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Transgenic Tobacco Plants Coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* Genes Display Altered Growth and Indoleacetic Acid Metabolism[1]

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ABSTRACT

Transgenic tobacco (*Nicotiana tabacum*) SR1 plants expressing the *Agrobacterium tumefaciens* nopaline transferred DNA *iaaH* gene were transformed with a 35S-*iaaM* construct. The transformants displayed several morphological aberrations, such as adventitious root formation on stem and leaves, dwarfism, epinastic leaf growth, increased apical dominance, and an overall retardation in development. In addition, xylem lignification was higher than in wild type. Free and conjugated indoleacetic acid (IAA) levels were quantified by gas chromatography-multiple ion monitoring-mass spectrometry in leaves and internodes of wild-type plants and two transformed lines with different phenotypes. Both transformed lines contained elevated levels of free and conjugated IAA, which was associated with increased transcription of the *iaaM* gene. The line with the highest IAA level also had the most altered pattern of growth and development. These IAA-overproducing plants will provide a model system for studies on IAA metabolism, IAA interactions with other phytohormones, and IAA roles in regulating plant growth and development.

IAA is a naturally occurring plant hormone known to stimulate cell enlargement, cambial activity, root initiation on stem cuttings, and lateral root development. IAA has furthermore been shown to be important in apical dominance, and it is also believed to be involved in tropistic responses (2). The precise mode of action of IAA, however, is still poorly understood. Studies in which endogenous IAA levels are correlated to different growth processes have been one way to determine a role for the phytohormone in different systems. This approach requires accurate and sensitive methods to measure the low amount of the compound present in plants. Immunological assays and the combined use of GC-MS are examples of such methods in current use for IAA quantifications (22). Other types of studies have also been performed in which IAA or other substances with IAA-like effects (auxins) have been exogenously applied to the plant material. However, such experiments are complicated by problems in standardizing parameters such as uptake, transport, and metabolic conversions of the applied substance, making results difficult to interpret and compare with other systems. Wound-induction responses and differences in tissue sensitivity are also factors to be considered when studying the effects of the hormone.

Two of the *Agrobacterium tumefaciens* Ti-plasmid T-DNA genes have been shown to encode a biosynthetic pathway for IAA production that is absent in plants. The *iaaM* gene encodes an enzyme converting tryptophan to IAM[2] (29, 31), which is subsequently hydrolyzed to IAA by the action of the *iaaH* gene product (23, 28). Plants expressing these IAA biosynthetic genes from promoters of different strength and tissue specificity could serve as a model system to manipulate endogenous IAA levels *in vivo*. Follin *et al.* (4) showed that tobacco plants expressing either the *iaaM* or the *iaaH* gene from their natural promoters displayed a normal phenotype. However, by substituting the natural promoter with the much stronger CaMV 19S promoter, Klee *et al.* (15) were able to show that transgenic petunia expressing a CaMV 19S-*iaaM* construct had a 10-fold IAA increase in leaf tissue. In addition, several morphological characteristics attributed to auxin effects were apparent. These included epinastic leaf growth and increased xylem production, adventitious root formation, and apical dominance. In the absence of an *iaaH* gene, the elevated IAA content of the 19S-*iaaM* plants was attributed to either the spontaneous hydrolysis of IAM or the enzymic conversion of IAM to IAA by a native enzyme with amidohydrolase activity.

The level of free IAA has been suggested to be regulated in several ways, such as by degradation, transport, synthesis, and inactivation through conjugation to sugars, amino acids, or peptides. Both ester and amide IAA conjugates have been identified in plants *in vivo*, as well as after exogenous application of IAA. Feung *et al.* (3) identified several amino acid conjugates in crown-gall tissues of *Parthenocissus cuspidata*. Ishikawa *et al.* (11) showed an increased rate of IAA-conjugate synthesis in carrot cultures transformed with a wild-type or a Cyt$^-$ pTiB6S3 plasmid. Sitbon *et al.* (24) observed an increase in free and conjugated IAA levels in transgenic

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[2] Abbreviations: IAM, indole-3-acetamide; CaMV, cauliflower mosaic virus; kb, kilobase; bp, base pair; CWR, cell wall residue; GC-MIM-MS, gas chromatography-multiple ion monitoring-mass spectrometry; MS medium, Murashige-Skoog medium; NAA, naphthalene acetic acid.

tobacco plants expressing the *iaaM* and *iaaH* genes from their natural promoters. These findings suggest the presence of an IAA regulatory system in plants that maintains IAA at a nontoxic or physiologically relevant level. Increased conjugation of IAA might be part of such a system. Transgenic IAA-overproducing plants could thus be used as a model system for the study of IAA down-regulation. This will require careful analysis of IAA levels in both wild-type and transformed plants. Furthermore, by using strong promoters to express the *iaaM* and *iaaH* genes in transgenic plants, the increased IAA biosynthesis might exceed the capacity of the IAA regulatory system, resulting in a change in phenotype. In this paper, we demonstrate effects on plant morphology and IAA metabolism obtained by substituting the *iaaM* natural promoter with the strong CaMV 35S promoter, and expressing this construct in *iaaH*-containing plants.

MATERIALS AND METHODS

All DNA manipulations were performed using standard techniques. Plasmids were propagated in *Escherichia coli* DH5α or GM99 (dam$^-$). Ti-plasmid vectors were introduced in the *Agrobacterium* 3101mp90RK strain (16) by electroporation.

Construction of a 35S-*iaaM* Gene Vector

A 3.5-kb *Eco*RI-*Pst*I fragment from the plasmid pG1 (10), comprising the *Agrobacterium tumefaciens* pTiC58 *iaaM* gene together with its untranslated 5′ and 3′ regions, was made blunt ended and cloned into the *Sma*I site of pUC19. The 5′ upstream region was deleted by Bal31 digestion until 23 bp remained in front of the translational start codon, and the resulting promoterless gene was excised as a 3-kb *Kpn*I-*Pst*I fragment, which was made blunt ended and linked with *Bcl*I linkers at both ends.

The pPCV 702 binary vector system used in this paper (16) contains a unique *Bam*HI cloning site in an "expression cassette," between a 850-bp CaMV 35S promoter and a polyA signal from the nopaline synthase gene, together with a kanamycin resistance marker. As the rG2 (*iaaH*) plants used in this study also were kanamycin-resistant, a new CaMV 35S vector was constructed with a hygromycin resistance marker, thus allowing selection for both *iaaM* and *iaaH* genes in doubly transformed plants. The 3-kb *Bcl*I *iaaM* fragment was inserted in both sense and antisense orientations into the *Bam*HI site of this vector giving rise to the 35S-*iaaM*(s) and 35S-*iaaM*(a-s) vectors, respectively.

Plant Materials

Tobacco (*Nicotiana tabacum* [L.] cv Petit Havana SR1) (17) and derived transformants were grown in a greenhouse with a day temperature of 25°C, a night temperature of 20°C, 50 to 70% RH, and a photoperiod of 18 h (natural daylight supplemented with Osram HQI-TS 400 W/DH lamps, giving a quantum flux density of 50–70 μE m^{-2} s^{-1}). Plants were grown in 4-L pots in peat, watered daily, and fertilized once a week with a 1:100 dilution of Superba S (Hydro Supra AB, Landskrona, Sweden). The N-P-K content of the fertilizer stock solution was 6.5, 1.0, and 4.7%, respectively.

Plants used in leaf-disc transformations were grown under sterile conditions in 1-L glass jars in 200 mL of half-strength MS salt medium (GIBÇO) containing 0.7% agar (Sigma).

Generation of Tobacco Plants Expressing Both *iaaM* and *iaaH* Genes

N. tabacum cv SR1 transformed with the *iaaH* gene expressed from its natural promoter and coupled to a kanamycin-resistance marker, denoted rG2 plants (4), were leaf-disc transformed (8) with the *A. tumefaciens* donor strains containing the 35S-*iaaM* vectors. In a first series of transformations, callus-induction medium consisted of K3-medium (20) supplemented with 0.5 μg/mL NAA (Sigma), 0.1 μg/mL benzyladenine (Sigma), 500 μg/mL cefotaxime (Hoechst), 25 μg/mL hygromycin (Sigma), and 0.7% agarose (Sea Kem, FMC BioProducts, Rockland, ME). Shoot-induction medium consisted of K3-medium with 0.1 μg/mL NAA and 0.5 μg/mL benzyladenine, plus antibiotics and agarose as above. In a second series of transformations, the callus-induction step was omitted and leaf-discs were placed directly on shoot-inducing medium when using the 35S-*iaaM*(a-s) vector, or on shoot-inducing medium without NAA when using the 35S-*iaaM*(s) vector. Resulting calli were cultivated on shoot-inducing medium for a period of 3 months and transferred to fresh medium every 3 weeks. Shoots were rooted on half-strength MS salt medium (GIBCO), and rooted shoots were transferred to peat and covered with plastic bags until shoot growth became visible. Seeds from self-fertilized primary transformants were selected on half-strength MS salt medium supplemented with 30 μg/mL hygromycin and/or 25 μg/mL kanamycin.

Southern and Northern Blot Analysis

Correct integration and expression of introduced genes was confirmed with Southern and northern blot analysis essentially as described by Sitbon *et al.* (24). The *iaaM* probe consisted of the 3-kb promoter-deleted *Bcl*I fragment, and the *iaaH* probe consisted of a 2.1-kb *Hind*III fragment subcloned from the pTiA6 plasmid.

Growth Measurements

Seeds were sown in peat without prior selection for the antibiotic resistance markers. After 2 to 3 weeks, the transformed phenotype was clearly distinguishable from the wild type by having smaller and more upright leaves. Seedlings were individually potted 3 weeks after sowing. Growth was measured periodically until the first flower was formed, and was recorded as total height, internode length and diameter, and leaf length and width. Leaves and internodes were numbered basipetally, with leaf number 1 being the first leaf longer than 15 mm. The radial widths of pith, xylem, and extraxylary tissues were measured in a transverse, handcut section made at the midpoint of selected internodes. In addition, a 5 mm internode segment was occasionally fixed in formalin:glacial acetic acid:95% ethanol:water (1:1:14:4; v/v/v/v), dehydrated through a tertiary butyl alcohol series, embedded in paraplast, and sectioned transversely or longi-

tudinally on a rotary microtome at 8 μm. Serial sections were mounted on chemically clean slides and stained with safranin and fast green (13).

Lignin Determination

The lignin content was measured both in internodes as a whole and in separate fractions representing extraxylary tissues, xylem, and pith. Three xylem fractions were collected by using a scalpel to scrape the exposed wood side with increasing intensity. The pith was sampled by splitting the internode longitudinally and scraping the exposed tissue.

Lyophilized samples were ground to a fine powder and washed three times with water, three times with 1 M NaCl + 2% Triton X-100, twice with ethanol-hexane (1:2 v/v), and once with 99.5% ethanol. Cell walls were sedimented after each washing step by centrifugation (1000*g*, 5 min). The final pellet was air dried and hydrolyzed in 2 M NaOH for 1 h under nitrogen to remove esterified phenolic acids bound to the cell wall. After acidification to pH 2.0 with 2 M HCl, the material was centrifuged (1000*g*, 5 min), washed with distilled water until pH was neutral, and oven dried. The lignin content of the final CWR was determined as described by Ingold *et al.* (9).

IAA Analysis

IAA and IAA-conjugates were quantified by GC-MIM-MS using [$^{13}C_6$]IAA as an internal standard as described by Sitbon *et al.* (24). Samples were collected when the plants had formed 11 nodes and were processed according to Sundberg (27) using 50 mM phosphate buffer, pH 7.0, for the extraction.

RESULTS

Generation of Tobacco Plants Expressing *iaaM* and *iaaH* Genes

In the first series of transformations of rG2 plants with the 35S-*iaaM* sense (s) and antisense (a-s) vectors, 35S-*iaaM* (s) calli developing on NAA-containing medium died within 3 weeks, whereas 35S-*iaaM* (a-s) calli remained healthy. In a second series of transformations, in which leaf-discs were placed directly onto shoot-induction medium and NAA was omitted when using the 35S-*iaaM*(s) vector, the calli survived independent of *iaaM* gene orientation. However, the majority of the 35S-*iaaM*(s)/*iaaH* calli had a different morphology compared to antisense and wild-type controls. They were pale in color, grew in a more compact manner, and the shoot-production frequency was only one-third of that in calli transformed with the *iaaM* gene in antisense orientation. About 40% of the shoots formed roots after transfer to root-induction medium, and rooted shoots were transferred to greenhouse conditions. In all, six 35S-*iaaM*(s)/*iaaH* and 18 35S-*iaaM*(a-s)/*iaaH* plants were obtained from about 120 calli of each genotype.

Two of the six primary 35S-*iaaM*(s)/*iaaH* transformants exhibited leaf curling or epinasty and a reduced growth rate early in development. One of these plants (denoted A) was grown to the 11-node stage and assayed for free and conjugated IAA in leaves and internodes (not shown). The axillary buds remaining at the stem base of plant A after harvest were not released from apical dominance as was the case in wild-type plants decapitated in the same way; hence, plant A was lost. The other plant (denoted B) was left intact and followed to anthesis. However, its flowers did not produce petals and stamens; thus, the plant died without forming seeds. An attempt to pollinate with wild-type pollen was unsuccessful. The remaining four plants lacked any obvious phenotypical alterations, and seeds were obtained after self-fertilization. However, after sowing these seeds (denoted T1 seeds), two lines (denoted C and D, Fig. 1, a and b) displayed an aberrant phenotype, and plants of these lines were used in the subsequent work. Plants from the remaining two T1 seed-lots appeared fully normal and were not further investigated.

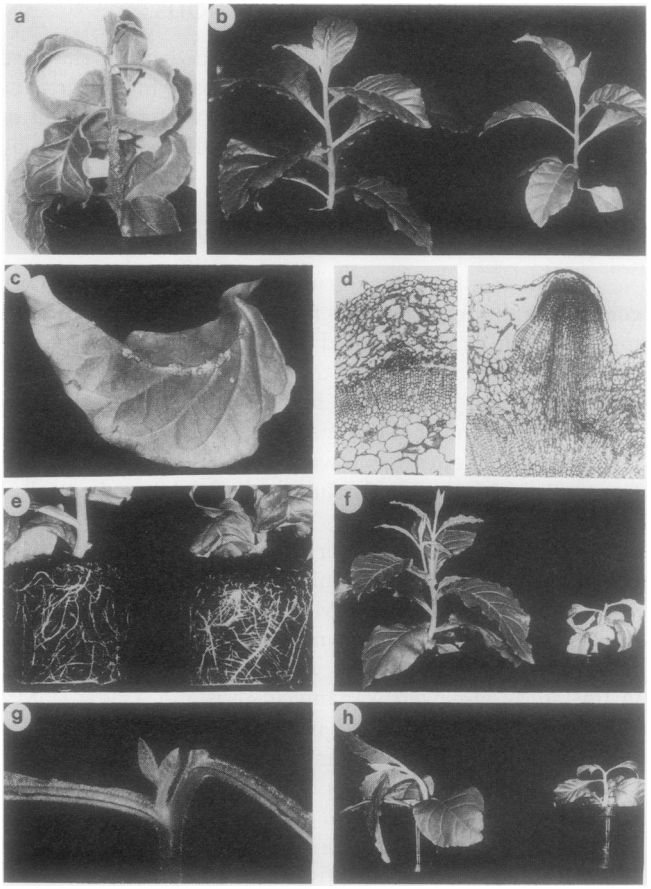

Figure 1. Morphology of 35S-*iaaM*/*iaaH* line C and line D plants. a, Transformant line C plant; b, wild-type SR1 (left) and transformant line D plant (right), both of the same age; c, adventitious root formation on midvein of a line C leaf; d, cross-sections of a line C stem showing adventitious root initiation in the cambial region (left) and an adventitious root after further development (right); e, root formation in wild-type (left) and line C (right) plants; f, appearance of wild-type (left) and line C (right) plants 21 d after decapitation (note the absence of elongating axillary shoots in the transformant); g, axillary shoot growth in the line C plant shown in f, 4 d after applying BA (1.5 μL of a 4 mM solution in 80% ethanol) to the leaf axil; h, positive phototropism in wild-type (left) and line C (right) plants after 48 h of exposure to unilateral light.

Growth and Development in Wild-Type and 35S-*iaaM*/*iaaH* Plants

Height growth and node formation rates were reduced in line C and D plants compared to wild type. The time taken to reach the height of 50 cm was 53 d in wild type, whereas line D required 60 d and line C 130 d. Similarly, the time required to produce 11 nodes was 50 d in wild type, but 56 d in line D and 80 d in line C. When wild-type and transformed plants with equal node numbers were compared, it was evident that the reduced height of transformants was attributable to a decrease in the elongation of every internode (Table I). Internode diameter was also reduced in the transformants (Table I). On a percentage basis, pith diameter was decreased in transformants, whereas the widths of xylem and extraxylary tissues were increased, particularly in line C plants (Table I). All transformants displayed epinastic leaf growth, and their leaves were narrower and shorter than those of wild type (Table I).

Adventitious root formation was prolific along the stem of transformants. This was most evident in line C (Fig. 1a), but also occurred in plants A and B, and occasionally in line D plants. Adventitious root formation was enhanced by increasing the air humidity. In line C, adventitious roots sometimes also developed on the leaf midveins (Fig. 1c). Formation of adventitious roots began at the stem base when plants had produced about six nodes, and proceeded acropetally as the plants aged. Initiation was normal, occurring in the cambial region (Fig. 1d). Lateral root production was also enhanced (Fig. 1e). Axillary bud outgrowth, which occurred in wild-type plants when they reached anthesis or were bent horizontally, was never detected in line C plants. Decapitation induced elongation of axillary buds within 1 week in wild-type SR1 and line D, but not in line C (Fig. 1f), even after 2 months. The inhibition of axillary bud outgrowth was not attributable to the absence of buds, because microscopical examination revealed their presence (not shown). However, a single application of the cytokinin benzyladenine, either to the apical cut surface or directly onto the leaf axil, induced axillary bud elongation in both wild-type and line C plants (Fig. 1g). When placed horizontally or illuminated unilaterally, gravitropic and phototropic responses were similar in line C and wild-type plants (Fig. 1h). However, the rigidity of line C stems prevented strict analysis of the rate and degree of response.

Flowering in wild-type SR1 is day-length neutral, and occurred when plants had formed 16 nodes, 60 d from sowing (Table II). Under conditions of high RH, line C plants died without flowering when they had formed 14 to 16 nodes and were 3 to 4 months old. However, flowering occurred in 30 to 40% of the plants if they were grown in low RH (10–30%), which suppressed adventitious root formation. Nevertheless, flowering was delayed and fewer flower buds were produced (Table II). Transformants were shorter than wild type, but had more nodes at the time of flowering (Table II). Transformant flowers were fertile, but occasionally line C flowers were sterile because of impaired pollen production. Offspring from both self- and back-crosses retained the transformed phenotype.

Table II. *Flowering in Wild-Type SR1, and 35S-iaaM/iaaH Line C and D Plants*

Plants were grown under standard conditions, with low RH (10–30%). Mean ± SD, $n \geq 6$.

Plant	Days to Flowering	Height	Number	
			Nodes	Flower buds
		cm		
SR1	60.5 ± 2.1	104.1 ± 4.4	15.7 ± 0.4	65.2 ± 15.3
Line D	68.3 ± 2.6	81.3 ± 6.2	16.1 ± 0.6	58.2 ± 6.6
Line C	154 ± 13	67.4 ± 8.4	26.7 ± 2.2	4.7 ± 1.7

Lignin Determinations

The lignin content of the whole internode was higher in line C than in wild type, the difference being most apparent in the apical internodes (Table III). Within the internode, the amount of lignin in line C was higher than in wild type in the xylem, lower in the extraxylary tissues, and the same in the pith. The content of lignin in the xylem increased centripetally.

Southern and Northern Analysis of Transformed Plants

When line C or line D seeds were selected for both hygromycin and kanamycin resistance, only plants with the aberrant phenotypes were obtained. However, two classes of plants were obtained after selecting for hygromycin resistance alone. One class showed the aberrant phenotype, whereas the other was similar to wild type. Southern analysis of both types of hygromycin-resistant plants showed that those with

Table I. *Internode and Leaf Size of Wild-Type SR1 and 35S-iaaM/iaaH Line C and D Plants*

Plants were measured at the 16-node stage. All internodes and leaves were measured, but only representative data are presented. Mean ± SD, n = 8, 4, and 6 for wild-type, line C, and line D plants, respectively.

Plant	Internode No. 9					Leaf No. 9	
	Length	Diameter	Percent of diameter			Length	Width
			Pith	Xylem	Extraxylary		
	mm	*mm*				*mm*	*mm*
SR1	68 ± 12	8.7 ± 0.9	80.4 ± 0.1	9.1 ± 0.1	10.5 ± 0.3	305 ± 27	141 ± 20
Line D	57 ± 15	7.8 ± 0.6	78.3 ± 2.7	9.9 ± 1.9	12.3 ± 1.8	291 ± 26	105 ± 16
Line C	17 ± 5	6.4 ± 0.4	62.6 ± 0.6	18.1 ± 0.5	19.3 ± 0.3	181 ± 15	82 ± 15

Table III. *Lignin Content in Wild-Type SR1 and in 35S-iaaM/iaaH Line C Plants*

A, Stem segments of plants with 11 nodes were divided into internodes 1 to 5, and 6 to 9, and the lignin content was determined in each part. B, Stems from plants with 12 nodes were subdivided radially into the tissue fractions listed. Xylem fraction 1 was composed of differentiating cells, whereas fractions 2 and 3 contained fully differentiated cells, those in fraction 2 being youngest. For each individual analysis (*n*), material was pooled from the indicated number of plants (m). Care was taken not to include tissues with adventitious roots. Mean ± SD.

Tissue	Lignin Content (A_{280} mg^{-1} CWR)					
	Wild type	*n*	m	Line C	*n*	m
A.						
Internode 1-5	0.114 ± 0.010	7	1	0.505 ± 0.007	5	3
Internode 6-9	0.221 ± 0.022	5	1	0.506 ± 0.042	2	3
B.						
Extraxylary	0.135 ± 0.007	4	4	0.070 ± 0.015	4	8
Xylem, fraction 1	0.143 ± 0.013	4	4	0.380 ± 0.094	2	8
Xylem, fraction 2	0.276 ± 0.063	4	4	0.669	1	16
Xylem, fraction 3	0.412 ± 0.037	4	4	0.528 ± 0.039	2	8
Pith	0.072 ± 0.007	3	4	0.072 ± 0.006	2	8

the aberrant phenotype contained both the 35S-*iaaM* and the *iaaH* genes (in five plants out of five), whereas those with the normal phenotype contained only the 35S-*iaaM* gene (in four plants out of four) (Fig. 2). Furthermore, the Southern analysis showed the expected internal *iaaM* *Hind*III fragments of 0.75 and 0.95 kb. Depending on the insertion in the plant chromosome, an additional band larger than 5.6 kb would also be expected. In line C, one such band (6.2 kb) was detected, indicating a single-gene insertion, whereas in line D, two bands were observed (6.6 and 9.6 kb, not shown), indicating a double-gene insertion. Analysis of the *iaaH* structure in transformed plants showed an internal *Eco*RI fragment (1.0 kb) and one fragment (6.4 kb) identical to the original rG2 plant.

Northern analysis revealed that the *iaaM* transcript levels increased acropetally in leaves and basipetally in internodes (Fig. 3). Transcript levels were similar in line C and D leaves, but clearly higher in line C internodes.

Free and Conjugated IAA Determinations

For both leaves and internodes, free and conjugated IAA levels were consistently higher in the transformed plants than in wild type (Fig. 4). In leaves, the largest increases in free IAA were 210% in line D and 400% in line C. In internodes, the maximal increases were 110% in line D and 1100% in line C. The free IAA contents in leaf and internode number 5 of line C hygromycin-resistant plants with a normal phenotype, containing the 35S-*iaaM* gene alone, were slightly increased compared with wild type (Table IV), whereas the free IAA levels in aberrant plants containing both 35S-*iaaM* and *iaaH* genes were elevated to a greater extent.

DISCUSSION

It is well known that the balance between cytokinin and auxin in the medium is crucial for the development of tobacco calli in culture (25). A high cytokinin/auxin ratio induces shoot production, whereas a low ratio promotes root formation. In the present study, doubly transformed calli had a relatively compact morphology, pale color, and low viability on auxin-containing medium, which suggests an increased IAA content (*cf.* 1). Shoot-formation occurred only in these calli when they were cultivated on a shoot-inducing medium that lacked auxin. These observations suggest the existence of an IAA "window," permitting shoot differentiation only in calli with a certain endogenous IAA level. Some of the primary transformants exhibited an aberrant phenotype (plants A and B), whereas others (plants C and D) appeared normal, but showed an abnormal phenotype after a self-cross. Presently, we have no explanation for the latter change in phenotype other than an altered expression of the 35S-*iaaM* or *iaaH* genes, possibly due to different methylation patterns of the T-DNA (6, 19, 32).

Plants with aberrant phenotypes appeared quite different from each another when mature. This variation might reflect position effects in the expression of the 35S-*iaaM* gene, because we observed a positive correlation between *iaaM* transcript levels and IAA content (Figs. 3 and 4). This suggests that position effects of the introduced genes as well as promoter-shifts might be used to create differences in IAA levels in transformed plants. The simultaneous expression of the 35S-*iaaM* and *iaaH* genes markedly elevated the levels of free and conjugated IAA (Fig. 4; Table IV), which were associated with reduced stem and leaf growth, delayed flowering, and increased adventitious and lateral root formation, leaf epinasty, apical dominance, and xylem lignification (Fig. 1; Tables I–III). Some of these effects were also observed in petunia transformed with a 19S-*iaaM* construct, which resulted in a 10-fold increase of free IAA in leaves (15). Such responses are well known from exogenous auxin application studies (2) and, thus, results obtained with exogenous auxin are confirmed for intact systems in which endogenous IAA is overexpressed. However, auxin typically is applied to excised plant segments, and the use of transgenic plants with an endogenous IAA overproduction should allow IAA effects to be studied in relation to the growth of intact plants.

The causal relationship between IAA overproduction and reduced growth in the transformed plants is not known. It is

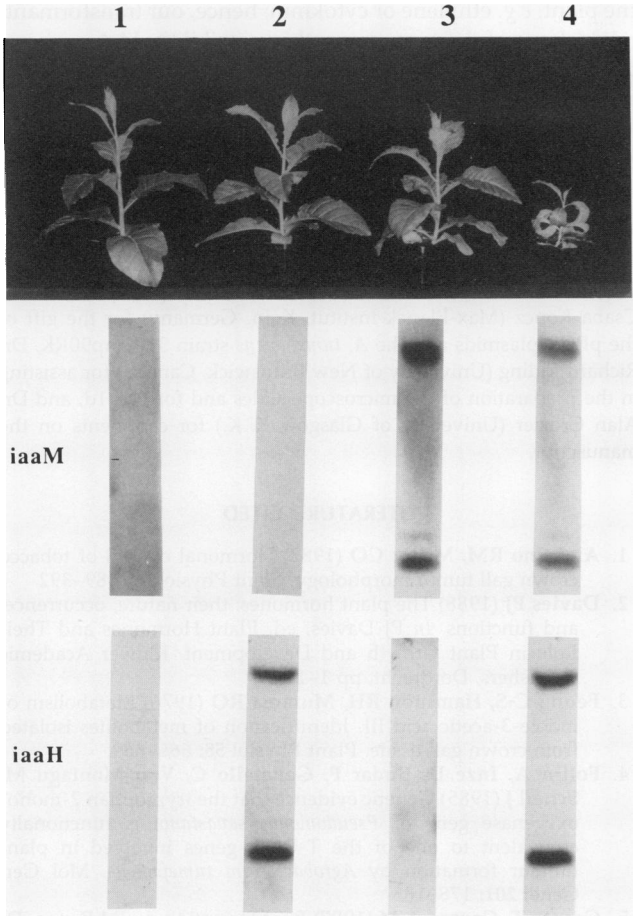

Figure 2. Southern analysis of wild-type SR1, rG2, and 35S-*iaaH*/*iaaM* line C plants. DNA in the upper row was digested with *Hind*III and hybridized to the *iaaM* gene. Estimated fragment sizes: 0.76, 0.95, and 6.2 kb. DNA in the lower row was digested with *Eco*RI and hybridized with the *iaaH* gene. Estimated fragment sizes: 1.0 and 6.4 kb. 1, Wild-type SR1; 2, rG2 plant, selected for kanamycin resistance; 3, line C plant, selected for hygromycin resistance, normal phenotype; 4, line C plant, selected for hygromycin resistance, aberrant phenotype.

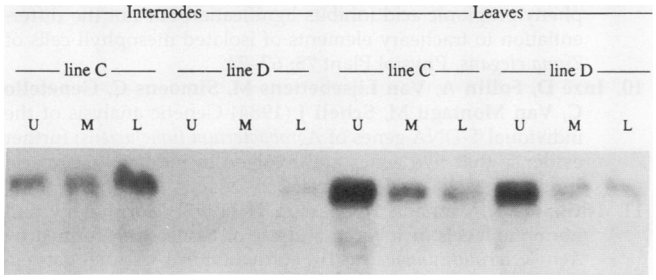

Figure 3. Northern analysis of *iaaM* transcription in leaves and internodes of 35S-*iaaM*/*iaaH* line C and D plants. Tissue was pooled from two plants, both selected for hygromycin and kanamycin resistance. RNA was extracted from upper (U), middle (M), and lower (L) stem parts of equal size, and from apex plus leaves 1 to 3 (U), leaf 6 (M), and leaf 9 (L). Thirty micrograms of total RNA were loaded in each lane. The *iaaM* transcript size was estimated to be 2.4 kb.

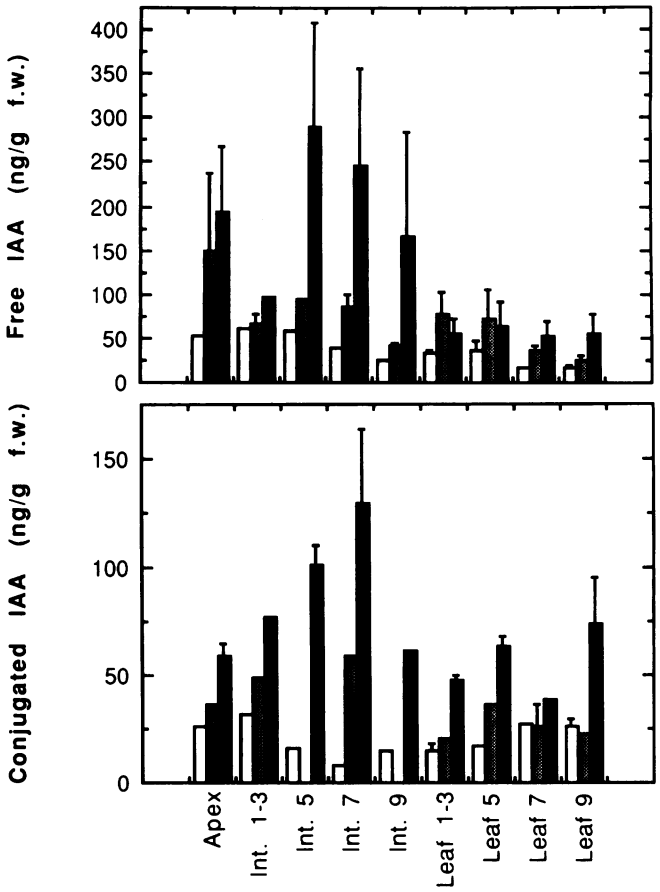

Figure 4. Free and conjugated IAA levels in wild-type SR1 and 35S-*iaaM*/*iaaH* plants. The presence of *iaaM* and *iaaH* genes in line C and D plants was verified by Southern analysis. Mean ± range of two wild-type and line D plants, and of three line C plants. The figure represents 120 measurements, of which 30 were made in duplicate. The average variation in duplicate samples was 10%. SR1, open bar; line D, shaded bar; line C, solid bar.

Table IV. *Free IAA Content in Wild-Type SR1 and Line C Plants*

Line C seeds were selected on hygromycin-containing medium, and resistant seedlings with normal or aberrant phenotypes were transferred to peat. Free IAA was assayed in plants with 11 nodes, the genotypes being verified by Southern analysis. Mean ± SD. n = 2 and 3 in SR1 and line C, respectively.

Plant	Genotype	Phenotype	Free IAA Content	
			Leaf No. 5	Internode No. 5
			ng g^{-1} fresh weight	
SR1	Wild-type	Normal	35 ± 11	57 ± 10
Line C	35S-*iaaM*	Normal	40 ± 21	78 ± 14
Line C	35S-*iaaM* *iaaH*	Aberrant	62 ± 29	291 ± 117

possible that increased lignification restricted cell growth, as was suggested for tomato plants treated with IAA (18). Another possibility is that the high IAA levels inhibited stomatal opening by decreasing root permeability (33). This is supported by our observation that line C plants, compared to wild type, had a decreased stomatal conductance and, thus, a decreased CO_2 assimilation rate (unpublished data).

Although it is well documented that exogenous auxin induces adventitious root formation (5, 12), there exist only limited data on the quantitative relationship between the endogenous IAA level and adventitious rooting (12). Therefore, it is interesting to note that these two parameters were positively correlated in the basal internodes of line C plants (Figs. 1a and 4). Furthermore, increased initiation of lateral roots was observed in line C and D plants (Fig. 1c), a phenomenon that also is known to be stimulated by exogenous auxin application (30).

The importance of IAA and cytokinin in controlling apical dominance is well established from feeding experiments (7). A high cytokinin/IAA ratio was associated with a shooty phenotype in tobacco plants transformed with the *A. tumefaciens ipt* gene (26). In contrast, two observations suggest that a low cytokinin/IAA ratio was induced in line C plants. First, axillary shoot outgrowth was inhibited, which was overcome by applying cytokinin to the bud (Fig. 1g). Second, apical dominance in line D plants, in which *iaaM* gene expression and IAA levels in the stem were lower than in line C plants (Figs. 3 and 4), was similar to wild type. The demonstration that plants expressing a 35S-*iaaL* construct had a reduced apical dominance associated with decreased free IAA levels due to the conjugation of IAA to lysine by the action of the *iaaL* gene product (21) further stresses the importance of the IAA level and the IAA/cytokinin ratio in apical dominance. However, nothing is yet known about the cytokinin content of our transformants.

Line C plants, which contained the highest IAA levels (Fig. 4), also exhibited the most pronounced changes in phenotype. We previously reported (24) that conjugated IAA increased more than free IAA in rG2 plants transformed with the natural *iaaM* gene. These plants had a normal phenotype and a relatively small (up to 60%) increase in free IAA, which suggests that conjugation plays a role in regulating the level of free IAA in tobacco, thereby maintaining a normal phenotype. However, when the amount of IAA produced is very large, as in our 35S-*iaaM*/*iaaH* transformants, the capacity of the conjugation process is presumably exceeded, resulting in high IAA levels and abnormal growth.

Whereas expression of a 35S-*iaaM* construct in tobacco SR1 elevated the free IAA level in leaf tissue by only 14% (Table IV), expression of a 19S-*iaaM* construct in petunia and tobacco cv Samsun caused tenfold and fivefold increases, respectively (15, 21). This may reflect between species variation, as hydrolysis of exogenous IAM to IAA has been shown to differ between plant species and cultivars (14). Furthermore, because our 35S-*iaaM* plants originally were selected also to contain the *iaaH* gene, we may indirectly have selected for a relatively low expression of the *iaaM* gene. We do not know if the changes in phenotype reported in this study are directly or indirectly caused by IAA. Increased IAA levels may influence the biosynthesis of other hormones in the plant, *e.g.* ethylene or cytokinin; hence, our transformants might be useful for studying this possibility. IAA-overproducing plants also would be valuable for studying particular aspects of normal IAA metabolism, such as feedback regulation of synthesis and induction of catabolism.

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