measured at 2 to 4 radii around the circumference of each section with an ocular measuring scale. The density and size of vessels and rays were determined in micrographs (100× magnification) covering the entire transverse section. A circular line was drawn across the zone of the most recently differentiated xylem. Vessels and rays crossed by the line were counted and measured on the micrographs in tangential direction, and for vessels also in radial direction, with the aid of a scale loupe (7× magnification). Only the vessels exceeding 40 μm in radial direction, i.e. twice the average size of a fiber, were taken into account to avoid possible confusion with fibers in the transgenic plants.

Southern and Northern Blot Analysis

Chromosomal DNA was isolated from leaf tissue according to Doyle and Doyle (1990). Ten milligrams of DNA were digested with the restriction enzymes *Hin*dIII or *Eco*RI for the analysis of the genes *iaaM* or *iaaH*, respectively. The DNA fragments were separated on a 0.9% agarose gel and transferred to a nylon Hybond-N membrane (Amersham) according to the manufacturer's instructions.

Total RNA was isolated from plants grown in sterile culture. Leaf and stem tissue were frozen in liquid N_2, homogenized in a mortar with a pestle, and extracted in 5 to 10 mL of GTC buffer (4 M guanidinium thiocyanate, 25 mM *tri*-natrium citrate dihydrate, pH 7.0, 20 mM EDTA, 0.5% Triton X-100, and 1.5% mercaptoethanol added just before use). The homogenate was passed through a Miracloth filter (Calbiochem), extracted with one-half to 1 volume of phenol:chloroform (50:50, v/v), and precipitated with ethanol. Thirty micrograms of the total RNA were denatured, separated on a formaldehyde-agarose gel (Sambrook et al., 1989), and transferred to a supported nylon Nytran-N membrane (Schleicher & Schuell).

Southern and northern hybridizations were performed under stringent conditions according to Sambrook et al. (1989) using randomly primed probes labeled to high specific activities. A 2093-bp *Bam*HI *iaaH* fragment and a 3358-bp *Bcl*I *iaaM* fragment, each spanning the entire coding sequence, were used as probes.

RESULTS

Regeneration of Transgenic Hybrid Aspen Occurs with High Efficiency

Hybrid aspen was transformed with a single binary vector (pPUV72012) or sequentially with two vectors (pPUV7201 and pPUV7022), in both cases enabling simultaneous expression of the two *A. tumefaciens* T-DNA IAA-biosynthetic genes, *iaaM* and *iaaH* (Fig. 1). Expression of the *iaaM* gene, encoding the first step in IAA biosynthesis from Trp, was under the control of the *mas* 1′ promoter. This promoter was chosen because of its relatively low level of expression (Harpster et al., 1988) and an expected specific-

ity of expression in vascular tissue (Langridge et al., 1989; Guevara-García et al., 1993). Earlier transformations with cauliflower mosaic virus 35S promoter-driven *iaaM* expression in a 35S:*iaaH* background resulted in a pronounced formation of callus, which could not be regenerated into shoots (F. Sitbon, unpublished data). Expression of the second gene, *iaaH*, was under the control of either the 35S (pPUV7022) or the *mas* 2′ promoter (pPUV72012).

Transformation with the single binary vector containing both the *iaaM* and *iaaH* genes on the same T-DNA (pPUV72012) resulted in regeneration of five independently transformed lines, denoted G1′2′ A to E. Transformation with the vector pPUV7201 resulted in 65 lines resistant to hygromycin. Out of these, three lines shown to contain the *iaaM* gene by Southern blot analysis (data not shown), denoted 58, 60, and 61, were selected to be transformed with the vector pPUV7022 containing the *iaaH* gene. Transformation of these lines resulted in regeneration of 13 doubly transformed lines, 58 A to E, 60 A to D, and 61 A to D.

Due to the relatively weak expression of the *mas* 1′ and 2′ promoters, the expression of both *iaaM* and *iaaH* genes could be verified by RNA gel analysis in only a few lines. These lines were 58A, 58C, 60C, 60D, 61D, and G1′2′D (Fig. 2). The lines 58A, 60C, 61D, and G1′2′D were selected for further characterization.

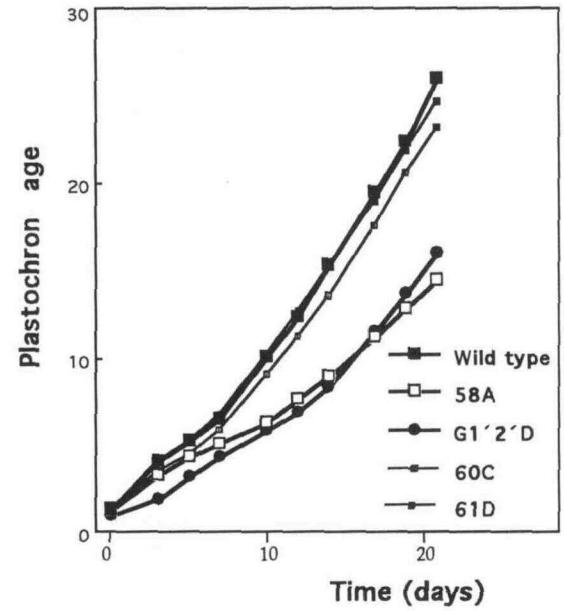

Figure 3. Growth rate of transgenic and wild-type hybrid aspen. Growth rate was followed by measuring plastochron age during the 24-d growth period in controlled conditions. Plastochron age, which reflects the number of leaves longer than 30 mm, was measured in 10 replicate plants of each line.

Expression of the IAA-Biosynthetic Genes Alters the Growth Pattern

To perform growth analysis and to obtain plant material for anatomical examination and IAA quantification, the selected lines were acclimatized in a growth chamber and raised under controlled conditions. As judged from the rate of leaf initiation and expansion, all lines attained a stable growth rate after an initial lag period (Fig. 3). However, the growth rate of the lines G1′2′D and 58A was much reduced. At the end of the growth period, morphological characteristics of the plants were measured. Compared to wild type, all of the transgenic lines were smaller, but to varying degrees (Figs. 3, 4, 5a). The line G1′2′D showed the most distinct phenotypic divergence, with a reduction in height, stem diameter, and leaf area (Fig. 4). The reduced height was associated with a reduced rate of leaf initiation, but not with a reduction in internode length (Figs. 3 and 4). In addition, the line G1′2′D had an aberrant root morphology, with little or no lateral root formation (Fig. 5b). The line 58A was similarly inhibited in height and diameter growth but not in leaf enlargement (Fig. 4). It had the same rate of leaf initiation as G1′2′D (Fig. 3) but longer internodes (Fig. 4), which resulted in an intermediate height in this line. The two other transgenic lines, 60C and 61D, diverged only slightly from the wild type. Both lines had less stem diameter, and the line 60C had longer internodes (Fig. 4). Due to the weak phenotypic expression, these lines were not further characterized.

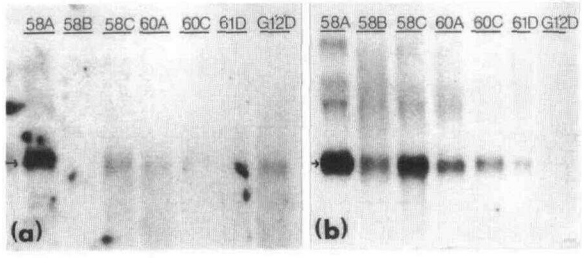

Figure 2. RNA gel blot of the transgenic hybrid aspen lines. a, Expression of the *iaaM* gene from the *mas* 1′ promoter. b, Expression of the *iaaH* gene from the cauliflower mosaic virus 35S promoter or the *mas* 2′ promoter (G1′2′D). Total RNA was isolated from plants in sterile culture, and 30 μg of RNA was loaded in each lane. The arrows indicate the bands corresponding to the expected transcript lengths of the *iaaM* and *iaaH* genes.

Expression of the IAA-Biosynthetic Genes from the *mas* Promoter Results in an Increased IAA Level in Mature Leaves and Roots

The plant material raised in the growth chamber was analyzed for free and conjugated IAA by GC-MS. The levels and proportions of free and conjugated IAA varied considerably among the analyzed parts of the plant: the apical part, mature leaves, mature stem, and roots (Fig. 6). In all lines, the highest concentrations of free IAA, but low concentrations of IAA conjugates, were found in the extraxylary tissues of the mature stem. The situation was reversed in mature leaves, where the highest concentrations of IAA conjugates, but very low concentrations of free IAA, were found. The apical parts and the roots contained intermediate proportions of free to conjugated IAA. The total IAA concentrations were lowest in the root samples.

Compared to the wild type, the transgenic lines G1′2′D and 58A showed elevated levels of free and conjugated

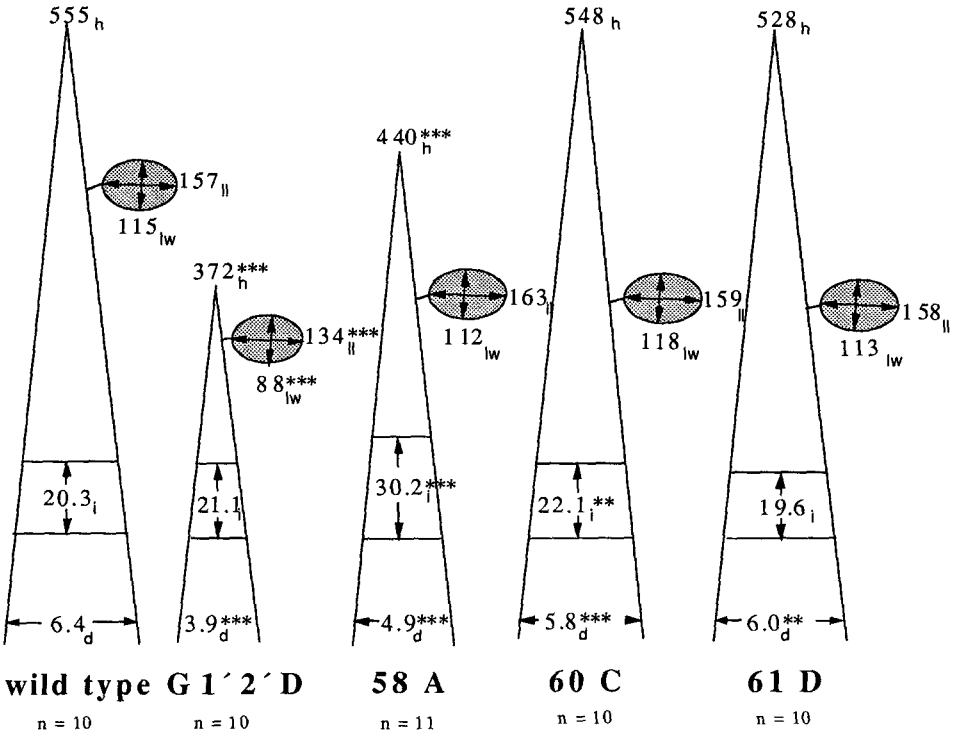

Figure 4. Schematic drawing of the morphology of transgenic and wild-type hybrid aspen. Plant height (h), basal stem diameter (d), length (ll), and width (lw) of mature leaves and mean internode length (i) are indicated in mm for each line. The measurements were taken at the end of the 24-d growth period. The asterisks indicate statistically significant differences (Student's *t* test) in the different growth characteristics between each transgenic line and the wild type: **P < 0.01; ***P < 0.001. *n*, Number of replicate plants.

IAA in mature leaves and roots (Fig. 6). The most pronounced increase was found in the roots of the line G1′2′D. In mature leaves, the increase in IAA level was statistically established for free IAA in the line G1′2′D and for conjugated IAA in the line 58A. In mature stem there was a reduction in free IAA concentration in the line 58A. In the apical part no differences in IAA levels were found between the transgenic and wild-type lines.

Overproduction of IAA Inhibits Release of Axillary Buds

The polar flow of IAA not only maintains cambial activity but also inhibits axillary bud outgrowth and leaf abscission. A major source of the polarly transported IAA along the stem axis is the apical shoot, and removal of this source by means of decapitation normally results in release of axillary buds (Cline, 1991). Likewise, reduction in the IAA flow through petioles causes abscission of the leaves (Osborne, 1989). The influence of decapitation on axillary bud release was investigated in the transgenic hybrid aspen lines 58A and G1′2′D in parallel with the wild-type plants. After decapitation, the number of released axillary buds was significantly lower in both transgenic lines than in the wild type (Table I; Fig. 5c). Again, the greatest difference was observed in the G1′2′D plants, in which no axillary buds were released in most of the replicate plants. During prolonged culture of the decapitated G1′2′D plants with no new shoots, the leaves did not abscise and the cambium remained active, resulting in pronounced stem growth in the basal part of the stump. These observations demonstrate that the transgenic plants were able to maintain polar IAA transport through the petioles and the vascular cambium in the decapitated shoots, thus confirming elevation in IAA level in the basal parts of the transgenic plants.

IAA Overproduction Results in Altered Xylem Formation

The effect of an altered IAA balance on xylem characteristics was investigated in plastic, embedded sections obtained from the basal stem of the transgenic hybrid aspen trees. Compared to wild type, the two transgenic lines, 58A and G1′2′D, had a reduced xylem width (Table II). In addition, they displayed alterations in the structure of the xylem (Fig. 7). This was most obvious in the most recently formed xylem of the line G1′2′D, with perturbations in the size and organization of the xylem elements (Fig. 7, a–c). Large fibers and small vessels gave the impression of a more uniform xylem with many intermediate-sized cells. In addition, cambial zone cells and xylem fibers were rounded (Fig. 7, a and c), the xylem contained fewer rays (Table II), and the radial files of fibers and ray cells were distorted, resulting in a disorganized xylem structure (Fig. 7a). In the tangential view, it was noted that the height of the rays was smaller than in the wild type, and biseriate rays were frequently observed (Fig. 7b). Moreover, the tangential width of the individual ray cells was greater

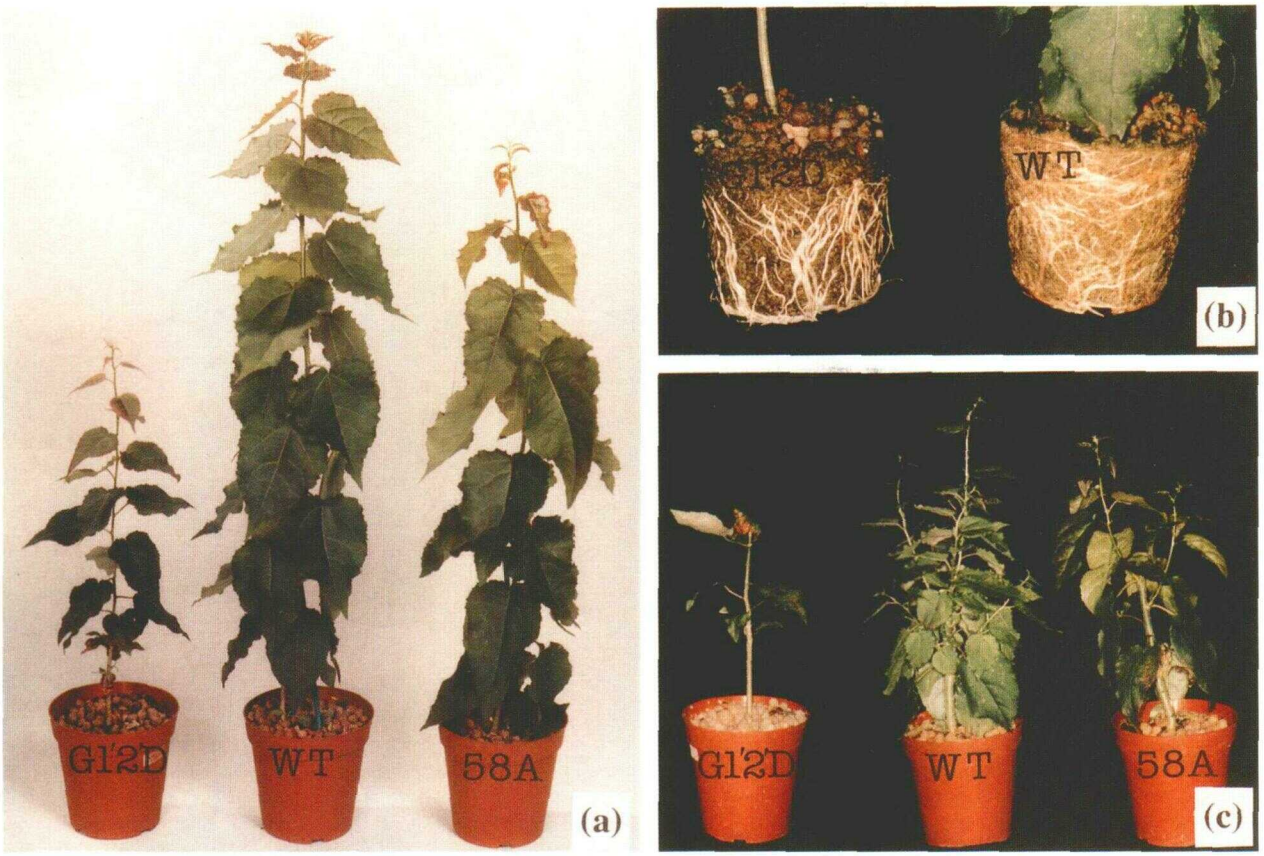

Figure 5. Phenotype of transgenic and wild-type hybrid aspen. a, Representative plants of the line G1'2'D, wild type (WT), and the line 58A at the end of the 24-d growth period. b, Comparison of the root system of the line G1'2'D (left) and the wild type (WT) (right). c, Phenotypic appearance of representative plants of transgenic lines G1'2'D and 58A and wild type (WT) 30 d after decapitation.

(Table II). Similar changes in xylem structure were observed in the line 58A, but to a lesser extent (Fig. 7, d–f).

Vessels were characterized in the most recently formed xylem in transverse sections. The transgenic line G1'2'D had a greater density of vessels, whereas the vessel density of the line 58A did not differ from that of the wild type (Table II). The radial and tangential diameter of the vessels was smaller in both transgenic lines compared to the wild type, the line G1'2'D being most affected (Fig. 8).

DISCUSSION

Transformation of hybrid aspen with *iaaM* and *iaaH* genes and subsequent regeneration resulted in several individually transformed lines that displayed different degrees of IAA-related phenotypic and developmental alterations. Common to several transgenic lines was inhibition of plant growth, affecting stem elongation, leaf enlargement, stem diameter growth, xylem formation, and axillary bud release (Figs. 3–5; Tables I and II). The two transgenic hybrid aspen lines showing the most pronounced phenotypic deviations from the wild type, 58A and G1'2'D, were analyzed for endogenous IAA levels and phenotypically characterized in more detail. Free and conjugated IAA levels were elevated in mature leaves of both of these transgenes and in roots of G1'2'D (Fig. 6). The observation of increased IAA levels in basal tissues is in agreement with the known spatial pattern of the *mas* 1' promoter activity (Langridge et al., 1989; Comai et al., 1990; Leung et al., 1991; Sitbon et al., 1992b; Guevara-García et al., 1993; Ursin and Shewmaker, 1993), which drives the expression of the first biosynthetic gene, *iaaM*, in our constructs. Expression of this gene has been shown to determine the extent of IAA overproduction and phenotypic changes in *iaaM/iaaH* doubly-transformed tobacco (Sitbon et al., 1992b). Furthermore, the extent of IAA increase correlated with the observed morphological changes among the different lines, the most pronounced phenotypic differences being observed in the line having the highest elevation of free IAA.

Ectopic expression of the IAA-biosynthetic genes in the transgenic hybrid aspen resulted in an altered size and proportion of the different xylem elements in the lower part of the stem. The newly differentiated vessel elements of the line G1'2'D showed smaller diameter and greater density than the wild type (Fig. 8; Table II). A similar trend was observed in our previous study with the IAA-overproducing tobacco (E.J. Mellerowicz, R.T. Riding, B. Sundberg, C.H.A. Little, unpublished data), and the published photographs of the IAA-overproducing petunia can be simi-

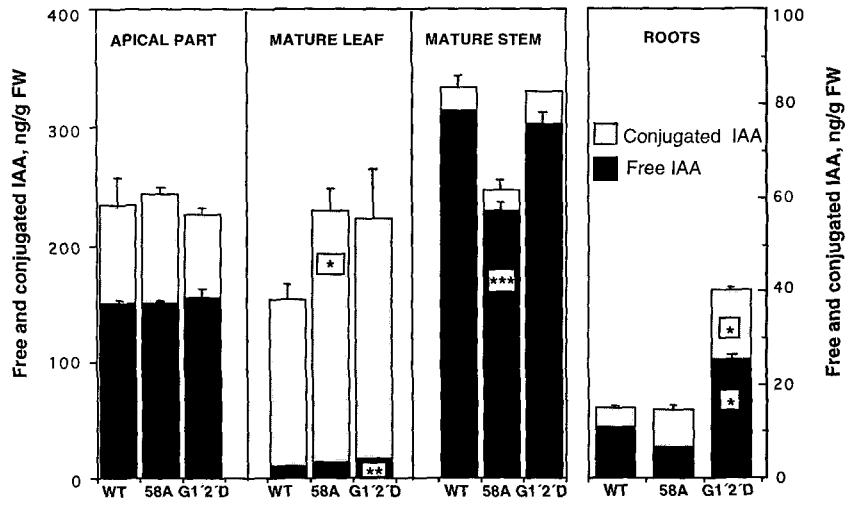

Figure 6. Free and conjugated IAA in transgenic and wild-type hybrid aspen. The columns represent the level of total IAA (free + conjugated) in different parts of the plants. The apical part consisted of all tissues above the leaf that had attained half the size of the fully expanded leaf. Mature leaf samples consisted of the two most basal full-sized leaves. Mature stem consisted of extraxylary stem tissues below the uppermost fully expanded leaf. Root samples consisted of 3- to 5-cm-long root tips. The values are the means of three independent samples, each sample consisting of pooled material from three plants. The asterisks indicate statistically significant differences (Student's *t* test) between each transgenic line and the wild type: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *n*, Number of replicate plants. Vertical lines indicate SE. FW, Fresh weight.

larly interpreted (Klee et al., 1987). These results are consistent with auxin application experiments in several broad-leaved trees, which demonstrate a positive correlation between auxin concentration and vessel density and size, up to a certain threshold level, beyond which auxin becomes inhibitory for vessel size but not density (Digby and Wareing, 1966; Doley and Leyton, 1968; Zakrzewski, 1983, 1991; Meicenheimer and Larson, 1985; Aloni, 1991). The reduction in vessel diameter, in combination with increased vessel density in the transgenic hybrid aspen line G1'2'D, is therefore indicative of endogenous IAA concentrations that are supraoptimal for vessel enlargement. In addition to vessel element differentiation, the pattern of ray development was altered in both transgenic hybrid aspen lines. These changes included smaller height of the rays, wider ray cells, reduced ray density, and the development of biseriate rays, which occurs only rarely in wild-type plants (Fig. 7; Table II). In many species, ray development is stimulated by wounding and application of ethylene in the form of ethrel, and it has been proposed to be counteracted by an axial signal, possibly IAA (Lev-Yadun and Aloni, 1995). However, application of exogenous auxin increased the proportion of ray tissue in the xylem of intact *Picea sitchensis* stems (Philipson and Coutts, 1980). Our results support the hypothesis that IAA has an inhibitory effect on ray initiation and a stimulatory effect on ray cell enlargement.

In addition to the qualitative changes of xylem properties, the transgenic hybrid aspen also had a smaller radial width of xylem compared to wild-type plants (Table II). This is not surprising, considering the general growth inhibition and the reduced stem diameter of the transgenic trees (Fig. 4; Table II). On the other hand, overproduction of IAA in transgenic 19S:*iaaM* petunia (Klee et al., 1987) and 35S:*iaaM*/*iaaH* tobacco transformants (Sitbon et al.,

Table I. *Release of axillary buds after decapitation in wild-type and transgenic hybrid aspen*

The average number of axillary buds released was determined 30 d after decapitation. n, Number of replicate plants.

Line	*n*	Mean No. of Released Buds
Wild type	5	4.8
58A	9	2.1[a]
G1'2'D	5	0.6[a]

[a] Indicates statistically significant difference (Student's *t* test) between each line and the wild type; $P < 0.01$.

Table II. *Xylem properties at the basal part of the stem in wild-type and transgenic hybrid aspen*

The measurements were taken from micrographs of transverse sections obtained from the basal part of the stem. Vessels and xylem rays crossed by a circular line drawn through the newly differentiated xylem were counted, and the density was calculated as the number of vessels or rays per unit length of the line. Tangential width of 20 randomly selected xylem ray cells was determined along the circular line of each transverse section. n, Number of replicate plants.

Line	*n*	Xylem Radius	Vessel Density	Ray Density	Ray Cell Width
		mm	*No./mm*	*No./mm*	*μm*
Wild type	6	2.2	6.8	10.9	10.5
58A	6	1.5[a]	6.2	10.8	12.2[b]
G1'2'D	7	1.1[a]	9.1[b]	5.5[a]	13.0[a]

[a] Indicates statistically significant difference (Student's *t* test) between each line and the wild type; $P < 0.001$. [b] Indicates statistically significant difference (Student's *t* test) between each line and the wild type; $P < 0.01$.

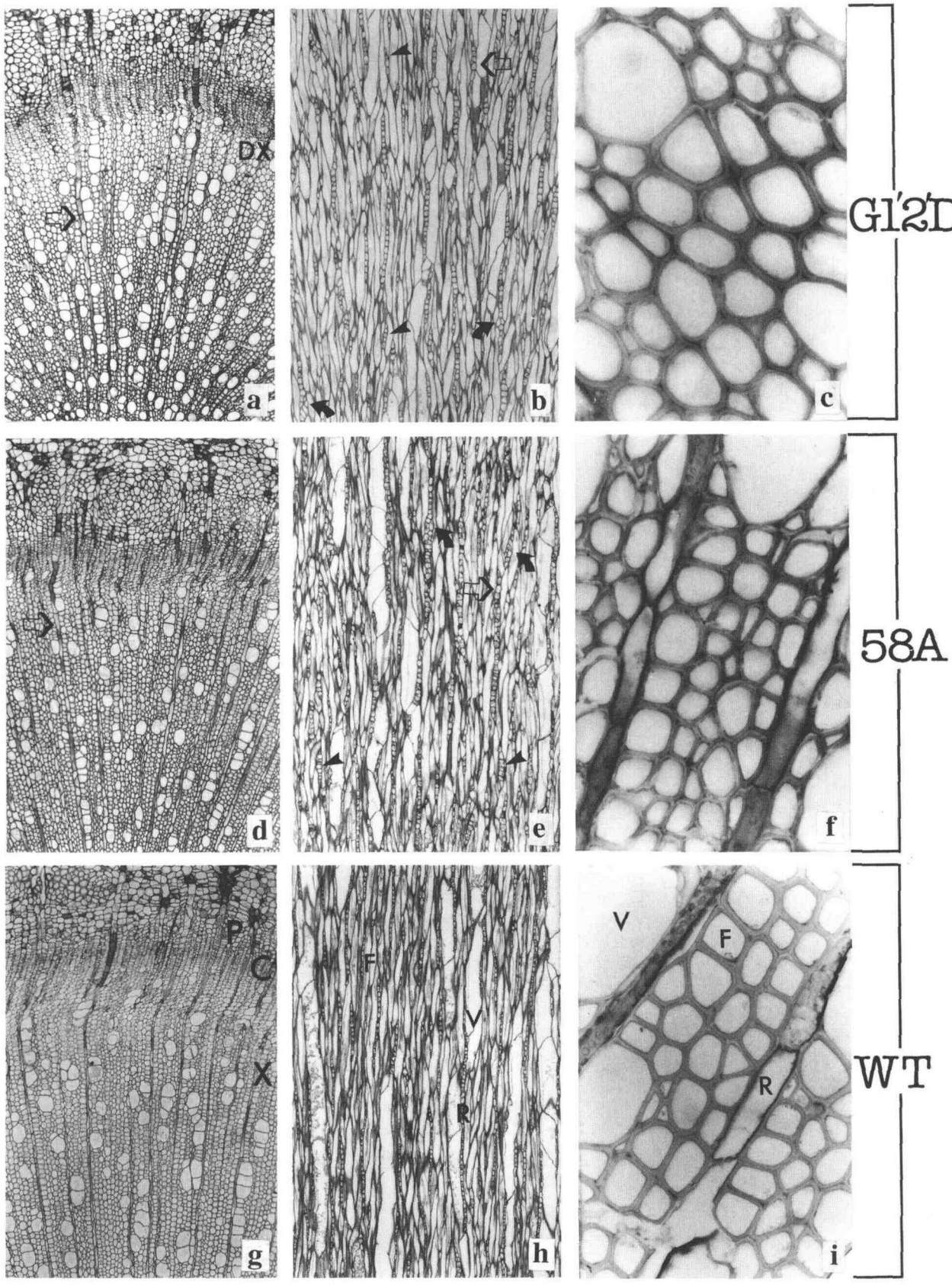

Figure 7. (Legend appears on facing page.)

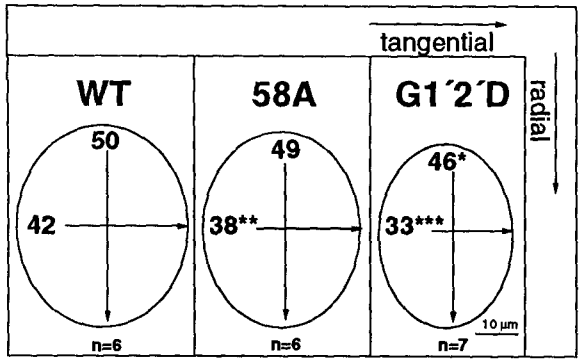

Figure 8. Vessel size at the basal part of the stem in transgenic and wild-type hybrid aspen. Radial and tangential vessel diameter (µm) was determined in vessel elements of the recently formed xylem. All vessels crossed by a circular line drawn on microphotographs from transverse sections were measured. Between 11 and 69 vessels were measured per transverse section. The asterisks indicate statistically significant differences (Student's *t* test) between each transgenic line and the wild type: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *n*, Number of replicate plants. WT, Wild type.

1992a) was reported to result in increased xylem width. However, interpretation of the results in tobacco is complicated because the internodes examined in the transgenic plants were older than those in the wild type, due to the slower growth rate of the transgenes. Thus, the observed increase in xylem width may have been a result of the longer period of production of secondary tissues. Nevertheless, exogenous auxin application directly on the stem has been demonstrated unambiguously to stimulate xylem production in several forest trees (Little and Savidge, 1987). In *Pinus sylvestris*, for example, a local elevation of the endogenous IAA level by exogenous application of IAA on intact trees was shown to increase tracheid production (Sundberg and Little, 1990; Little and Sundberg, 1991). Similarly, in several broad-leaved tree species, xylem production was stimulated by auxin that was applied to young stem explants (Digby and Wareing, 1966; Doley and Leyton, 1968; Zakrzewski, 1983). These observations indicate a potential to increase the rate of xylem production, using transgenic trees overproducing IAA. However, it is important to direct the overproduction in specific tissues, and in this way reduce interference with other growth processes. Therefore, xylem production in transgenic plants should be further investigated by directing the expression of the IAA-biosynthetic genes to the stem tissues responsible for cell division and differentiation, the vascular cambium.

The changes in xylem differentiation at the basal stem of the transgenic hybrid aspen lines were not correlated with an increased IAA level in that part of the plant (Fig. 6). In fact, the line displaying intermediate morphological changes, 58A, showed a decreased level of free IAA in the mature stem. This decrease may be a result of reduced IAA biosynthesis by the native gene products. This is quite likely, since both of the transgenic lines had reduced rates of leaf initiation (Fig. 3) and therefore fewer developing leaves, which are major potential sources of native IAA for the subjacent stem (Sundberg and Little, 1987; Rinne et al., 1993). On the other hand, the known induction pattern of the *mas* 1′ promoter, driving the expression of the first IAA-biosynthetic gene, suggests that there should be basipetally increasing IAA overproduction in the transgenes (Langridge et al., 1989; Sitbon et al., 1992b). This is also supported by the observed growth pattern of the decapitated transgenic plants (Table I). Thus, we suggest that the decreased supply of IAA from the native genes in the transgenes is compensated for by the basal expression of the T-DNA genes in the line G1′2′D and, to a lesser extent, in the line 58A. However, both the overall concentration of IAA and the distribution pattern of IAA within and between tissues are important for the regulation of xylem production. The occurrence of a steep radial concentration gradient of IAA over the cambial meristem and its differentiating derivatives has recently been demonstrated in our laboratory (C. Uggla, T. Moritz, G. Sandberg, B. Sundberg, unpublished data). This radial gradient is most likely involved in the control of cambial growth. Ectopic expression of the IAA biosynthesis genes could disturb the normal distribution pattern of IAA over the cambium at the basal stem. This would not necessarily be reflected in the IAA concentration of multi-tissue samples, but would change the hormone balance in specific cell layers, resulting in the observed alterations in xylem formation. Taken together, the distribution and concentration of IAA affecting xylem formation arise from complex vertical and radial patterns of ectopic expression of the IAA-biosynthetic genes, the expression of the native IAA-biosynthetic genes, and their mutual interference.

In conclusion, we have demonstrated an altered phenotype in a forest tree as a consequence of transformation with the *A. tumefaciens* T-DNA IAA-biosynthetic genes. The pattern of wood formation was affected in several ways, offering information about its regulation. The structure and composition of the component xylem cells is a primary determinant for wood quality, and the results presented here indicate potential future applications for genetic engineering in breeding for this com-

Figure 7. (On facing page.) Xylem anatomy at the basal part of the stem in transgenic and wild-type hybrid aspen. a, d, and g, Transverse sections, magnification 100×. b, e, and h, Tangential sections, magnification 50×. c, f, and i, Transverse sections, magnification 500×. Note the distortion of the radial files and the occurrence of intermediate-sized xylem elements and rounded fibers in the transgenic lines, most obviously in the recently formed xylem (DX) of the line G1′2′D (a), and larger ray cells (indicated by open arrows), decreased height of the rays (arrowheads), and tendency to biseriate rays (filled arrows) in both transgenic lines (a, b, d, and e) compared to the wild type (g, h). X, Xylem; C, cambium; P, phloem; V, vessel; F, fiber; R, ray.

mercially important trait (Cheliak and Rogers, 1990; Whetten and Sederoff, 1991).

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