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Increased Endogenous Auxin Production in *Arabidopsis thaliana* Causes Both Earlier Described and Novel Auxin-Related Phenotypes

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Abstract

Transgenic *Arabidopsis thaliana* plants harboring the *Agrobacterium tumefaciens* phytohormone-biosynthetic genes *iaaM* and *iaaH* display an altered development indicative of elevated auxin levels. These plants exhibit an increased apical dominance, increased hypocotyl and petiole length and epinastic leaves and cotyledons. In addition, the transgenic plants display the formation of necrotic spots on leaves and bracts in soil and an altered behavior of axillary buds in tissue culture. Despite transcriptional activity of the *iaaM* and *iaaH* promoters in the vasculature of root tissue, root development of young *iaaM/iaaH* transgenic plants is identical to wild type. However, transgenic *iaaM/iaaH* plants grown under tissue culture conditions for a prolonged period display both vigorous and ectopic root growth. The level of free IAA in the rosette of the *iaaM/iaaH* transgenic plants is increased approximately 2-fold compared to wild type. The number of cells comprising the vascular tissue in hypocotyls and inflorescence stems of the transgenic plants is reduced and these plants exhibit a reduced basipetal polar auxin transport in the inflorescence stems. This reduced polar auxin transport probably accounts for the rapid auxin effect on the aerial part of the transgenic plants and the late effect on root development.

Key words: Apical dominance; *Arabidopsis*; Auxin overproduction; Epinasty; *iaaH*, *iaaM*; Necrotic spots; Polar auxin transport; Transgenic; Vascular differentiation

Introduction

The plant hormone auxin (IAA) influences a wide variety of developmental pathways such as apical dominance, cell elongation, the establishment and maintenance of the root meristem, the initiation of lateral organs and the formation of vascular tissue (Costa and Dolan 2000; Reinhardt and others 2000;

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Swarup and others 2000). Together with the plant hormone cytokinin, auxin influences cell division and morphogenesis (Eklof and others 2000). The levels of free IAA are regulated by a complex balance among biosynthesis, conjugation, catabolism and efflux (Bartel and others 2001; Delarue and others 1999; Ljung and others 2002). To study the role of auxin in plant development mutants overproducing auxin can be exploited. Several such mutants have been described for *Arabidopsis thaliana*. The *sur*1/*alf*1/*rty*/*hsl*3 (Boerjan and others 1995), *sur*2 (Barlier and others 2000) and its stronger allele *rnt* (Bak and others 2001) and *yucca* (Zhao and others 2001) mutants display similar developmental alterations correlated with increased auxin levels. These include an increased apical dominance, increased root formation, increased cell elongation and the formation of epinastic cotyledons and leaves. In contrast, the auxin-overproducing *bus*1 (Reintanz and others 2001) and the allelic *sps* mutant (Tantikanjana and others 2001) exhibit the formation of bushy shoots, retarded onset of vascularization and upwards curling leaves. However, *sps* furthermore contains increased cytokinin levels (Tantikanjana and others 2001), which might explain the *bus*1/*sps* phenotype deviating from the other auxin-overproducing mutants.

Alternatively, endogenous auxin levels can be manipulated by the expression of the *Agrobacterium tumefaciens* phytohormone-biosynthetic genes *iaaM* and *iaaH*, encoding the *TRYPTOPHAN MONOOXYGENASE* and the *INDOLEACETAMIDE HYDROLASE* gene, respectively (for review, Gaudin and others 1994; Hamill 1993). Such genetic alterations cause a continuous presence of elevated auxin levels in the affected plant, which allows *in vivo* studies of its effects on plant development. Such studies have been performed in many plant species such as tobacco, petunia and potato, where elevated auxin levels caused an increased adventitious root formation, dwarfism, formation of epinastic leaves and an increased apical dominance (Gaudin and others 1994; Hamill 1993). The extent of the alteration in development was shown to be dependent on the relative strength of the promoters used to drive the expression of the *iaaM/iaaH* genes (Sitbon and others 1991; 1992b). Furthermore, free IAA levels could not be increased withouts limits because 'excess'-free IAA was converted into inactive auxin-conjugates (Sitbon and others 1992a; 1993).

To date, only one *Arabidopsis thaliana* transgenic line exhibiting an altered development caused by the expression of *Agrobacterium* phytohormone-biosynthetic genes has been described in detail. In this 19S-*iaaM* transgenic, the expression of the *iaaM* gene was controlled by the constitutive CaMV 19S promoter (Romano and others 1993), which resulted in clear auxin-related alterations in development such as epinastic leaves, elongated hypocotyl length and increased apical dominance. These effects were shown to be independent of putative secondary ethylene effects resulting from auxin-induced ethylene production (Romano and others 1993). Although the expression of both the *iaaM* and *iaaH* genes is normally required for increased IAA levels, the expression of the *iaaM* gene alone in this transgenic line apparently caused increased IAA levels either by spontaneous hydrolysis of IAM to IAA or by low affinity enzymatic conversion (Klee and others 1987).

We have now generated transgenic *Arabidopsis thaliana* lines harboring both the *Agrobacterium tumefaciens* phytohormone-biosynthetic genes *iaaM* and *iaaH* under the transcriptional control of their natural promoters. The expression of the *iaaM* and *iaaH* genes caused an increase in IAA levels. The effects on development, which included a number of phenotypic alterations that have not been associated with increased auxin levels before, will be described.

Materials and Methods

Plant Material

The *iaaM/iaaH*, *iaaM*:*GUS* and *iaaH*:*GUS* plant transformation vectors used (kindly provided by Clement and Hoge, unpublished) were based on the pBin19 vector (Bevan 1984) and contained the kanamycin resistance gene as plant selectable marker. The *iaaM/iaaH* construct harbored the *iaaM* and *iaaH* genes from the *Agrobacterium tumefaciens* octopine plasmid pTiAch5 driven by their natural promoters. The *iaaM*:*GUS* and *iaaH*:*GUS* constructs contained the promoters of the *iaaM* and *iaaH* genes, respectively, fused to the *GUS* reporter gene.

Plant Transformation and Growth Conditions

The transgenic lines described in this study were generated using a leaf transformation method as described before (van der Graaff and Hooykaas 1996) in the *Arabidopsis thaliana* ecotype C24. Transgenic shoots were regenerated on kanamycin selective medium (50 mg/L). The T_2 generation was obtained from this T_1 generation using glass tubes for seed set in tissue culture. The regeneration of shoots from the leaf explants transformed with the *iaaM/iaaH* construct required alteration of the standard phytohormone levels by increasing the

BAP concentration in the shoot induction medium from 1 mg/L to 5 mg/L and the reduction of the NAA concentration from 0.1 mg/L to 0.01 mg/L. Plant growth analysis and the assay for hypocotyl length of dark grown plants in the presence of the phytohormone BAP were performed as described (van der Graaff and others 2001). The experiments described in this study were performed with transgenic seeds derived from the third and fourth (T_3 and T_4) generation obtained by seed set in soil.

PCR Analysis

The presence of the *iaaM/iaaH* T-DNA in the transgenic lines was detected by PCR as described (van der Graaff and others 2001) using primers for the coding region of the kanamycin gene producing a 574 bp fragment upon amplification. Primers for the *GLYCERALDEHYDE 3P-DEHYDROGENASE CYTOSOLIC* gene (*GapC*) producing a 1300 bp fragment from genomic DNA (van der Graaff and others 2001) were used to analyze the efficiency of the PCR amplification.

IAA Measurement

For IAA measurements, samples were extracted, purified and analyzed by GC-SRM-MS (gas chromatography-selected reaction monitoring-mass spectrometry) as previously described (Bhalerao and others 2002). Calculation of isotopic dilution factors was based on the addition of 50 pg [^{13}C6]IAA/mg tissue.

Root Growth

Surface sterilized seeds were sown and pushed into half strength Murashige and Skoog agar medium (Murashige and Skoog 1962), ensuring root growth in the medium, and grown for 14 days. The petri dishes were inclined 60° to allow the roots to grow in a straight line. The length of the roots was measured along a millimeter scale and lateral roots were counted using a binocular microscope.

Anatomical Analysis

Glutaraldehyde-fixed material was embedded in epon, sectioned and stained as described before (van der Graaff and others 2000).

Auxin Transport

Segments (16 mm long) were removed from the inflorescence stems of bolting wild-type and *iaaM/iaaH*-40 transgenic plants and incubated in an inverted orientation on agar blocks containing 10^{-7} M^{3}H -IAA for 2.5 hours for measurement of basipetal polar auxin transport (PAT). After incubation the segments were cut in three parts: 1) lower part (4 mm) of segment, which was in full contact with the ^{3}H-IAA containing agarblock: 2) mid-part (4 mm) of segment and 3) top part (8 mm) of segment. The amount of ^{3}H-IAA accumulated in these parts was determined by a scintillation counter. For each experiment 8 different flowerstalks were assayed.

Plant Crosses

To obtain *iaaM/iaaH*ipt* double transgenic plants, pollen from homozygous transgenic *ipt*-161 plants (van der Graaff and others 2001) was transferred to the stigma of the transgenic *iaaM/iaaH*-40 plants, displaying the strong auxin phenotype. The progenies of these crosses were screened for plants displaying development that deviated from either of the single parents. Such plants were allowed to set seed and the resulting progenies were used to analyze the combined effect of an increased cytokinin and auxin production on *Arabidopsis* development.

Results

Expression Conferred by *iaaM* and *iaaH* Promoters

The expression pattern of the *iaaM* and *iaaH* genes in *Arabidopsis* was examined using transgenic lines harboring *iaaM* and *iaaH* promoter *GUS* fusions. This analysis showed that the *iaaM* promoter (16 independent lines) is active in the vascular tissue of root and hypocotyl (Figure 1A–E). Despite differences in the levels of GUS activity displayed by the independent *iaaM*:*GUS* transgenic lines, the spatial expression patterns were comparable (Figure 1A–C). GUS activity was furthermore detected at the shoot apex and leaf primordia (Figure 1A, 1C and 1E), whereas cotyledons and rosette leaves exhibited no detectable GUS activity (Figure 1A, 1B and 1D). The *iaaH* promoter (18 independent lines) conferred an expression pattern similar to the *iaaM* promoter. However, the activity of the *iaaH* promoter was lower (data not shown).

Generation of *iaaM/iaaH* Transgenic Lines

The transformation of *Arabidopsis* leaf discs with the *iaaM/iaaH* plasmid resulted in abundant callus formation at wound sites on the infected leaf discs.

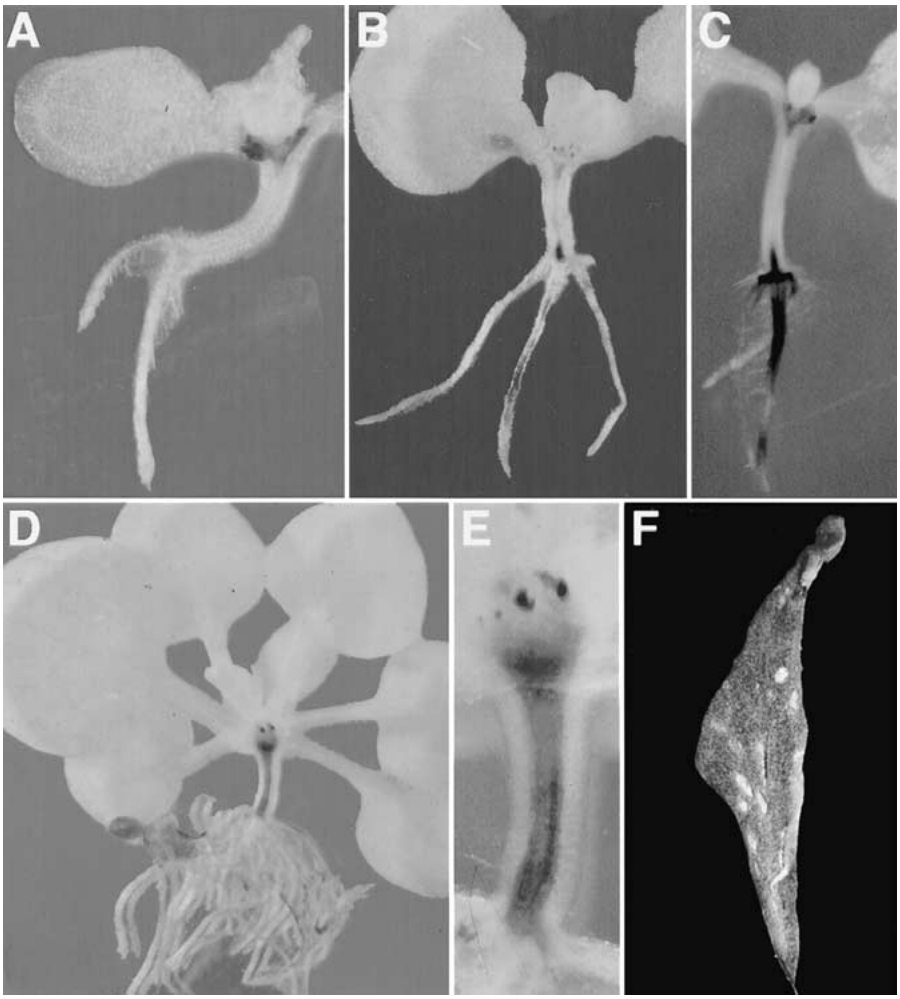

Figure 1. Expression conferred by the *iaaM* and *iaaH* promoters. *GUS* expression pattern conferred by the *iaaM* promoter in 9-day-old (**A–C**) and 21-day-old (**D–E**) transgenic lines grown in tissue culture. (**A**), (**B**) and (**C**) weak, medium and strong levels of GUS activity in the *iaaM*:*GUS* lines 18, 20 and 56, respectively. (**D**) *iaaM*:*GUS* line 21 exhibiting medium levels of GUS activity. (**E**) Detail from **D** showing restriction of GUS activity to vascular tissue in hypocotyl. (**F**) Necrotic spot formation on representative rosette leaf from 6-week-old *iaaM/iaaH*-40 transgenic plant grown in soil.

Occasionally root-like structures differentiated from this callus, which resembled the callus structures formed by the *rooty callus* lines described by Frank and others (2000). Regeneration of shoot-like structures was never observed using the standard hormone regime in the leaf disc transformation. However, by increasing the cytokinin to auxin ratio in the shoot induction medium (see Materials and Methods) callus formation became less abundant and occasionally small green leaf-like structures differentiated. Separation of such structures from the callus and subsequent growth on medium with an increased cytokinin to auxin ratio, resulted in the regeneration of several shoots.

The *iaaM/iaaH* Transgenic Lines Display Both Earlier-Described and Novel Auxin-Related Phenotypic Changes

Eventually, seeds were obtained for 9 transgenic lines harboring the *iaaM* and *iaaH* genes. In tissue culture the progenies from 3 lines (*iaaM/iaaH*-32, 40 and 41) exhibited a clear auxin phenotype (Figures 2B and 2C), including a rapid elongation of the hypocotyl (Table 1 and Figure 2B), elongated petioles and epinastic leaves and cotyledons (Figure 2C). The development of the lines *iaaM/iaaH*-32, 40 and 41 proved to be indistinguishable from each other and *iaaM/iaaH*-40 was chosen as the representative line. Using PCR, this transgenic was shown to contain the *iaaM/iaaH* T-DNA construct (data not shown). Upon flowering, the *iaaM/iaaH*-40 transgenic plants produced unbranched flower stalks indicating an increased apical dominance (Figure 2G). An extensive root growth was observed in 8-week-old seedlings and occasionally ectopic root formation was encountered on flowering transformants (Figure 2G). Sometimes callus formation was observed on the elongated hypocotyl of such plants (data not shown). When inflorescence segments, including bracts (Figure 2D), were grown on hormone-free medium, the axillary buds were able to form shoots exhibiting the auxin phenotype (Figure 2E and 2F). Such bud outgrowth was never

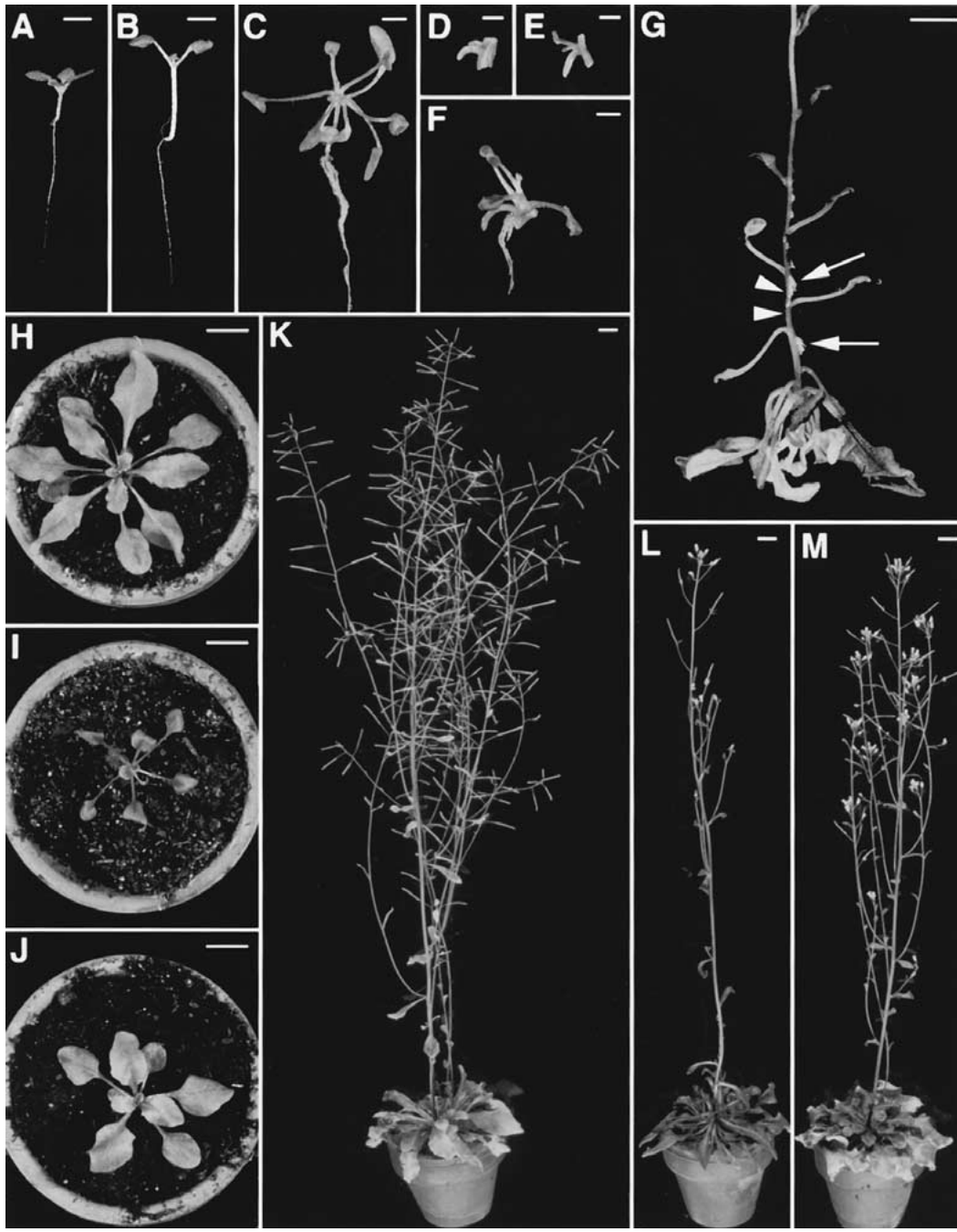

Figure 2. Development of the *iaaM/iaaH* transgenic lines. The plants shown in **A–G** were grown in tissue culture and those shown in **H–M** in soil. Development of 1-week-old wild type (**A**) and *iaaM/iaaH*-40 seedling (**B**), respectively. Note the rapid elongation of the hypocotyl and petioles in **B**. (**C**) Development of 3-week-old *iaaM/iaaH*-40 seedling. Segments grown in tissue culture for 1 day (**D**), 1 week (**E**) and 3 weeks (**F**), respectively, after removal from an *iaaM/iaaH*-40 inflorescence stem. (**G**) Flowering *iaaM/iaaH*-40 transgenic plant. Note that the primary root system has been removed. Arrows indicate the ectopic root growth on the flowerstalk and arrowheads indicate the segments cut from the flowerstalks which were subsequently grown in tissue culture. Development in soil of 3-week-old wild type (**H**), *iaaM/iaaH*-40 (**I**) and *iaaM/iaaH*-18 (**J**) seedling, respectively. Inflorescence structure from 8-week-old wild type (**K**), *iaaM/iaaH*-40 (**L**) and *iaaM/iaaH*-18 (**M**), respectively. Note that the *iaaM/iaaH* inflorescence stems are shorter than wild type because of the later onset of bolting. Scale bars in **A–F** = 2 mm; **G–M** =1 cm.

Table 1. Hypocotyl Length of Wild Type and the *iaaM/iaaH*-40 Transgenic Line Grown for 18 Days in Tissue Culture

	Wild type		*iaaM/iaaH*-40	
	Length in mm	n	Length in mm	n
Light	2.1 (0.2)	102	5.1 (1.3)	48
Dark	18.4 (1.3)	46	18.5 (3.8)	39

Seedlings were grown in either light or dark. The standard deviation is given in parenthesis, n = number of seedlings analyzed.

observed for wild-type inflorescence segments treated similarly (data not shown). The hypocotyl length of the *iaaM/iaaH*-40 transgenic line grown in the light was increased approximately 2.5-fold compared to wild type. However, no additional auxin effect on the hypocotyl length was observed for the *iaaM/iaaH*-40 transgenic line grown in the dark (Table 1), which is in agreement with earlier observations (Jensen and others 1998). The *iaaM/iaaH*-40 transgenic line furthermore showed a normal response to the application of the cytokinin BAP during growth in the dark (Table 2).

After germination and subsequent growth in soil, the *iaaM/iaaH*-32, 40 and 41 lines displayed epinastic leaves and elongated hypocotyl and petioles (Figure 2I). However, callus formation and ectopic root growth were not observed. Furthermore, these lines exhibited retarded senescence, the formation of necrotic spots on all rosette leaves and bracts after the onset of bolting (Figure 1F), retarded onset of flowering and increased apical dominance (Figure 2L). The internode length of the unbranched inflorescence was increased compared to wild-type plants resulting in an increased height of the inflorescence (data not shown).

The development of the progenies from the remaining six lines (*iaaM/iaaH*-3, 10, 11, 18, 19 and 20) was identical to wild type in tissue culture (data not shown). Upon germination and growth in soil these lines displayed a wild-type development (Figure 2J). However, for several lines, retarded senescence, the formation of brown necrotic spots on the rosette leaves and bracts after the onset of bolting, retarded onset of flowering, increased apical dominance (Figure 2M) and increased internode length were observed, although to a lesser extent than seen in the lines *iaaM/iaaH*-32, 40 and 41. Therefore, this phenotype is referred to as a weak auxin phenotype, whereas the auxin-related phenotype displayed by the *iaaM/iaaH*-32, 40 and 41 transgenic lines is referred to as a strong auxin phenotype.

Table 2. Effect of Cytokinin on the Hypocotyl Length of Wild Type and *iaaM/iaaH*-40 Transgenic Line Grown for 18 Days in the Dark

Concentration	Wild type		*iaaM/iaaH*-40	
BAP (in μM)	Length in mm	n	Length in mm	n
0.5	11.6 (1.2)	47	12.7 (2.4)	40
1	11.1 (1.7)	49	12.4 (1.6)	36
5	10.2 (1.9)	50	11.0 (2.3)	43
10	9.5 (1.6)	53	10.3 (2.6)	42
20	9.0 (1.1)	49	8.3 (1.8)	40
30	7.5 (1.2)	47	7.2 (1.7)	44
50	7.0 (0.8)	43	7.2 (1.6)	39

The standard deviation is given in parenthesis. n = number of seedlings analyzed.

The *iaaM/iaaH*-40 Transgenic Line Contains Elevated IAA Levels in the Rosette

When grown in tissue culture the *iaaM/iaaH*-40 transgenic line accumulated 1.8-fold higher levels of free IAA (Table 3, Experiment A), which is in the medium range increase of IAA levels compared to the strong IAA overproducing mutants *sur*1/*rty* (King and others 1996) and *sur*2 (Barlier and others 2000). This same increase in free IAA levels was also observed for young leaves of 4- and 7-week-old transgenic plants grown in soil (Table 3, Experiments B and C). In old leaves of plants grown in soil, the levels of free IAA decreased. However, this decrease was less pronounced for the transgenic plants and the difference in auxin levels between the *iaaM/iaaH*-40 transgenic line and wild type increased to 3.5 fold (Table 3, Experiments B and C).

The *iaaM/iaaH*-40 Transgenic Line Displays Wild-type Root Development

Based on the expression patterns conferred by the *iaaM* and *iaaH* promoters in *Arabidopsis*, a clear auxin effect on root development might be expected in the *iaaH/iaaM* transgenic lines. Instead, in young *iaaM/iaaH*-40 seedlings a rapid auxin effect was observed on the development of the aerial part without an obvious alteration in root development. Except for a slight increase in root length, neither the 'basal' root development nor the response towards exogenously applied NAA was altered compared to wild type (Figure 3). Furthermore, the *iaaM/iaaH*-40 transgenic line exhibited a wild-type response towards the exogenously applied auxin 2,4-D and the cytokinin BAP. Other *iaaH/iaaM* transgenic lines (*iaaM/iaaH*-3, 10, 18, 19 and 41) also exhibited wild-

Table 3. Free IAA Levels in Wild Type and *iaaM/iaaH*-40 Transgenic Line

		Wild type		*iaaM/iaaH*-40		
Experiment	Age leaves	Mean	n	Mean	n	Increase[1]
A	n.a.[2]	7.2 (0.9)	8	12.7 (1.5)	10	1.8
B	Young	21.2 (2.0)	4	38.0 (3.6)	5	1.8
	Old	4.9 (1.1)	6	17.0 (2.7)	7	3.5
C	Young	16.7 (2.6)	6	31.2 (4.9)	6	1.9
	Old	6.2 (1.1)	7	21.6 (3.6)	8	3.5

[1] *Increase of IAA levels in iaaM/iaaH-40 compared to wild type.*
[2] *n.a. = not applicable.*
*The levels of free IAA (pg/mg fresh weight) were measured in (**A**) 16-day-old seedlings grown in tissue culture, (**B**) 4-week-old and (**C**) 7-week-old plants grown in soil. The standard deviation is given in parenthesis. n = number of individual biological replicates.*

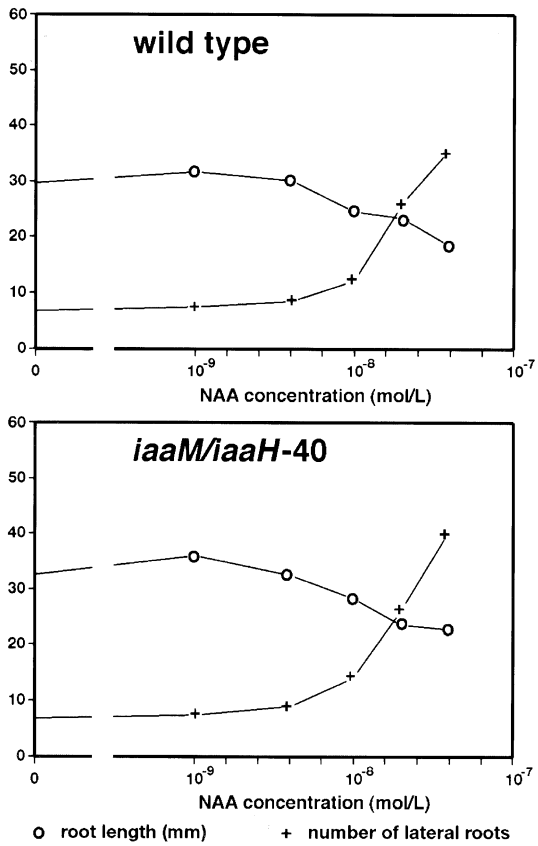

Figure 3. Root growth of wild type and *iaaM/iaaH*-40 transgenic line on medium supplemented with different concentrations of NAA. Average values for 40 seedlings per NAA concentration are shown.

type root development in response to exogenously applied NAA, 2,4-D and BAP (data not shown).

Basipetal PAT is Reduced in the *iaaM/iaaH*-40 Transgenic Line

Interestingly, we were not able to obtain homozygous progenies from the *iaaM/iaaH* transgenic lines exhibiting the strong auxin phenotype. Approximately one-third of the seedlings exhibiting an auxin phenotype segregated in the progeny of hemizygous plants from such transgenic lines failed to form a proper inflorescence structure. Instead, these plants formed pin-like structures comparable to those formed by the *pin*1 mutant or wild-type plants grown on polar auxin transport (PAT) inhibitors (Palme and Galweiler 1999). Together with the lack of a rapid auxin effect on root development this suggests that the *iaaM/iaaH* transgenic lines are disturbed in basipetal PAT. Measurements of basipetal PAT using ^{3}H-IAA in inflorescence stem segments from hemizygous *iaaM/iaaH*-40 transgenic plants indeed showed a 50% reduction in basipetal PAT compared to wild type (Figure 4B). The reduced basipetal PAT in the *iaaM/iaaH*-40 transgenic line was not caused by an increased acropetal PAT (Figure 4C).

Vascular Tissue Formation is Disturbed in the *iaaM/iaaH*-40 Transgenic Line

Anatomical analysis showed that the overall cellular structure of leaves, that is, the number of cell layers and cell morphology, was comparable to wild type (Figure 5A and 5B). However, the lower epidermal cell size of the *iaaM/iaaH*-40 transgenic plants was significantly reduced at the leaf margins where the leaf epinasty was most severe (data not shown).

Longitudinal sections of hypocotyls showed that the increase in length for the *iaaM/iaaH*-40 transgenic line was caused by an increase in cell length rather than an increase in the number of cells (Figure 5E and 5F). This increase in cell length was most evident for the epidermal and cortex cells and apparently took place without an increase in cell volume, which is evident by a smaller cell diameter (Figure 5C, 5D, 5E and 5F). In addition, the diameter of the hypocotyls was reduced and the neat

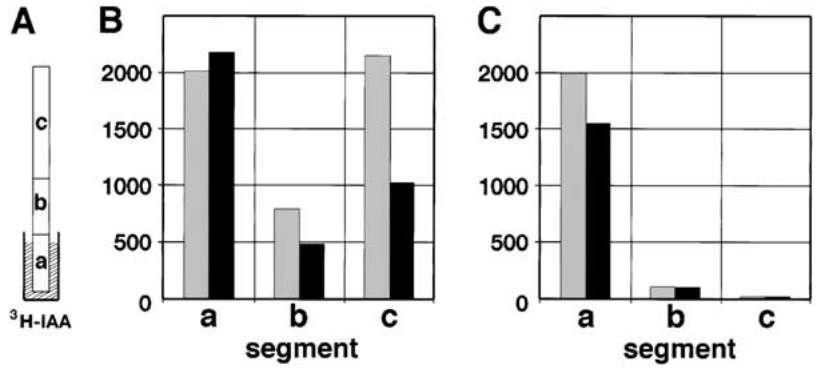

Figure 4. PAT in the inflorescence of wild-type (grey bars) and *iaaM/iaaH*-40 transgenic plants (black bars) exhibiting the strong auxin phenotype. (**A**) Transport of auxin is reflected by the accumulation of ^{3}H-IAA in segments b and c. (**B**) Inflorescence segments were incubated in an inverted orientation to measure active (basipetal) PAT. (**C**) Inflorescence segments were incubated in normal orientation to measure acropetal PAT. Numbers reflect radioactivity counts indicative of ^{3}H-IAA.

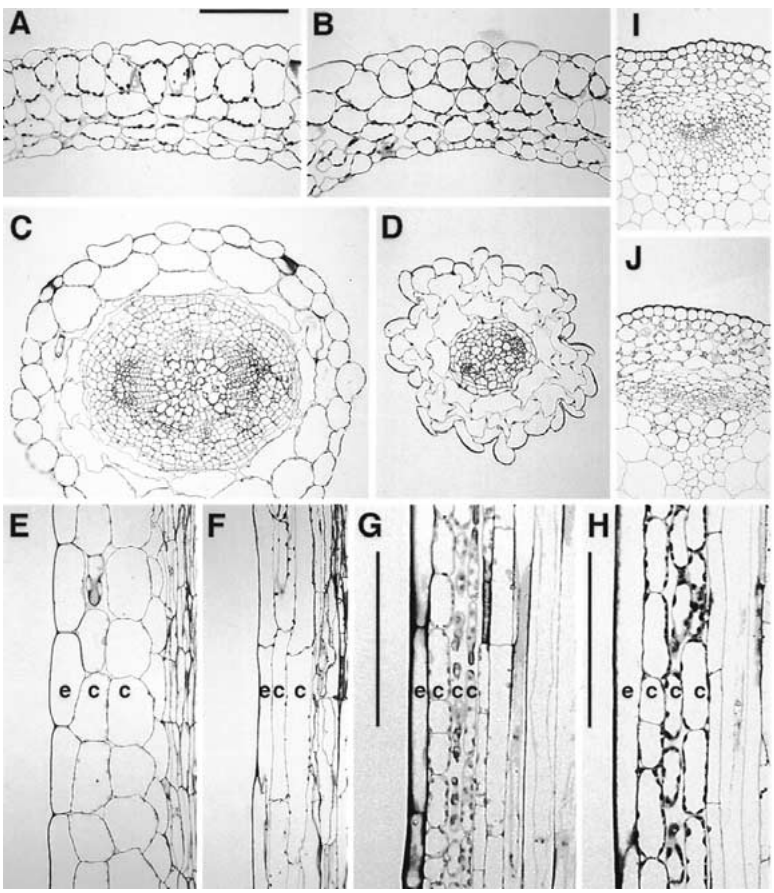

Figure 5. Anatomy of wild type and *iaaM/iaaH*-40. Sections were made from leaves and hypocotyls of 2-week-old seedlings grown in soil and inflorescence stems of 6-week-old seedlings grown in soil. (**A**, **C**, **E**, **G** and **I**) wild type and (**B**, **D**, **F**, **H** and **J**) *iaaM/iaaH*-40. (**A**,**B**) Transverse sections of leaves. Note the smaller size of the lower epidermal cells in **B**. (**C**,**D**) Transverse section of hypocotyl. (**E**,**F**) Longitudinal section of hypocotyl. (**G**,**H**) Longitudinal section of inflorescence stem. (**I**,**J**) Transverse sections of inflorescence stem. Scale bars = 100 μm. e, epidermis; c, cortex.

ordering of the cell arrays as observed in wild type was disturbed (Figure 5C and 5D). Furthermore, cell length was increased in *iaaM/iaaH*-40 inflorescence stems. This was most prominent for the epidermal cells of which the length increased by 3.8-fold, whereas the cell length of the cortex cell layers was moderately affected (Figure 5G and 5H).

In addition to the increased cell length in hypocotyls and inflorescence stems of the *iaaM/iaaH*-40 transgenic line, the size of the vascular tissue was altered in those organs. In hypocotyls, vascular tissue size was decreased (Figure 5C and 5D) due to a decrease in cell number for all cell types comprising the vasculature. In contrast, the size of the vascular

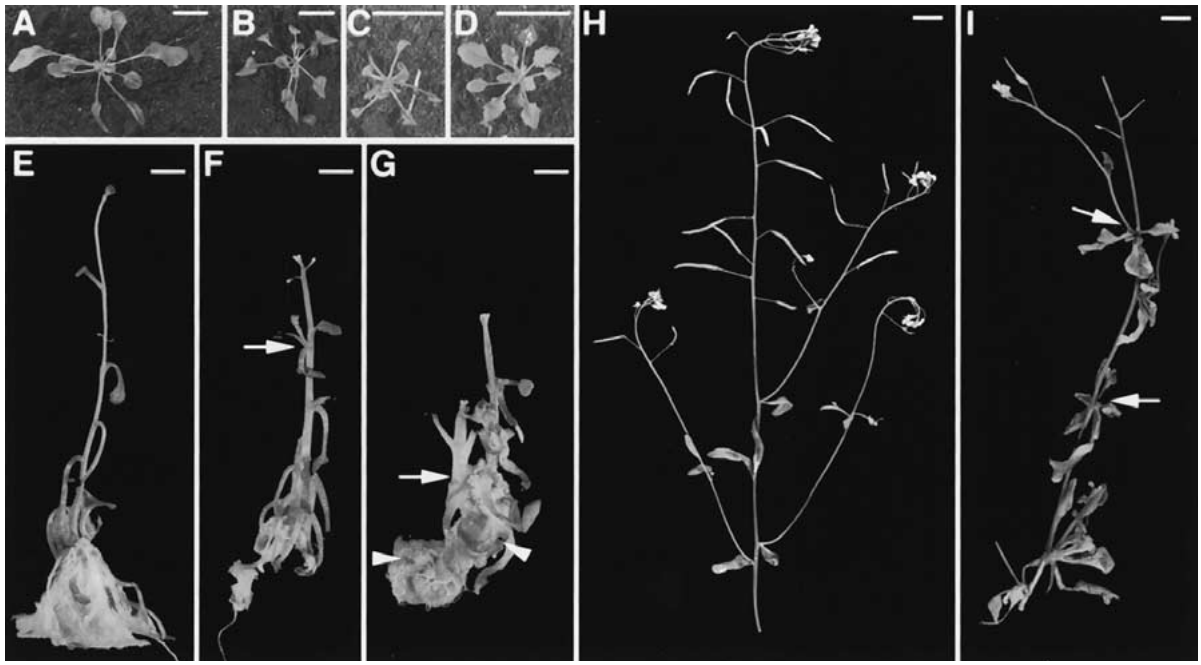

Figure 6. Development of the *iaaM/iaaH*-40**ipt*-161 double transgenic plants. All plants were grown in soil except those shown in **E–G**. Development of 3-week-old wild type (**A**), *iaaM/iaaH*-40 transgenic (**B**), *iaaM/iaaH*-40**ipt*-161 double transgenic (**C**) and *ipt*-161 transgenic plants (**D**), respectively. Inflorescence structures of the *iaaM/iaaH*-40 transgenic (**E**) and *iaaM/iaaH*-40**ipt*-161 double transgenic plants (**F**,**G**). Arrow in **F** indicates the aerial rosette formed on the *iaaM/iaaH*-40**ipt*-161 inflorescence. Arrowheads in **G** indicate the vigorous callus formation at the base of the hypocotyl and the arrow indicates the formation of a shoot from the callus produced just below the rosette leaves. Inflorescence structure from wild type (**H**) and *iaaM/iaaH*-40**ipt*-l61 double transgenic plants (**I**), respectively. Arrows in **I** indicate the aerial rosettes formed on the inflorescence. Scale bars = 1 cm.

bundles was slightly increased in the inflorescence stem of such transgenics (Figure 5I and 5J). However, the number of vascular bundles was smaller in the inflorescence stems of the *iaaM/iaaH*-40 transgenic line (8) compared to wild type (12). Thus, the number of cells comprising the vascular tissue in hypocotyl and inflorescence stem of the *iaaM/iaaH*-40 transgenic line was decreased. Analysis of root structure showed that neither cell length nor vascular tissue size was affected compared to wild type. Furthermore, the *iaaM/iaaH*-40 transgenic line displayed no apparent alteration in vascular tissue patterning and size in the leaves (data not shown). Thus, the alteration in vascular tissue is specific for hypocotyl and inflorescence stem.

Combined Effect of Cytokinin and Auxin Overproduction Causes Formation of Aerial Rosettes

To study the combined effect of an increased cytokinin and auxin production on *Arabidopsis* development, crosses were made between the *ipt*-161 (van der Graaff and others 2001) and *iaaM/iaaH*-40 transgenic lines. The *ipt*-161 transgenic line exhibited a strong cytokinin phenotype consisting of the formation of highly serrated leaves that were smaller in size and pointed in shape (van der Graaff and others 2001). In addition, the *ipt* transgenic line formed very short roots, and callus formation was occasionally observed at the base of the hypocotyl. The *iaaM/iaaH*-40**ipt*-161 double transgenic plants obtained from these crosses showed a phenotype (Figure 6C) that was in most respects the sum of the phenotypes displayed by the parental transgenic lines (Figure 6B and 6D). Compared to *iaaM/iaaH*-40 the double transgenic plants showed a reduced hypocotyl length and petiole elongation. Leaves were smaller and slightly serrated like those of the *ipt*-161 transgenic line, but still showed epinasty (Figure 6C). Furthermore, root length was intermediate between that of the parental transgenic lines (data not shown). Bolting double transgenic plants grown in tissue culture produced callus on the hypocotyl just below the rosette leaves as observed for *iaaM/iaaH*-40. However, instead of roots, shoots differentiated from the callus formed on the double transgenic plants (Figure 6G). In addition, callus was formed at the base of the hypocotyl as observed for *ipt*-161, but these calli frequently de-

veloped more vigorously (Figure 6G). Flowering double transgenic plants grown in soil formed aerial rosettes (Figure 6I), like those observed for the *Sy*-0 ecotype described by Grbic and Bleecker (1996). These aerial rosettes were also occasionally observed on double transgenic plants grown in tissue culture (Figure 6F), but were never observed on either one of the parental lines.

Discussion

Increased Endogenous Auxin Production Brought About by the *iaaM* and *iaaH* Genes Results in Both Earlier Described and Novel Auxin-related Phenotypes

The altered development displayed by the *Arabidopsis iaaM/iaaH* transgenic lines is indicative of elevated auxin levels and included leaf epinasty, increased cell elongation and increased apical dominance. The levels of free IAA were increased 2-fold in rosettes of the *iaaM/iaaH*-40 transgenic line. The phenotype exhibited by this line resembled, in general, the phenotypes observed for the *Arabidopsis* 19S-*iaaM* and CYP79B2 overproducing transgenic lines (Romano and others 1993; Zhao and others 2002), the *Arabidopsis* auxin overproducing mutants *sur*1 (Boerjan and others 1995), *sur*2/*rnt* (Bak and others 2001; Barlier and others 2000) and *yucca* (Zhao and others 2001) and those seen in *iaaM/iaaH* transgenic lines of other plant species (Gaudin and others 1994; Hamill 1993). There are significant differences in the mechanism by which the increase in IAA levels are brought about in these different *Arabidopsis* lines. The *CYP83B1* gene mutated in *sur*2 and *rnt* is expressed in all tissues with the highest expression in roots (Mizutani and others 1998). The overexpression of the *CYP79B2* gene resulting in an auxin overproduction phenotype was driven by the CaMV 35S promoter (Zhao and others 2002), while the *yucca* phenotype caused by activation tagging could be phenocopied by overexpression of the *YUCCA* cDNA from the CaMV 35S promoter. Both this 35S and the 19S CaMV promoter used in the 19S-*iaaM* transgenic line (Romano and others 1993) confer strong constitutive expression. Therefore, in these mutants and transgenic lines the IAA levels can be expected to be affected in all tissues. In contrast, the *iaaM* and *iaaH* promoters used to generate the *iaaM/iaaH* transgenic lines described in this study confer expression to the vasculature of roots and hypocotyl. Subsequently, the IAA levels in the *iaaM/iaaH* transgenic lines can be expected to be affected in the basal tissues of the plants only. This could explain differences in phenotypes among the *iaaM/iaaH*-40 transgenic line, the 19S-*iaaM* transgenic line, the *CYP79B2* overproducing transgenic line and the *Arabidopsis* auxin overproducing mutants. Small differences in phenotype could furthermore reflect small differences in the levels of free IAA (Chen 2001; Weijers 2002) or differences between *Arabidopsis* ecotypes.

In addition to the aforementioned earlier developmental alterations, which are correlated with elevated levels of auxin, the *iaaM/iaaH* transgenic lines formed necrotic spots on leaves and bracts. Furthermore, inflorescence stem segments of the *iaaM/iaaH*-40 transgenic line could be propagated in tissue culture due to outgrowth of the axillary buds. These two features have not been reported before for auxin overproducing transgenic lines.

A large number of *Arabidopsis* mutants have been described that exhibit spontaneous formation of necrotic spots or lesions (Dietrich and others 1994; Greenberg 1996; Weymann and others 1996). Interestingly, a similar phenotype was described for the auxin overproducing *atr*4 mutant and this lesion phenotype in *atr*4 was likely caused by salicylic acid (Smolen and Bender 2002). However, the formation of necrotic spots was not observed for other auxin-overproducing mutants, including *sur*2 (Barlier and others 2000) and *rnt* (Bak and others 2001), which are allelic to *atr*4. Recently, we isolated a T-DNA tagged mutant displaying the formation of necrotic spots on the leaves, similar to those formed by the *iaaM/iaaH* transgenic lines. The necrotic spots formed by this *spontaneous necrotic spots* (*sns*) mutant were shown to be caused by increased programmed cell death as visualized by the TUNEL assay (van der Graaff 1997).

The outgrowth of axillary buds is under the strong control of auxin. Apically applied auxin inhibits bud outgrowth, whereas auxin applied to the base of stems releases this inhibition (Chatfield and others 2000). In the *iaaM/iaaH*-40 transgenic line, elevated IAA levels resulted in increased apical dominance, but surprisingly the inhibition of axillary bud outgrowth was released. This peculiar behavior of the axillary buds was furthermore altered by the combined effect of both the *ipt* and the *iaaM/iaaH* genes. The inflorescence stems of the *iaaM/iaaH*-40**ipt*-161 double transgenic plants formed aerial rosettes, which were never observed on either the parental *ipt*-161 or *iaaM/iaaH*-40 transgenic lines.

The combined effect of both the *ipt* and *iaaM/iaaH* genes in general resulted in additive cytokinin and auxin effects on the development of such double transgenic plants. At the sites where callus was

formed in either the parental *ipt*-161 or *iaaM/iaaH*-40 transgenic lines, this callus formation was more pronounced in the double transgenic plants. However, spontaneous callus formation was never observed. In the *iaaM/iaaH*-40*ipt*-161 double transgenic plants both elevated cytokinin and auxin levels can be expected given the fact that these double transgenic plants display the sum of developmental alterations exhibited by the parental lines. Recently, such double transgenic plants were studied in tobacco. Despite a clear combined effect of both auxin and cytokinin on the development of the double transgenic plants, the levels of these hormones were comparable to wild type, whereas clearly increased hormone levels were detected in both parental lines (Eklof and others 2000).

Vascular Tissue Formation and Basipetal PAT are Disturbed in the *iaaM/iaaH*-40 Transgenic Line

The *iaaM/iaaH*-40 transgenic line exhibited a 50% reduction in basipetal PAT in inflorescence segments. Furthermore, the cell number for all cell types comprising the vascular tissue was reduced in the hypocotyls and inflorescence stems. Interestingly, the *ilf* mutant showing a reduced secondary xylem and interfascicular fiber formation was defective in PAT (Zhong and Ye 2001). In this *ilf* mutant a reduced basipetal PAT was evident before morphological alterations were visible in the vasculature and, therefore, the vascular defect in *ilf* is a secondary effect of reduced PAT (Zhong and Ye 2001). The growth of *iaaH* transgenic plants on IAM resulted in increased levels of free IAA and decreased basipetal PAT in the inflorescence stem (Oka and others 1999), suggesting that increased auxin levels negatively regulate basipetal PAT. Unfortunately, the anatomy of such plants was not studied and the mechanism by which the basipetal PAT was reduced remains unknown.

Auxin biosynthesis is complex and takes place in cotyledons (Avsian-Kretchmer and others 2002), young leaves, which, however, are first auxin sinks before becoming auxin sources (Avsian-Kretchmer and others 2002), roots (Ljung and others 2001) and flower buds (Okada and others 1991). Locally high auxin levels result in auxin diffusion, initiating polar auxin flow. Through complex feedback mechanisms this flow is canalized through narrow files of specialized cells resulting in the formation of the vasculature (Aloni 2001). The promoters of the *iaaM* and *iaaH* genes are active in the vasculature of roots, hypocotyls and further in the shoot apex and leaf primordia. Therefore, IAA production is increased in plant organs that are located basally from the main source of IAA production in young plants (cotyledons and young leaves). This could result in a feedback regulation leading to a reduction in the basipetal auxin flow, thereby compensating for the increased IAA production in the basal parts of the *iaaM/iaaH* transgenic lines. Subsequently, this reduced polar auxin flow might cause reduced vascularization as a secondary effect similar to that in the *ilf* mutant. Alternatively, the altered IAA production in the *iaaM/iaaH* transgenic lines could directly influence the vascular differentiation causing a reduction in the number of cells responsible for PAT, thereby leading to a reduced basipetal PAT as a secondary effect.

It can be envisaged that the reduced basipetal PAT resulted in less of the IAA produced by the cotyledons and young leaves being transported to the root system. Consequently, an 'excess' of IAA will therefore be present in the aerial part of these transgenic lines. This excess can migrate into the surrounding cortex and epidermal cell layers, which act as a sink for free IAA (Sanchez-Bravo and others 1991), eventually resulting in elevated IAA levels in the shoot. The inability to obtain homozygous progenies for the *iaaM/iaaH* transgenic lines exhibiting the strong auxin phenotype could therefore be explained by a stronger inhibition of PAT and/or an increased auxin accumulation in homozygous versus hemizygous transgenic plants.

In roots, IAA is transported basipetally from the apex in the epidermis and cortex cells. This root basipetal PAT is important for the initiation of lateral root primordia, while shoot-derived IAA transported acropetally in the root vasculature is important for the emergence of lateral root primordia (Bhalerao and others 2002; Casimiro and others 2001). It is thought that later in development, the root itself starts producing IAA, rendering it independent from shoot-derived IAA (Bhalerao and others 2002). Despite the fact that the *iaaM* and *iaaH* promoters conferred expression to the root vasculature, young *iaaM/iaaH*-40 seedlings displayed a near wild-type root development, while only late in development did this transgenic line display both an increased and ectopic root formation. This unexpected lack of a clear auxin effect on root development in young transgenic plants reflects the complex mechanisms regulating auxin homeostasis throughout plant development.

The *Arabidopsis iaaM/iaaH*-40 transgenic line described in this article has been deposited in the NASC stock center as *aux*-40 and is available to the research community under the accession number N116.

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