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IAA8 expression during vascular cell differentiation

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Abstract

We report the characterization of a member of the auxin-induced *IAA* gene family from zinnia, designated *zIAA8*, which is expressed by mesophyll cells differentiating as tracheary elements *in vitro*. Transcription of *zIAA8* is up-regulated within 3 h after cell isolation in inductive medium, indicating that cells perceive and respond to growth factor stimulus early in culture. Transcript levels of *zIAA8* remain high through 72 h of culture in medium containing auxin and cytokinin or auxin alone, but low in medium containing only cytokinin or control medium lacking growth factors, demonstrating auxin-specific induction and consistent with lack of desensitization to prolonged auxin stimulation. *In situ* localization shows *zIAA8* is localized to primary vasculature, root tips, and nascent leaves in zinnia seedlings. The observation that *zIAA8* is expressed during vascular development *in planta* supports the hypothesis that expression early in culture reflects early events during normal vascular differentiation. The promoter of *Arabidopsis IAA8* drives expression of the GUS reporter in a pattern in *Arabidopsis* similar to that for *zIAA8* in zinnia, suggesting conservation of *cis* regulatory elements between the species and confirming the results from *in situ* localization. The vascular expression pattern of the *IAA8* promoter in leaves mirrors the developmentally regulated auxin gradient in expanding leaf blades. The expression patterns of *zIAA8* and *IAA8* yield new insight into vascular development *in vitro* and *in planta*, and provide much needed markers for early vascular differentiation.

Abbreviations: TE, tracheary element

Introduction

The developmental processes underlying vascular development include provascular cell function, tissue patterning, and the differentiation of specialized cell types including the tracheary element (TE). Auxin has been implicated in all of these processes, but the distinct roles of auxin during each diverse process remain unclear. The zinnia cell culture system supports the differentiation of TEs from mesophyll cells mechanically isolated from leaves of zinnia seedlings (Fukuda and Komamine, 1980). This synchronous *in vitro* differentiation of TEs is induced by exogenous auxin and cytokinin, and provides a system for investigating the role of auxin on specific stages of vascular cell differentiation.

The differentiation of zinnia TEs *in vitro* follows a predictable process: cells expand, synthesize an elaborate secondary cell wall, initiate a programmed cell death, and autolyse (Groover *et al.*, 1997; Groover and Jones, 1999). Although these stages of differentiation are increasingly well understood at the morphological, biochemical, and gene expression levels, they occur relatively late during differentiation and early differentiation events remain poorly defined. Auxin is the earliest known signal inducing TE differentiation *in vitro*, but the process by which cells become competent to respond to auxin and initiate differentiation is not known, and currently markers defining specific stages of auxin perception and response are lacking. In addition to auxin, wounding may also influence early culture events. In planta, severed vas-

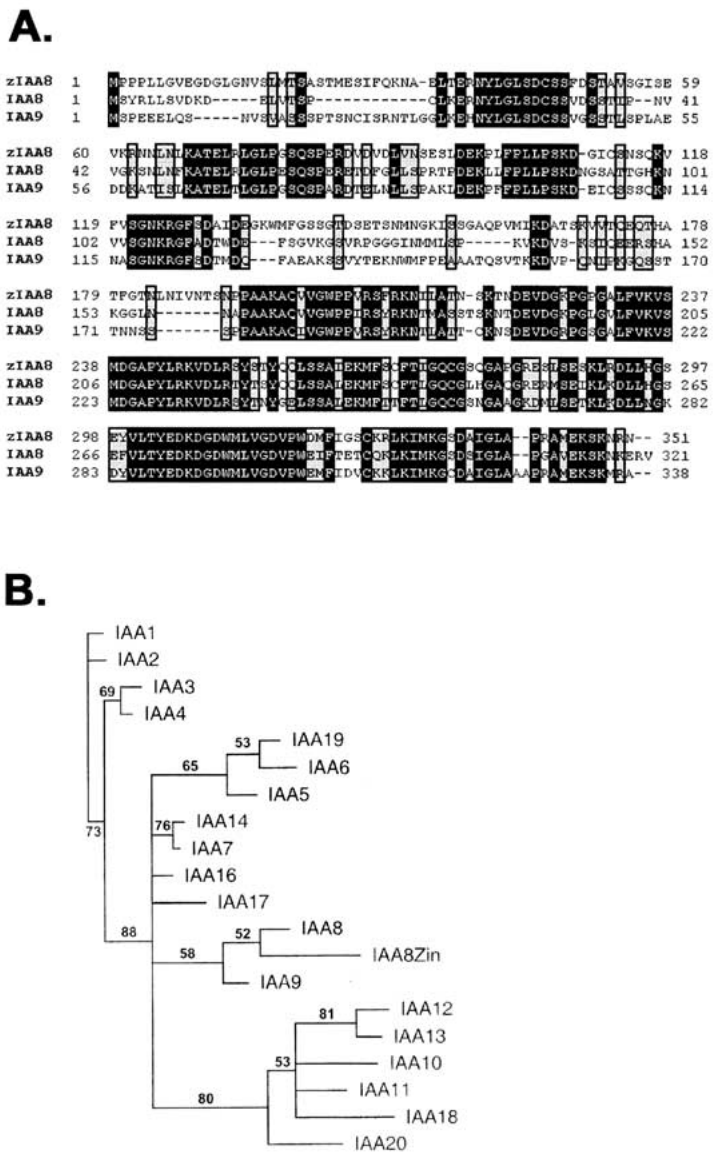

Figure 1. Sequence analysis of *zIAA8*. A. Alignment of the derived protein coding sequence of *zIAA* (Accession AAM12952) and the two most closely related members of the IAA protein family from *Arabidopsis,* IAA8 (accession number AAL24387.1) and IAA9 (AAL49854.1). zIAA shares 61% and 57% identity with IAA8 and IAA9, respectively. Alignment was performed using CLUSTALw (http://www2.ebi.ac.uk/clustalw/), and manually optimized with BioEdit (Hall, 1999). B. Phylogenic analysis with the PAUP program on an informative set of the *IAA* gene family in *Arabidopsis*. The tree is rooted with IAA1; numbers represent bootstrap values. The region of the zIAA8 protein.

cular bundles can be rejoined by the differentiation of surrounding parenchyma as wound-induced TEs (Sachs, 1981). The morphology of these 'wound' TEs is similar to those that differentiate *in vitro*. Since the zinnia mesophyll cells are isolated through mechanical dissociation from leaves, these cells experience a significant wounding that may act as an inductive signal. Alternatively, cultured cells might require a recovery period from wounding before becoming competent to respond to other inductive signals, including auxin and cytokinin. The identification of molecular markers defining early culture events could elucidate the exact nature of early culture processes including the timing of auxin perception and the influence of wounding.

With the zinnia system, the timing of responses to auxin and cytokinin has been assayed indirectly

through the timing and extent of TE differentiation in response to various growth factor treatment regimes. Fukuda and Komamine (1985) found that isolated mesophyll cells do not respond to differentiation-inducing exogenous growth factors until after 12 h in culture, and are only slightly delayed in TE differentiation if exogenous growth factors are not supplied until after 24 h in culture. These results raise the possibility that the cultured cells are not capable of perceiving or responding to growth factor-induced stimulus to differentiate early in culture. Church and Galston (1988) concluded that cells become determined to differentiate as TEs after 56 h exposure to auxin, and after 24 h exposure of cytokinin. More recently, Milioni *et al.* (2001) found that cells cultured in medium lacking growth factors become maximally responsive to growth factors between 46 and 50 h of culture. During this period, growth factor exposure for as little as 10 min is sufficient to determine cell fate as TE (Milioni *et al.*, 2001). Importantly, differentiation has been initiated many hours to days prior to the morphological changes assayed in these studies, as revealed by biochemical and genetic markers of TE differentiation that precede morphological changes (Demura and Fukuda, 1994; Ye and Droste, 1996; Beers and Freeman, 1997; Miloni *et al.*, 2001). Another factor limiting the interpretation of these studies is that the endogenous growth factor levels of the cultured cells are not known.

The cloning of genes whose expression is rapidly induced by auxin has provided molecular markers of early auxin perception and auxin responses. The *IAA* gene family from *Arabidopsis* consists of genes that are rapidly transcriptionally induced by exogenous auxin (Abel *et al.*, 1995). In addition, several members of the gene family are primary response genes, and are directly transcriptionally induced in response to auxin without translation of intervening protein components (Abel *et al.*, 1995). Nuclear localization and similarity to Arc and MetJ DNA-binding proteins support the hypothesis that IAA proteins are involved in transcription (Abel *et al.*, 1994). Conserved domains within IAA proteins mediate homodimerization and heterodimerization between IAA proteins and heterodimerization with auxin-response factor (ARF) proteins (Kim *et al.*, 1997; Ulmasov *et al.*, 1997). Experiments using protoplast transfection assays suggest that *IAA* genes act to repress transcription of auxin-responsive genes (Tiwari *et al.*, 2001). Stability of IAA proteins is decreased in the presence of increasing auxin concentrations, which may directly affect derepression

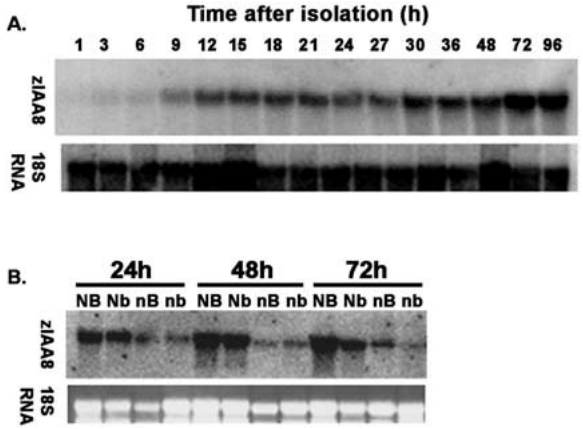

Figure 2. Expression of *zIAA8* during culture. A. The top panel shows a time course of *zIAA8* expression in inductive medium containing both auxin and cytokinin, as determined by northern analysis (Materials and methods). *zIAA8* is transcriptionally up-regulated within 3 h after cell isolation. The bottom panel shows results of reprobing of the same blot with total cDNA. B. The top panel shows expression of *zIAA8* by cells cultured in different media types, as determined by Northern analysis. *zIAA8* expression is shown for cells cultured for 24, 48, or 72 h in either medium containing inductive levels of both auxin and cytokinin (NB), inductive levels of auxin but no cytokinin (Nb), no auxin but inductive levels of cytokinin (nB), or medium lacking exogenous growth factors (nb). *zIAA8* is induced in medium containing auxin (NB and Nb) irrespective of TE differentiation, and transcript levels remain elevated throughout culture in medium containing auxin. The bottom panel shows ethidium bromide staining of the gel blotted for analysis.

of early auxin responsive genes (Tiwari *et al.*, 2001). IAA proteins are targeted for proteasome degradation by SCF^{TIR1} ubiquitin-ligase (Gray *et al.*, 2001).

We report here the cloning and partial characterization of an auxin-responsive gene from zinnia, designated *zIAA8*, which is most similar to the *IAA8* gene of *Arabidopsis*. We use this molecular marker to determine the timing of auxin responses in cultured zinnia cells, and correlate expression with early stages of TE differentiation *in vitro* and *in planta*.

Materials and methods

Cell culture and analysis

Plant cultivation, cell isolation, and cell culture were as described previously (Groover and Jones, 1999). For RNA isolation from cultured cells, cells were pelleted by centrifugation and ground using a mortar and pestle in liquid nitrogen. The ground cells were shaken in extraction buffer (100 mM Tris pH 7.5, 1.5 M NaCl, 2% CTAB, 50 mM EDTA, 50 mM DTT), followed by

extraction with chloroform/isoamyl alcohol 24:1 and centrifugation at 4 °C. The resulting aqueous phase was removed and RNA precipitated by addition of 1/4 volume 4 M LiCl and 2-mercaptoethanol to 1%. After overnight precipitation at −20 °C, RNA was pelleted by centrifugation at 4 °C for 45 min. The resulting pellet was rinsed with 70% ethanol, resuspended in water, and extracted once with phenol/chloroform/isoamyl alcohol 25:24:1, and once with chloroform/isoamyl alcohol 24:1. Finally, RNA was precipitated with 0.1 vol 3 M sodium acetate and 2 vol 100% ethanol. The resulting pellet was resuspended in 10 mM Tris pH 8, 0.1 mM EDTA. Electrophoresis of RNA on denaturing formaldehyde gels, blotting onto nylon membranes, and hybridization with DNA probes were as described (Sambrook *et al.*, 1989). The probe used for northern analysis corresponds to nucleotides 744–1034 of GenBank Accession AY090553.1.

Synthesis of cDNA, PCR, polyacrylamide gel electrophoresis, and recovery of amplified products were as described by manufacturer's protocols (GeneHunter, Brookline, MA). Synthesis of cDNA was primed with 5′-TTTTTTTTTTTTMN-3′ (M = A, C, or G; N = A, C, G, or T). Primer sequences used for PCR amplification of *IAA* cDNAs were A1 (5′-RTIGTIGGITGGCCICCIRT-3′) and B1 (5′-ATRCTYCTRTTYCTRCCICT-3′). 5′- and 3′-RACE were performed on total RNA isolated with the Qiagen RNAeasy procedure with primer IAA_R-A, 5′-AGCTCCCTGCGATCCGCATT-3′ for 5′-RACE and primer 5′-GCAACATCAAAGGTTGTGACACAGG-3′ as described by manufacturer's protocols (SMART Race, Clonetech, Palo Alto, CA).

In situ *hybridization*

For *in situ* hybridization, plasmid pA1B1 containing a *zIAA8* cDNA fragment (corresponds to nucleotides 744–1034 of GenBank accession number AY090553.1) was digested with *Xba*I (sense probe) and *Hin*dIII (antisense probe). Probe synthesis was carried out with the anti-DIG RNA labeling kit (Boehringer Manheim) with T3 polymerase (sense probe) and T7 polymerase (antisense probe). *Zinnia* seeds were grown for 2 days in the dark on wet filter paper. The seedlings were fixed in 50% ethanol, 5% acetic acid, 3.7% formaldehyde for 16 h, dehydrated through ethanol and xylenes series, and paraffin-embedded. The embedded tissue was sectioned at a thickness of 8 μm. Slides were dewaxed with two 10 min incubations with Histoclear, rehydrated, treated with 0.96 μg/ml proteinase K for 30 min, and dehydrated in an ethanol series. The slides were air-dried and hybridized with 0.2 μg of probe per milliliter of hybridization solution (200 μl/slide) for 16 h at 45 °C. Slides were then washed as described by Cho and Kende (1998). Immunological detection was carried out using the anti-DIG RNA detection kit from Boehringer Manheim according to the manufacturer's specifications.

IAA8 *promoter analysis*

For assembly of the *IAA8* promoter:GUS fusion construct, the 3 kb upstream of the ATG translation start of *IAA8* on chromosome II (GenBank accession number AC006340) was amplified from *Arabidopsis* (Columbia ecotype) with primers 5′-GGAGTTGGGTTGTAATGGGTTAATGG-3′ and 5′-CGCTCTAGACAGATCTTCAATATGCAGGACTTG-3′. The resulting PCR product was cut with *Xba*I and cloned into pGPTV-BAR containing the GUS reporter gene. Orientation of the insert was determined by *Hin*dIII digest. Transformed plants carrying the construct were identified by resistance to BASTA herbicide (Liberty herbicide, AgrEvo). GUS staining of seedlings was performed by vacuum-infiltrating seedlings with GUS staining solution (100 mM sodium phosphate pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.5 mg/ml X-Glucuronide, and 100 μg/ml chloramphenicol) with 1 mM or 2 mM potassium ferricyanide and potassium ferrocyanide. After staining for two days at 37 °C, seedlings were cleared with several changes of 70% ethanol. Similar staining patterns were observed for four independently transformed lines.

Results

zIAA8 *is a member of the* IAA *gene family and reports early auxin response in cultured zinnia cells*

Mesophyll cells isolated from seedlings of *Zinnia elegans* (cv. Green Envy) show morphological evidence of differentiation as TE after about 72 h of culture in liquid medium containing auxin (α–naphthalene-acetic acid, 0.1 mg/l) and cytokinin (6-benzylaminopurine, 0.2 mg/l), as previously described (Groover *et al.*, 1997; Groover and Jones, 1999). Other workers have indirectly inferred cultured cell response to exogenous auxin by determining the timing of cell differentiation after exposure to various hormone

regimes (Fukuda and Komamine, 1985; Church and Galston, 1988; Milioni *et al.*, 2001). Auxin-induced subcellular changes begin well before the appearance of secondary cell walls, however, making it difficult to precisely judge the timing of auxin responses based on morphological changes alone. We thus sought to identify a molecular marker that would allow precise determination of auxin responses by cloning of cDNAs from the large family of auxin-regulated *IAA* gene family.

A member of the *IAA* gene family was isolated for use as a molecular marker of early auxin responses in cultured zinnia cells. Degenerate primers recognizing conserved features of the *IAA* gene family were used to amplify cDNAs derived from mesophyll cells cultured for 6 h in medium containing inductive levels of both auxin and cytokinin (Materials and methods). Amplification products were fractionated on a 6% denaturing polyacrylamide gel, from which a ca. 350 bp product was recovered, cloned, and sequenced. Primers were designed based on the sequence of the partial cDNA clone and used for 5′- and 3′-RACE (Materials and methods). The resulting RACE products were cloned and sequenced. The overlapping 5′- and 3′-RACE sequences were aligned to produce a full-length mRNA of 1499 bp containing an open reading frame encoding a protein of 351 amino acids, 197 bp of 5′-UTR, and 246 bp of 3′-UTR (GenBank accession number Y090553). A BLAST search of the protein database at National Center for Biotechnology Information (NCBI) indicated that the predicted protein shares high sequence identity with IAA proteins from a variety of species. In *Arabidopsis*, the most similar proteins identified by protein BLAST analysis are IAA8 and IAA9. As shown in Figure 1A, alignment of the predicted zinnia IAA protein sequence with IAA8 and IAA9 (Materials and methods) indicates the zinnia protein is of similar size and amino acid content, and shares conserved domains with IAA8 and IAA9. The zinnia IAA protein sequence is most similar to IAA8 (61% identity) but also highly similar to IAA9 (57% identity). Figure 1B shows the overall phylogenetic relationship of the zinnia IAA protein with IAA proteins of *Arabidopsis*, indicating that IAA8 and IAA9 form a distinct clade with the zinnia protein. The cloned cDNA represents the most likely zinnia orthologue of *Arabidopsis IAA8* and was therefore tentatively designated *zIAA8*. It is cautioned that confirmation of this tentative assignment will require sequencing and characterization of all related members of the *IAA* gene family from zinnia.

The expression of *zIAA8* reports early auxin responses in zinnia cultures. RNA was isolated from cells cultured under different hormone regimes to determine the hormone response and timing of *zIAA8* induction. As shown in Figure 2A, cells cultured in medium containing levels of auxin and cytokinin that induce TE differentiation (inductive medium, NB) induce expression of *zIAA8* within 3 h after cell isolation. Transcript levels for *zIAA8* remain high though 96 h of culture in inductive medium (Figure 2A), suggesting that the cells do not become desensitized to constant auxin stimulus. The expression profile for *zIAA8* was determined for cells cultured in inductive medium (NB), medium containing inductive levels of auxin but without cytokinin (Nb), medium containing no auxin but inductive levels of cytokinin (nB), and control medium containing no auxin or cytokinin (nb), as shown in Figure 2B. Transcript levels for *zIAA8* remained low in cells cultured in medium nb or nB through 72 h of culture (Figure 2B). In contrast, transcript levels for *zIAA8* were high for cells cultured in NB or Nb medium at 24, 48, and 72h (Figure 2B). Because TE differentiation is induced only by NB and not Nb medium, this result indicates that *zIAA8* transcription can be induced by auxin in the absence of TE differentiation.

zIAA8 *is expressed in vascular tissues*

Auxin is required to induce TE differentiation and *zIAA8* expression reports early auxin responses in cultured cells. However, inductive signals other than auxin (e.g. cytokinin) are required to support TE differentiation, and it is not clear if *zIAA8* expression could report auxin responses related to wounding *in vitro* not directly related to normal vascular development *in planta*. To resolve this issue, *in situ* hybridizations were undertaken in zinnia seedlings to determine if *zIAA8* is normally expressed during vascular development.

The *zIAA8* transcript is localized to developing vasculature and nascent leaves in zinnia seedlings, and expression precedes the appearance of secondary cell walls in TEs. Anti sense and negative control sense probes derived from *zIAA8* were hybridized to sections of zinnia seedlings (Materials and methods). As shown in Figure 3A, antisense probe localizes *zIAA8* expression to primary vasculature and young leaves in young zinnia seedlings. *zIAA8* is expressed in cells that appear to be precursors to tracheary elements (Figure 3D). Expression is not restricted to a spe-

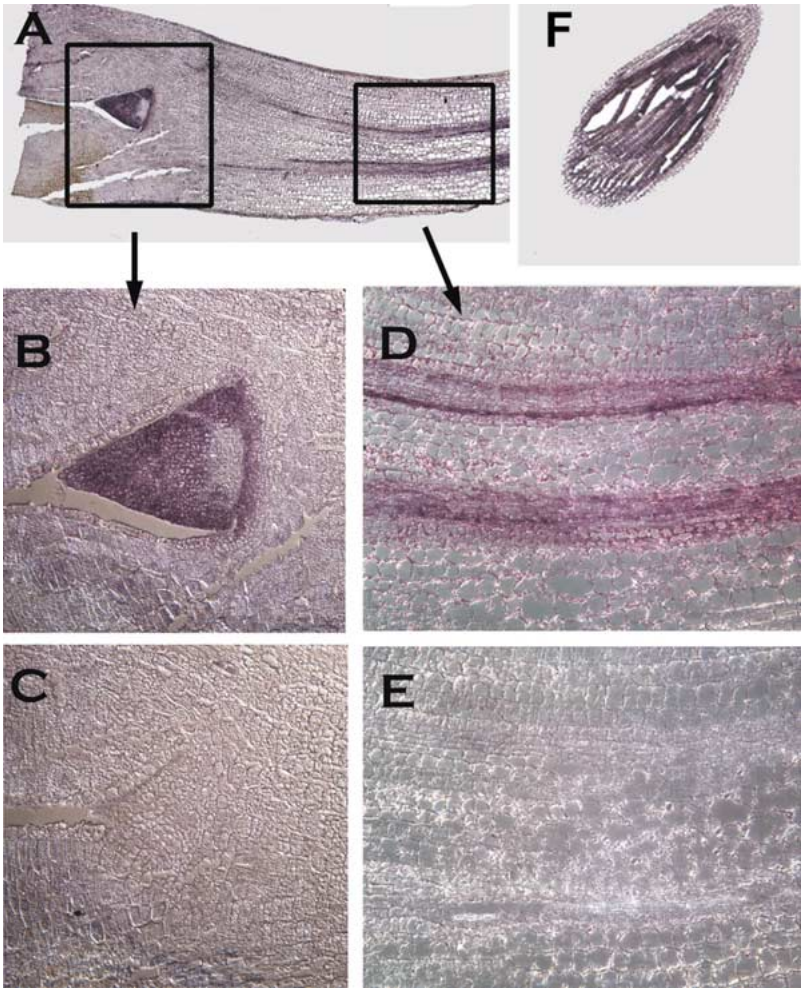

Figure 3. Expression pattern of *zIAA8* in zinnia seedlings revealed by *in situ* hybridization.
Light-grown seedlings were fixed, sectioned, and probed as described in Materials and methods. A. Expression of *zIAA8* is found in primary vascular tissues in the hypocotyl and recently formed leaves. Note IAA8 expression precedes maturation of vessel members in the hypocotyl. The boxed regions in A are shown at higher magnification in B–E. B. Expression is found within young leaves and the tunica region of the shoot apical meristem. D. expression in the hypocotyl is restricted to the vascular tissues. C and E are probed with the sense probe as controls. F. Expression is found in the root tip.

cific organ, but rather is found in the root, hypocotyl, and shoot. Interestingly, *zIAA8* is also expressed in meristematic regions of the shoot and root apices (Figure 3B, F). No signal was detected using negative control sense probes (Figure 3C, E).

The Arabidopsis IAA8 *promoter drives marker gene expression in vascular tissues*

The zinnia culture system is currently limited by the lack of genetic tools. Therefore, to further correlate results from *zIAA8* expression in culture to gene function *in planta*, we examined the expression of *IAA8*, the *Arabidopsis* gene most similar to *zIAA8*.

The *Arabidopsis IAA8* promoter drives marker gene expression in vascular tissues and apices similar to *zIAA8* expression in zinnia (as established by *in situ* hybridization of *zIAA8*). The promoter region of the *Arabidopsis IAA8* gene was cloned upstream of the coding sequence for the GUS reporter and introduced as T-DNA via *Agrobacterium*-mediated transformation into ecotype Columbia (Materials and methods). Seedlings grown for 7 days on standard MS plates were histologically stained to reveal patterns of GUS expression (Materials and methods). As shown in Figure 4, the pattern of GUS expression driven by the *IAA8* promoter in *Arabidopsis* includes developing

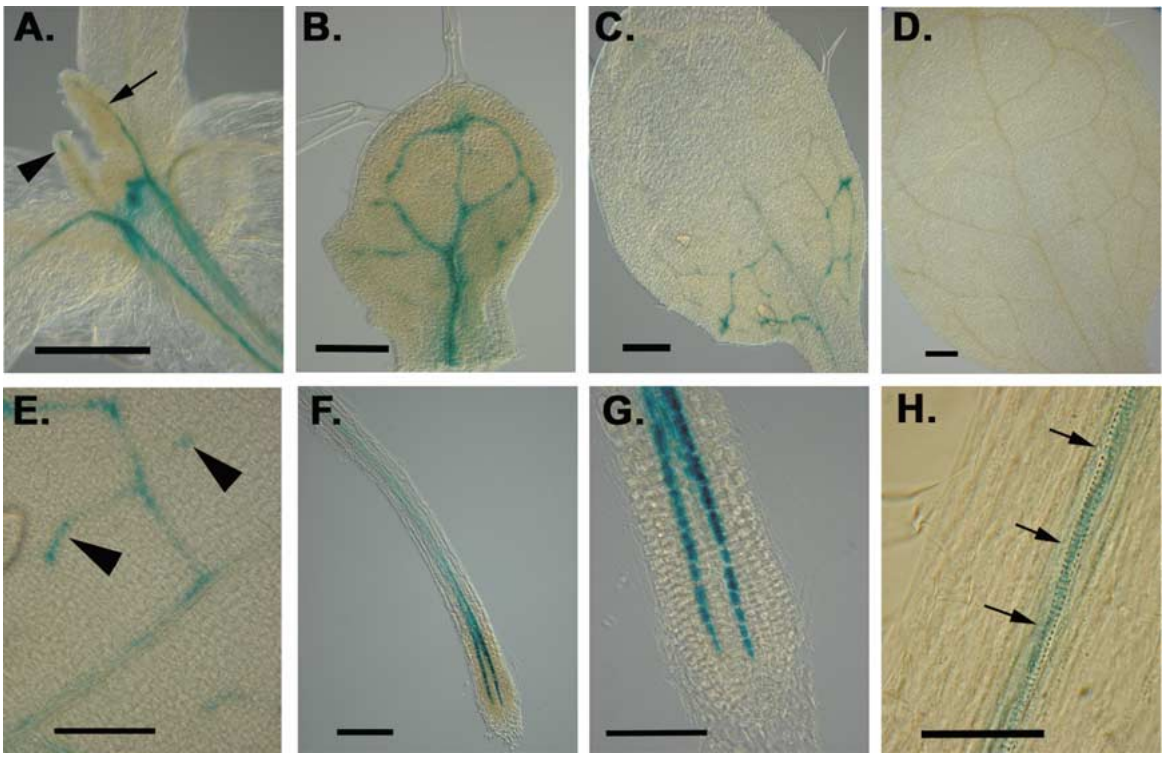

Figure 4. Expression pattern of *Arabidopsis IAA8* promoter in *Arabidopsis* seedlings revealed by *IAA8* promoter::GUS fusion. A–D. Developmental series of leaves from a single plant stained to reveal GUS activity. Expression is first found in the distal hydathode region of young leaves (arrowhead, A). Slightly older leaves show GUS expression in the midvein and incipient leaf trace (arrow, A). B. In leaves with second-order vein development, GUS expression is found in vascular tissues throughout the leaf blade. C. In older leaves establishing higher-order vasculature, GUS expression is limited to the vascular tissues in the proximal regions of the leaf blade. D, All GUS expression is lost from the leaf blade in later stages of development. E. GUS expression can be detected in vascular cells of free-ending veinlets. F. In the root, expression is limited to the vascular tissue of the root tip, and expression is not found in older portions of the root. G. Provascular cells in the root tip show expression of GUS far in advance of cell differentiation. H. in the region of cell differentiation, nascent TEs which have not initiated or completed autolysis can be found that express GUS (arrows). I. Expression in the stem is associated with individual cells within vascular bundles.

vasculature of the shoot apex, hypocotyl, and root tip, and is similar to the expression of *zIAA8* revealed by *in situ* hybridization in zinnia (cf. Figure 3). Marker gene expression in leaves as driven by the *IAA8* promoter is developmentally regulated, as illustrated by GUS expression in leaves from a single transgenic plant (Figure 4A–D). Expression in youngest leaves is restricted to the distal hydathode region (arrowhead Figure 4A) and later includes the nascent midvein/leaf trace (arrow Figure 4A). Expression is found in primary veins in slightly older (ca. 1 mm long) leaves, and is uniformly expressed in vasculature from tip to base (Figure 4B). Older, more elongated leaves (ca. 2 mm long) show expression increasingly restricted to the proximal region of the leaf (Figure 4C) until finally all expression is lost from the leaf blade (Figure 4D). Interestingly, this pattern mimics the proposed developmentally regulated auxin gradient in leaf blades (Chen *et al.*, 2001; Ljung *et al.*, 2001). Within the expanding leaf blade, expression can be found at the developing termini of freely ending veinlets (Figure 4E).

The developing vasculature in the *Arabidopsis* root tip is arranged in cell files that represent a developmental series (Dolan *et al.*, 1993). Provascular cells at the root tip range to fully mature cell types through the zones of cell maturation and elongation. As shown in Figure 4F–G, the *IAA8* promoter drives GUS expression in provascular cells in the root tip prior to any morphological evidence of tracheary element differentiation. In regions containing differentiating cell types, nascent tracheary elements containing secondary cell wall thickenings that are either still living

or just initiating autolysis were found that express GUS (Figure 4H).

Discussion

Auxin is a comprehensive regulator of vascular development, and molecular markers of specific auxin responses are required to discern the roles of auxin during the diverse events underlying vascular tissue patterning, provascular cell function, and vascular cell differentiation. Towards that goal, we isolated and characterized related members of the *IAA* gene family from both zinnia (*zIAA8*) and *Arabidopsis* (*IAA8*) that serve as early markers of auxin-regulated vascular development. Using the zinnia cell culture system, we assayed the precise timing of auxin-induced expression of *zIAA8* during the *in vitro* differentiation of a specific vascular cell type, the TE, and determined the time at which cultured cells respond to exogenous auxin. Using genetic techniques afforded by *Arabidopsis*, we determined the *Arabidopsis IAA8* promoter's expression pattern in whole plants. Comparison of the results from these two distinct systems allowed synergistic insights into *IAA* gene function, provided markers of vascular development and auxin response, and established relationships of zinnia culture events to events *in planta*.

At ca. 72 h of culture in medium containing inductive levels of auxin and cytokinin, cultured zinnia mesophyll cells display obvious morphological features of TEs, most notably the presence of secondary cell wall (Groover *et al.*, 1997). The time at which cultured cells actually perceive and respond to auxin by initiating TE differentiation has remained unclear. The timing of induction by auxin has been examined through media shift experiments in which the response to exogenous auxin was ultimately assayed indirectly through assay of morphological evidence of differentiation (Fukuda and Komamine 1985; Church and Galston, 1988; Milioni *et al.* 2001). Fukuda and Komamine (1985) concluded that cells do not respond to differentiation-inducing exogenous growth factors until after 12 h in culture, and are only slightly delayed in TE differentiation if exogenous growth factors are not supplied until after 24 h in culture. Differentiation is initiated well in advance of morphological events, however, and molecular markers are thus required to more precisely determine the timing of auxin responses. Ye and Varner (1994) characterized a zinnia cDNA, p48h-10, which marks an auxin-induced gene expressed after 24 h of culture in medium containing inductive levels of auxin and cytokinin. It is not known if p48h-10 marks an auxin response that is initiated after 24 h of culture, or if it is a late-expressed marker of auxin response from earlier time points of culture. Together, these results raised the possibility that there is a refractory period early in culture when cells are not capable of perceiving or responding to growth factor-induced stimulus to differentiate.

Our results indicate that cells are able to perceive and respond to auxin stimulus within only 3 h after isolation. This suggests that cells do not undergo a lengthy 'recovery' process from wounding during isolation before becoming competent to respond to inductive signals, but is roughly consistent with the ca. 12 h 'recovery' period proposed by Fukuda and Komamine (1985). Our results are distinct from the auxin-responsive gene p48h-10 described above, which is specifically transcriptionally up-regulated by auxin only after a lengthy 24–48 h culture in medium containing auxin. That *zIAA8* is specifically up-regulated by auxin early (within 3 h) in culture suggests that either p48h-10 is far downstream of initial auxin perception, or that there are multiple pathways which are activated by auxin at different times in culture. Expression of *zIAA8* (and the most similar *Arabidopsis* gene, *IAA8*) is found in pre-vascular cells *in planta*, supporting the notion that early auxin response in culture reported by *zIAA8* expression represents early events in vascular development and is not an artifact of cell isolation or culture.

It should be noted that the northern assays used in our experiments examined populations of cells and would not have detected variation in responses among individual cells. Notably, the fate of cells that do not differentiate as TE in culture is unclear. Although it is possible that they retain their identity as leaf mesophyll cells, it is plausible that they differentiate into a cell type less morphologically conspicuous as a TE. For example, it is possible that some cells differentiate as xylem parenchyma that, in the absence of chloroplast loss, would be difficult to distinguish from leaf mesophyll in culture.

The *Arabidopsis* gene *IAA8*, which is the most similar to *zIAA8* at the protein sequence level, shows a similar expression pattern in developing vasculature and is consistent with similar function in the two species. The vascular expression pattern of the GUS marker gene driven by the *Arabidopsis IAA8* promoter mimics the proposed auxin gradient in ex-

panding leaves (Chen *et al.*, 2001; Ljung *et al.*, 2001). It thus appears that expression of *IAA8* is regulated at three levels: by auxin, by tissue or cell type, and by developmental stage.

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References

Abel, S., Oeller, P. and Theologis, A. 1994. Early auxin-induced genes encode short-lived nuclear proteins. Proc. Natl. Acad. Sci. USA 91: 326–330.

Abel, S., Nguyen, M. and Theologis, A. 1995. The PS-IAA4/5-like family of early auxin-inducible mRNAs in *Arabidopis thaliana*. J. Cell Biol. 251: 533–549.

Beers, E. and Freeman, T. 1997. Proteinase activity during tracheary element differentiation in *Zinnia* mesophyll cultures. Plant Physiol. 113: 873–880.

Chen, J.-G., Shimomura, S., Sitbon, F., Sandberg, G. and Jones, A. 2001. The role of auxin-binding protein 1 in the expansion of tobacco leaf cells. Plant J. 28: 607–617.

Cho, H. and Kende, H. 1998. Tissue localization of expansins in deepwater rice. Plant J. 15: 805–812.

Church, D. and Galston, A. 1988. Kinetics of determination in the differentiation of isolated mesophyll cells of *Zinnia elegans* to tracheary elements. Plant Physiol. 88: 92–96.

Demura, T. and Fukuda, H. 1994. Novel vascular cell-specific genes whose expression is regulated temporally and spatially during vascular system development. Plant Cell 6: 967–981.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. 1993. Cellular organization of the *Arabidopsis thaliana* root. Development 119: 71–84.

Fukuda, H. and Komamine, A. 1980. Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. Plant Physiol. 65: 57–60.

Fukuda, H. and Komamine, A. 1985. Cytodifferentiation. In: I.K. Vasil (Ed.) Cell Culture and Somatic Cell Genetics of Plants, vol. 2, Academic Press, New York, pp. 149–212.

Gray, W., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. 2001. Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature 414: 271–276.

Groover, A., DeWitt, N., Heidel, A. and Jones, A. 1997. Programmed cell death of plant tracheary elements differentiating in vitro. Protoplasma 196: 197–211.

Groover, A. and Jones, A. 1999. Tracheary element differentiation uses a novel mechanisms coordinating programmed cell death and secondary cell wall synthesis. Plant Physiol. 119: 375–384.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41: 95–98.

Kim, J., Harter, K. and Theologis, A. 1997. A protein-protein interaction among the Aux/IAA proteins. Proc. Natl. Acad. Sci. USA 94: 11786–11791.

Ljung, K., Bhalerao, R. and Sandberg, G. 2001. Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. Plant J. 28: 465–474.

Milioni, D., Sado, P., Stacey, N., Domingo, C., Roberts, K. and McCann, M. 2001. Differential expression of cell-wall-related genes during the formation of tracheary elements in the *Zinnia* mesophyll cell system. Plant Mol. Biol. 47: 221–238.

Sachs, T. 1981. The control of the patterned differentiation of vascular tissues. Adv. Bot. Res. 9: 152–262.

Sambrook, J., Fritsch, E. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Tiwari, S., Wang, X., Hagen, G. and Guilfoyle, T. 2001. AUX/IAA Proteins are active repressors, and their stability and activity are modulated by auxin. Plant Cell 13: 2809–2822.

Ulmasov, T., Hagen, G. and Guilfoyle, T. 1997. ARF1, a transcription factor that binds to auxin response elements. Science 276: 1865–1868.

Ye, Z. and Droste, D. 1996. Isolation and characterization of cDNAs encoding xylogenesis-associated and wounding-induced ribonucleases in *Zinnia elegans*. Plant Mol. Biol. 30: 697–709.