

# Early Gene Expression Associated with the Commitment and Differentiation of a Plant Tracheary Element Is Revealed by cDNA–Amplified Fragment Length Polymorphism Analysis<sup>[W]</sup>

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**Isolated mesophyll cells from *Zinnia elegans* are induced by auxin and cytokinin to form tracheary elements (TEs) in vitro with high synchrony. To reveal the changing patterns of gene expression during the 48 h of transdifferentiation from mesophyll to TE cell fate, we used a cDNA–amplified fragment length polymorphism approach to generate expression profiles of >30,000 cDNA fragments. Transcriptional changes of 652 cDNA fragments were observed, of which 304 have no previously described function or sequence identity. Sixty-eight genes were upregulated within 30 min of induction and represent key candidates for the processes that underlie the early stages of commitment and differentiation to a TE cell fate.**

## INTRODUCTION

A central goal in plant developmental biology is to understand how embryonic cells differentiate into the 40 or so cell types that constitute plants. Meristems of shoot and root are a source of cells that must be maintained in a proliferative state, often regarded as undifferentiated, so one approach has been to study mutants in which meristematic function has been compromised (Haecker and Laux, 2001). A second approach has been to study mutants in which a clear developmental phenotype for a particular cell type can be identified, for example, root hairs (Parker et al., 2000), trichomes (Hulskamp and Kirik, 2000), or xylem (McCann and Roberts, 2000). Together, these two approaches have led to the identification of key regulatory genes involved in either the maintenance of an undifferentiated state or the promotion of a differentiated state. In the future, global gene expression technologies may permit the dissection of downstream events through comparisons of mutants in these pathways; however, to date, only a few genes have been identified that are specific to particular cell types. Many genes involved in vascular cell fates have been identified in two large-scale cDNA-sequencing projects using material derived from young xylem tissue of loblolly pine (Allona et al., 1998) and poplar (Sterky et al., 1998). These EST databases are derived from many vascular cell types and from asynchronously differentiating cells; consequently, the cell specificity of each EST and the order of differentiation-related

events are difficult to establish. However, tissue-specific transcript profiles have been obtained using DNA microarray analysis of 3000 ESTs of poplar (Hertzberg et al., 2001).

The use of a remarkable synchronized in vitro cell system, the *Zinnia elegans* mesophyll cell system (Fukuda and Komamine, 1980), allows us to establish the chronology of molecular and biochemical events during the commitment and differentiation to a specific cell fate (McCann and Roberts, 2000; Milioni et al., 2001). Cells isolated from the leaves of *Zinnia* cv Envy, an ornamental plant, are put into liquid culture and supplied with two plant growth factors, auxin and cytokinin. Cells that were already differentiated as photosynthetic mesophyll cells in the leaf now transdifferentiate—change cell fate—to become tracheary elements (TEs) (Fukuda and Komamine, 1980). Sheets of epidermal cells also can be induced to transdifferentiate to TE fate (Church and Galston, 1989). TEs are cells, dead at maturity, that in the plant form a series of connected tubes that transport water and dissolved minerals from the root to the shoot.

During the formation of TEs, hoops of secondary cell wall material are deposited and subsequently stiffened and waterproofed by the deposition of lignin. Finally, the end wall of the cell is broken down and the cell contents are autolysed. The transdifferentiation of mesophyll or epidermal cells in vitro to a TE fate is analogous to the ability of cortical cells in wounded stems to transdifferentiate into xylem elements and reestablish vascular continuity (Sinnott and Bloch, 1945). Thus, TE fate is terminal. The *Zinnia* mesophyll cell system has several key advantages for studying xylogenesis in vitro rather than in planta. First, the entry into a new developmental pathway is induced by adding plant growth factors that act as a molecular switch to start the process of transdifferentiation. Second, the cells that form TEs do so with remarkable

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synchrony, making it possible to stage more precisely the events involved in building a TE.

We have shown that the exogenous growth factors, auxin and cytokinin, are not required in the first 48 h after isolation of *Zinnia mesophyll* cells; furthermore, as little as 10 min of exposure to the growth factors at 48 h is both necessary and sufficient to commit cells to the TE differentiation pathway (Milioni et al., 2001). These findings suggest that the first 48 h of culture represents a time in which the cells adapt to liquid culture and acquire the competence to respond to the inductive signals (McCann, 1997; Milioni et al., 2001). At 48 h, the auxin and cytokinin act as a switch to start the developmental pathway of TE formation. Other signals then are required for cells to progress through the subsequent stages of differentiation leading ultimately to cell death. By delaying the addition of auxin and cytokinin for 48 h, we have improved the synchrony of the culture significantly, so that up to 60% of the cells transdifferentiate to TEs in just 48 h after the addition of growth factors (Milioni et al., 2001). The precise start point, good synchrony, and compressed time scale of the transdifferentiation process provide a new and improved context in which to discover the earliest genes involved in switching on the developmental program.

To determine the range of genes involved in this complex process, we require sequence information on both high- and low-abundance transcripts. We selected five time points at which to sample mRNA populations: they reflect early changes in gene expression (30 min) and immediate downstream events (4 h), the time at which secondary wall synthesis begins (24 h), and the time at which TEs begin to deposit lignin and autolyse their cellular contents (48 h). We then performed an extensive cDNA-amplified fragment length polymorphism (cDNA-AFLP) study (Bachem et al., 1996; Durrant et al., 2000) in which we systematically characterized the amplification products whose abundance changed during the 48 h in which the cells transdifferentiate. Of the ~30,000 cDNA fragments inspected, 652 (~2.1%) exhibited altered expression profiles. A subset of 68 of these 652 ESTs was accumulated within 30 min of adding the auxin and cytokinin, and these represent a set of candidate genes whose expression is involved in the very earliest events in cell commitment and differentiation.

## RESULTS

### A cDNA-AFLP Screen Identified 652 Differentially Accumulated ESTs during the Time Course of TE Formation

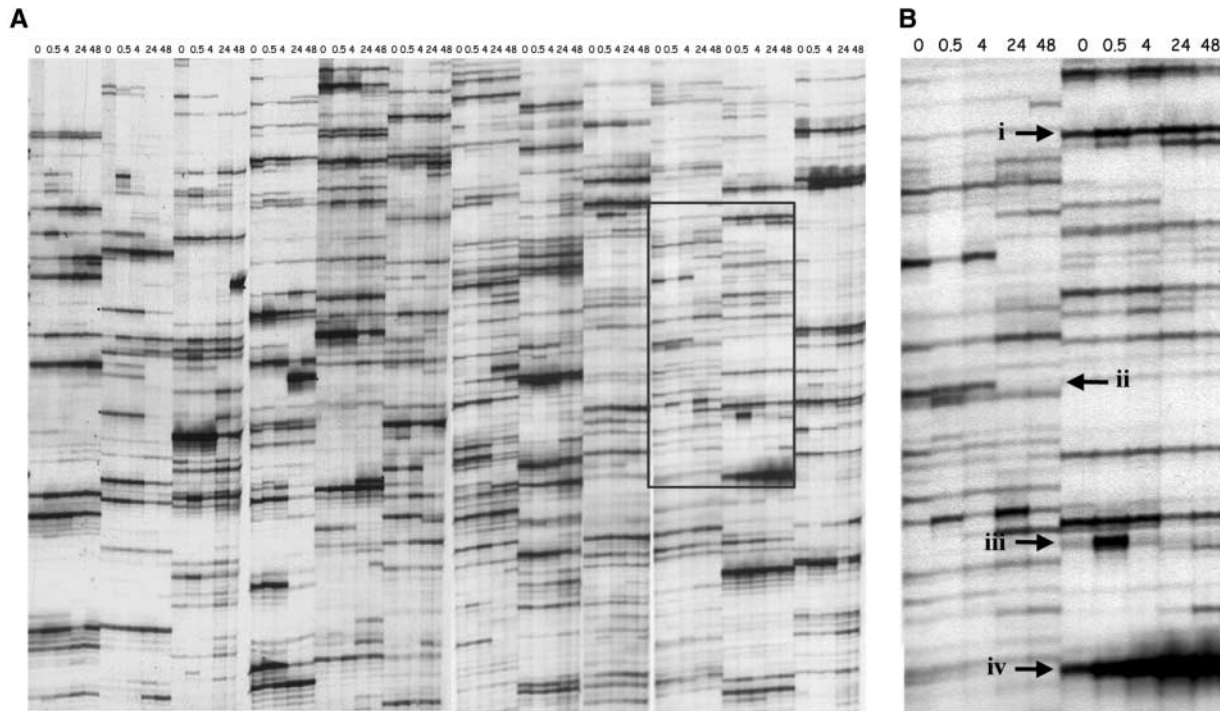
The addition of auxin and cytokinin to *Zinnia mesophyll* cells, after they have been cultured in maintenance medium for 48 h, is followed by TE differentiation of ~60% of the cell population between 96 and 120 h (Milioni et al., 2001). Poly(A<sup>+</sup>) RNA was extracted from *Zinnia* cells at 48 h without growth factors

(regarded as time 0) and then at 30 min, 4 h, 24 h, and 48 h after the addition of the growth factors at time 0 and used for cDNA-AFLP analysis, as described in Methods. The cDNA expression profiles were determined by PCR selective amplification using 512 different primer combinations, and we screened ~30,000 cDNA fragments. Comparison of fingerprints obtained from these five cell populations identified transcript-derived fragments (TDFs) from genes that were accumulated differentially (Figure 1). As anticipated, the majority of the TDFs showed similar levels of accumulation in all five samples. To examine the reproducibility of the fragment banding patterns, reaction products that derived from two sets of independently prepared samples of mRNA were compared. Using 12 combinations of primers, we detected ~800 cDNA fragments. The differential amplification of the TDFs was reproducible (see supplemental data online).

A total of 652 differentially accumulated TDFs, ranging in length from 50 to 450 bp, were recovered from gels and reamplified, subcloned, and sequenced. Each sequence was identified by homology search using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1997) against the GenBank nonredundant public sequence database. A total of 349 fragments (53.5%) of the differentially expressed genes showed close matches (BLASTX expectation values [*E*] of <10<sup>-5</sup>) to database entries with assigned identities. We classified these sequences into 13 groups based on functional categories established for Arabidopsis (Arabidopsis Genome Initiative, 2000), and the proportion of genes in each category is shown in Figure 2. The major group is involved in primary and secondary metabolism and energy generation (19.2%), whereas a slightly higher proportion (8%, compared with 5.6% in Arabidopsis) is cell wall related, reflecting the restructuring of the primary wall and the deposition of secondary cell wall during TE formation. An additional 9.7% of the TDFs are involved in information processing and constitute genes involved in transcriptional control and signal transduction. Interestingly, 12.4% of the sequences share significant similarity to unknown or hypothetical genes with no assigned function from various genome projects (Figure 2). These fragments represent new candidate proteins involved in cell fate determination, differentiation, cell wall remodeling, and cell death. Furthermore, no function could be assigned to 34% of the clones, because they showed no or only poor sequence similarity to any database entries. They may represent either previously uncharacterized genes or fragments too short to reveal any significant identity. The distribution of our 652 sequences by putative function is similar to that for the loblolly pine and poplar EST databases (Allona et al., 1998; Sterky et al., 1998).

### Validation of Expression Patterns by Reverse Transcriptase-PCR Analysis in the *Zinnia* System

To validate the cDNA-AFLP expression profiles, reverse transcriptase (RT)-PCR and/or RNA gel blot analysis were



**Figure 1.** A cDNA-AFLP Autoradiogram Showing the Accumulation Patterns of TDFs from Zinnia Cultured Cells during TE Formation.

**(A)** Templates were derived from Zinnia cells cultured for 48 h in medium with no growth factors (lane 0) and 0.5, 4, 24, and 48 h after the addition of auxin and cytokinin. Lanes are in groups of five: each group was amplified using a different primer combination with two selective nucleotides.

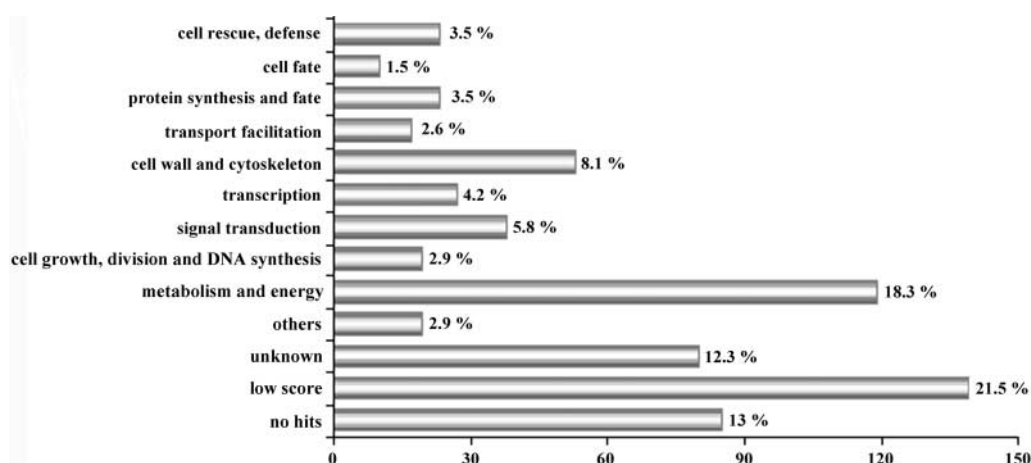
**(B)** Enlarged view of the boxed region in **(A)**. Various expression patterns can be detected across the time course: constitutive expression (i), downregulation (ii), transient expression (iii), and upregulated expression (iv).

used to study 41 differentially accumulated TDFs using mRNA populations harvested from cells at the same time points used for the cDNA-AFLP analysis. Of these TDFs, 12 were selected for their similarity to genes implicated previously in the formation of vascular tissues (Figure 3). The cDNA-AFLP sequences were named Ze (for *Zinnia elegans*) followed by the number corresponding to the AFLP fragment. Zinnia cDNA sequences Ze203, Ze349, and Ze622 were similar to *MONOPTEROS* ( $E < 10^{-24}$ ) (Berleth et al., 2000), *PIN1* ( $E < 10^{-26}$ ) (Gälweiler et al., 1998), and *REVOLUTA/IFL1* ( $E < 10^{-10}$ ) (Talbert et al., 1995; Ratcliffe et al., 2000) from Arabidopsis, respectively. All of them were accumulated or induced within 4 h and have been identified in Arabidopsis mutant screens with vascular phenotypes. Two TDFs, Ze452 and Ze414, similar to *CESA* ( $E < 10^{-31}$ ) (Richmond, 2000) and *CSL* ( $E < 10^{-16}$ ) (Richmond and Somerville, 2001), respectively, were shown to be accumulated differentially in differentiating TEs of Zinnia (data not shown).

Marker genes isolated previously from the Zinnia system also were identified in our screen, demonstrating that the cDNA-AFLP results are fully comparable with data obtained

from alternative methods of differential display. The TDFs Ze68, Ze572, and Ze374 were most similar to the molecular markers *TED2* ( $E < 10^{-8}$ ), *TED3* ( $E < 10^{-20}$ ), and *TED4* ( $E < 10^{-8}$ ), of which *TED3* is expressed preferentially in cells that transdifferentiate into TEs (Demura and Fukuda, 1994). Ze370 was similar to *ZePEL1* ( $E < 10^{-12}$ ), which is involved in remodeling the cell wall (Domingo et al., 1998), whereas Ze293 was similar to *ZeEXP1* ( $E < 10^{-14}$ ), which is expressed in developing TEs in vitro (Im et al., 2000). Ze519 and Ze94 were similar to *ZCAD1* ( $E < 10^{-36}$ ) (Sato et al., 1997) and *LACCASE*-like ( $E < 10^{-8}$ ) (LaFayette et al., 1999), respectively, which are known to be expressed during lignification. The accumulation patterns observed by cDNA-AFLP for these selected genes were confirmed by semi-quantitative PCR (Figure 3).

Various numbers of PCR cycles were tested to ensure that the reactions had not reached the plateau, as described in Methods. In addition, RNA gel blot analysis was used to confirm the accumulation patterns of the Ze121, Ze198, Ze48, Ze138, and Ze266 cDNA-AFLP fragments. Ze121, Ze198, and Ze48 showed similarity to *PG1* ( $E < 10^{-5}$ ) and *ZeEXP1* ( $E < 10^{-14}$ ) and to a ripening-related protein-like



**Figure 2.** Classification of Differentially Accumulated Zinnia ESTs during in Vitro TE Formation.

On the basis of BLASTX expectation ( $E$ ) values of  $<10^{-5}$ , 348 sequences were assigned to functional categories, 80 sequences shared homology with hypothetical proteins or proteins of unknown function, and 224 sequences showed no or low similarity to existing sequences.

gene ( $E < 10^{-5}$ ) from Arabidopsis, respectively. *Ze138* and *Ze266* were most similar to Arabidopsis putative endo- $\beta$ -1,4-glucanase ( $E < 10^{-36}$ ) and peroxidase ( $E < 10^{-16}$ ) genes, respectively (see supplemental data online). RNA gel blot analysis was performed using the differentially amplified cDNA fragments as digoxigenin-labeled probes. The comparisons described above showed that 32 of the 41 cDNAs examined had the same expression profiles as on the original cDNA-AFLP analysis (data not shown). The remaining nine cDNAs all showed differential expression patterns on subsequent RT-PCR or RNA gel blot analysis, but these differed from the original cDNA-AFLP patterns (see supplemental data online). These results indicate that the original cDNA-AFLP pattern was validated in 78% of the cases, so the general approach is a reliable method for identifying differentially expressed genes in the Zinnia system.

#### Sixty-Eight ESTs Are Expressed within 30 min of the Addition of Growth Factors to the Zinnia System

The differentially expressed genes identified in this screen can be divided into two main categories: 43% of the 652 ESTs are expressed at 48 h (before the addition of auxin and cytokinin), whereas the remainder are expressed at very low or undetectable levels and then upregulated. From the second category, we identified 68 cDNA-AFLP fragments that correspond to genes that are newly expressed within 30 min of adding auxin and cytokinin. Table 1 lists all of the sequences within that category, together with three genes downregulated within 30 min that show high similarity ( $E < 10^{-5}$ ) to sequences in the public databases. Some of the induced sequences, such as those for *Ze620* (IAA3-like), *Ze92* (GH3-like), and *Ze235* (NAC4-like), were similar to those of

auxin-induced genes. The *IAA/AUX* and *GH3* gene families are known to be induced very early by auxin. The proteins encoded by the *IAA/AUX* family are short-lived nuclear proteins that are involved in auxin signaling (Ouellet et al., 2001). NAC domain-containing proteins (for petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2) are a family of transcription factors, specific to plants, that play a role in a diverse set of developmental processes. Sequences corresponding to proteins involved in metabolic processes such as ion, nucleotide, and amino acid transportation, energy production, and protein metabolism are induced. Because relatively little is known about basic metabolic processes during xylogenesis, information on the induction of these genes will help our understanding of the coordination of metabolism during vascular development. Furthermore, early changes in the expression profiles of 10 other genes with no previous functional description were observed. These 10 genes, and others, represent new candidate genes involved in the early stages of the TE developmental pathway.

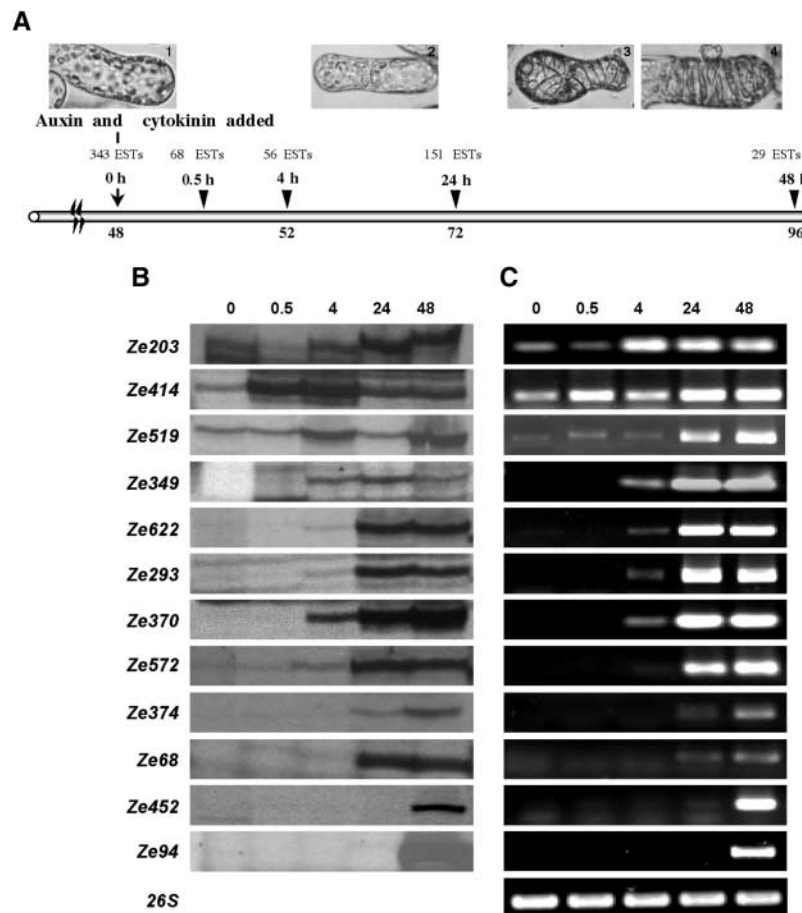
#### The Zinnia System Provides a Set of Marker Genes for Xylogenesis

To confirm that the cDNA-AFLP screen provides reliable potential markers for vascular development, we chose three differentially accumulated TDFs representative of unknown proteins predicted from the Arabidopsis sequencing project. These were selected by their cDNA-AFLP expression patterns: *Ze469* was expressed very early (30 min), *Ze126* was expressed later in the time course (24 h), and *Ze86* was expressed at 48 h. The expression of these genes was studied in a more extensive time course by semiquantitative RT-PCR (Figure 4). RNA was reverse transcribed from cells cul-

tured for 48 h in the absence of growth factors (i.e., at time 0) and from cells at six time points (30 min and 4, 24, 48, 60, and 72 h) after the addition of auxin and cytokinin. Additionally, expression profiles were determined in cells exposed for 72 and 96 h to either auxin or cytokinin only. There was no detectable transcript accumulation in Zinnia cells cultured for 48 h in the absence of the plant growth factors (i.e., at time 0). *Ze469* mRNA accumulation (first detected at 30 min after the addition of auxin and cytokinin to the culture) reached a peak level 48 h later and started declining thereafter (Figure 4). Transcripts of *Ze126* were first observed at

high levels 24 h after the addition of auxin and cytokinin. *Ze86* was seen at 48 h after the addition of the growth factors and then declined; it was not detected in cells exposed to either auxin or cytokinin alone (Figure 4). The expression of both *Ze469* and *Ze126* was upregulated by auxin only, whereas the expression of the former also was induced by cytokinin alone.

To determine the spatial patterns of expression of *Ze469*, *Ze126*, and *Ze86*, we performed in situ RNA hybridization experiments in transverse sections of stem tissues from the second internodes of 4-week-old Zinnia plants (Figure 5).



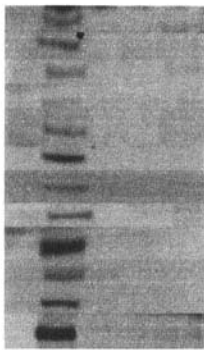
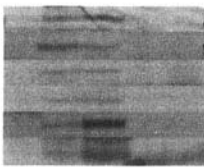
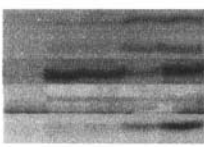
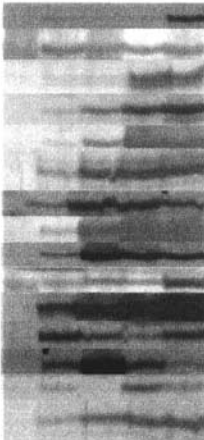
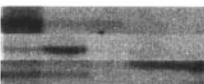
**Figure 3.** Comparison of Expression Patterns of Selected Genes by Both cDNA-AFLP and RT-PCR.

**(A)** Time course of Zinnia mesophyll cells transdifferentiating into TEs, and the corresponding number of ESTs first accumulated at each time point.

**(B)** and **(C)** Comparison of expression patterns of 12 cDNA-AFLP fragments, similar to genes known to be involved in vascular differentiation, by cDNA-AFLP **(B)** and RT-PCR **(C)**. RNA templates were derived from Zinnia cells cultured in noninductive medium for 48 h (lane 0) and cells left for 30 min, 4 h, 24 h, and 48 h (lanes 0.5, 4, 24, and 48, respectively) after the addition of auxin and cytokinin. *Ze203* (*MONOPTEROS*-like), *Ze519* (*ZCAD1*-like), and *Ze414* (*CSL*-like) transcripts were present already before the addition of auxin and cytokinin. *Ze349* (*PIN1*-like), *Ze622* (*REVOLUTA/IFL1*-like), *Ze572* (*TED3*-like), *Ze370* (*PEL1*-like), and *Ze293* (*EXP1*-like) were upregulated at the 4-h time point. The mRNAs for genes similar to *Ze452* (*CESA*-like), *Ze374* (*TED4*-like), *Ze68* (*TED2*-like), and *Ze94* (*LACCASE*-like) were upregulated at later stages of the developmental pathway.



**Table 1.** cDNA-AFLP Fragments Differentially Expressed within 30 min of Addition of Growth Factors to the Zinnia System

AFLP BAND	LENGTH	ACCUMULATION PATTERN <sup>a</sup>					ACCESSION NUMBER	SEQUENCE SIMILARITY	BLAST X SCORE
		0	0.5	4	24	48h			
<i>Ze62</i>	135						AJ316087	Similar to amp binding protein from rice (X94625)	2e-10
<i>Ze129</i>	119						AJ316088	Alkaline/neutral invertase from Arabidopsis (At5g22510)	4e-08
<i>Ze192</i>	114						AJ316089	Putative carboxyl-terminal peptidase from Arabidopsis (At2g44210)	4e-07
<i>Ze536</i>	153						AJ316090	Amino acid transporter c from Arabidopsis (At5g23810)	2e-05
<i>Ze609</i>	118						AJ316091	Thylakoid luminal 25.6 KDa protein from Arabidopsis (At3g55330)	1e-11
<i>Ze16</i>	221						AJ316092	Putative protein predicted from genome sequence of Arabidopsis (At5g08400)	4e-05
<i>Ze25</i>	163						AJ316093	PRKASE precursor from <i>Mesembryanthemum crystallinum</i> (gi:125578)	1e-23
<i>Ze89</i>	123						AJ316094	Similar to chaperonin 21 precursor from tomato (AF233745)	8e-06
<i>Ze234</i>	107						AJ316095	Unknown protein predicted from genome sequence of Arabidopsis (At5g53550)	2e-10
<i>Ze336</i>	133						AJ316096	Granule-bound starch synthase from <i>Perilla frutescens</i> (AF210699.1)	5e-13
<i>Ze542</i>	129						AJ316097	Unknown protein predicted from genome sequence of Arabidopsis (At5g48050)	2e-05
<i>Ze109</i>	198						AJ316098	Arginase from <i>Pinus taeda</i> (AF130440.1)	1e-22
<i>Ze169</i>	166						AJ316099	Unknown protein predicted from genome sequence of Arabidopsis (At2g42580)	2e-10
<i>Ze282</i>	152						AJ316100	LEA14-A protein from cotton (gi:7484727)	3e-12
<i>Ze373</i>	99						AJ409102	Similar to glucosyltransferases from Arabidopsis (At1g24100)	2e-06
<i>Ze487</i>	118						AJ316101	Hypothetical protein predicted from genome sequence of Arabidopsis (AT3g61980)	1e-10
<i>Ze597</i>	256						AJ316102	Stress-induced cysteine proteinase from <i>Lavatera thuringiaca</i> (AF007215.1)	1e-34
<i>Ze598</i>	250						AJ316103	Nuclear RNA binding protein A from <i>Spinacia oleracea</i> (AAF14244.1)	1e-10
<i>Ze104</i>	108						AJ316104	Unknown protein predicted from genome sequence of Arabidopsis (At5g63670)	3e-05
<i>Ze335</i>	117						AJ316105	Auxin-induced protein from Arabidopsis (At1g60680)	7e-06
<i>Ze492</i>	168						AJ316106	Unknown protein predicted from genome sequence of Arabidopsis (At4g24160)	1e-09
<i>Ze509</i>	159						AJ316107	Unknown protein predicted from genome sequence of Arabidopsis (At2g05170)	8e-21
<i>Ze366</i>	70						AJ316108	Unknown protein predicted from genome sequence of Arabidopsis (At2g05170)	6e-06
<i>Ze367</i>	149						AJ316109	Nitrate reductase apoenzyme from rice (gi:128186)	4e-20
<i>Ze500</i>	208						AJ316110	ClpA protein from <i>Spinacia oleracea</i> (AF043539.1)	2e-22
<i>Ze39</i>	127						AJ316111	Spermine synthase from Arabidopsis (At5g19530)	7e-12
<i>Ze155</i>	123						AJ316112	CTP synthase like protein from Arabidopsis (At4g20320)	3e-09
<i>Ze387</i>	120						AJ316114	Putative chloroplast prephenate dehydratase from Arabidopsis (At3g44720)	2e-05
<i>Ze620</i>	185						AJ316115	IAA3 protein, induced by auxin, from Arabidopsis (At1g04240)	2e-16
<i>Ze482</i>	196						AJ316116	Putative senescence-associated protein from <i>Pisum sativum</i> (AB049723)	3e-17
<i>Ze250</i>	175						AJ316117	Endo1,4 β-D-glucanase from <i>Fragaria × ananassa</i> (AJ006349)	6e-24
<i>Ze519</i>	286						AJ316113	Mannitol dehydrogenase from <i>Petroselinum</i> (P42754)	1e-35
<i>Ze469</i>	116						AJ316118	Hypothetical protein predicted from genome sequence of Arabidopsis (At2g35760)	3e-12
<i>Ze152</i>	174						AJ316119	Myosin-like protein from sunflower (U94781.1)	5e-23
<i>Ze235</i>	199						AJ316120	NAC4 protein from rice (AB028183.1)	5e-25
<i>Ze92</i>	127						AJ316121	Auxin-responsive GH3-like protein from Arabidopsis (At2g14960)	1e-15
<i>Ze533</i>	191						AJ316122	Putative serine protease from genome sequence of Arabidopsis (At2g47940)	7e-25
<i>Ze450</i>	182						AJ316123	Similar to a member of the PFJ00903 glyoxalase family of Arabidopsis (At1g15380)	1e-06
<i>Ze35</i>	281						AJ316124	Glycine hydroxymethyltransferase from Arabidopsis (At4g13930)	3e-10
<i>Ze275</i>	107						AJ316125	ATP synthase gamma chain precursor from tobacco (X63606.1)	1e-06
<i>Ze514</i>	111						AJ316126	Photosystem I reaction centre subunit III precursor from <i>Lactuca sativa</i> (AF162201.1)	2e-15

<sup>a</sup> Zinnia cell cultures were supplied with auxin and cytokinin for the times (in hours) indicated within the accumulation pattern column.

Sections were hybridized with *Ze469*, *Ze126*, and *Ze86* anti-sense and sense probes. No hybridization signals were detected using sense probes (data not shown). Transcripts of *Ze469* were localized to young xylem tissue within the vascular bundle (Figures 5A and 5B). The presence of *Ze126* transcripts could be seen in both young xylem tissue and phloem fiber cells (Figures 5C and 5D). Because fibers, like vessel elements and TEs, deposit thick lignified secondary walls, it is likely that common genes are involved in secondary wall thickening in both cell types. There was a strong *Ze126* signal in alternating vascular bundles around the circumference of the stem, with no signal detected in the leaf trace bundles (Figure 5D). Transcripts of *Ze86* (Figures 5E and 5F) were restricted to xylem parenchyma cells. These data demonstrate that *Ze469*, *Ze126*, and *Ze86* transcripts are under temporal and spatial regulation and are all correlated with vascular cell differentiation.

## DISCUSSION

The main advantage of using the Zinnia system and the cDNA-AFLP approach over the existing EST databases is in being able to establish the precise chronology of changing gene expression events during transdifferentiation. The improvements that we made previously to the synchrony of the time course (Miloni et al., 2001) permit a precise mapping of cytological, biochemical, and molecular markers, whereas the cDNA-AFLP screen gives a reasonable indication of the temporal expression profiles of relevant genes. The formation of TEs involves several processes fundamental to plant development, including cell division, local cell signaling, cell elongation, cell fate specification, cell wall synthesis and deposition, lignification, and programmed cell death, involving the activity of hundreds of genes (McCann, 1997).

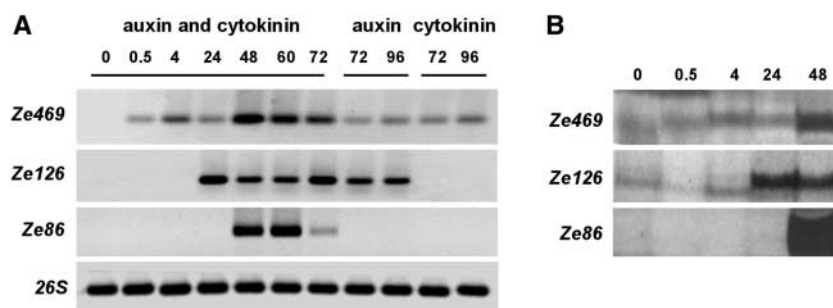
In this article, we report the isolation of 652 differentially accumulated ESTs across the time course of TE formation in the Zinnia mesophyll cell system using cDNA-AFLP analysis. We estimate that we have sampled two-thirds of the original mRNA populations using one pair of restriction enzymes and all possible primer combinations and that the total number of genes involved in TE formation could be ~1000. We inspected the sequences of 47 Zinnia mRNAs and found that 66% contained suitable *ApoI*-*MseI* fragments of between 50 and 450 bp. This finding suggests that 66% of the genes expressed could be visualized using this enzyme combination. Some genes may be expressed constitutively and yet play a role in the transdifferentiation process. Others may show differential expression and yet not be involved directly in xylogenesis. The reliability of the expression patterns as displayed by the cDNA-AFLP approach was verified. The reproducibility was high even when samples of mRNA prepared from independent batches of starting material were used. A more detailed analysis of a subset

of the 652 genes, by RT-PCR and RNA gel blotting, showed that all members of the subset displayed differential expression patterns and that 78% of them matched exactly the cDNA-AFLP pattern. Therefore, we conclude that the cDNA-AFLP screen reliably detects differential patterns of gene expression in the Zinnia system.

Twelve of the differentially accumulated cDNA-AFLP sequences to which we can assign an identity by comparison with the public databases are from genes already known to be involved in processes related to TE formation (McCann and Roberts, 2000). Studies indicate that the various members of the *CESA* gene family have specialized roles in primary and secondary wall formation (Taylor et al., 1999; Williamson et al., 2001). The *Ze452* (*CESA*-like) gene reported here has highest sequence similarity to Arabidopsis, cotton, and poplar sequences implicated in secondary wall formation. The *REVOLUTA/IFL1* gene encodes a homeodomain-Leu zipper protein essential for the normal differentiation of interfascicular fibers and secondary xylem in the inflorescence stem (Zhong and Ye, 1999). Local increased levels of auxin may control cell specification in cambial cells in poplar (Uggla et al., 1996) and in the basal embryo and in the cells surrounding the initials of the seedling root in Arabidopsis (Sabatini et al., 1999). The *PIN1* gene encodes an auxin efflux-carrier protein that establishes polar auxin transport in the embryo by the polar localization of PIN1, later becoming localized on the basal side of xylem parenchyma cells (Gälweiler et al., 1998). *Ze203* (*MONOPTEROS*-like) and *Ze349* (*PIN1*-like) TDFs are accumulated or induced within 4 h of the addition of auxin and cytokinin in the Zinnia system. Auxin transport inhibitors can phenocopy the mutant *monopteros*, which fails to form an interconnected vascular network. *MONOPTEROS* is a transcription factor similar to ARF1, an auxin response factor that binds to auxin-responsive elements in the promoters of auxin-responsive genes (Berleth and Sachs, 2001).

Auxin and cytokinin have long been implicated in the process of vascular development. Recent genetic and molecular analyses have confirmed the role of auxin as a coordinating signal across the plant (Berleth and Sachs, 2001) and implicated auxin as a positional signal in pattern formation (Uggla et al., 1996). Cytokinin is known to promote the induction of TE differentiation in many tissue culture systems (Blee et al., 2001; Miloni et al., 2001), including differentiation of xylem and phloem tissues in tobacco pith explant cultures in vitro (Boucheron et al., 2002) and in *Eucalyptus* tumors (Azmi et al., 2001).

Zinnia cells can be committed to a TE fate even by 10 min of exposure to auxin and cytokinin; they then differentiate to TEs even when the growth factors have been removed. Thus, differentiation events after 10 min clearly are independent of exogenous auxin and cytokinin (Miloni et al., 2001). Sixty-eight TDFs are switched on within 30 min of the addition of auxin and cytokinin to Zinnia cultures; the expression of these genes is the earliest known event in the signaling networks that result in TE formation. The sequences of



**Figure 4.** Expression of *Ze469*, *Ze126*, and *Ze86* cDNAs in Cells Cultured in Vitro.

**(A)** The lanes contain total RNA from cells cultured in noninductive medium for 48 h (lane 1), from cells at 30 min and 4, 24, 48, 60, and 72 h (lanes 2, 3, 4, 5, 6, and 7, respectively) after the addition of auxin and cytokinin at 48 h, from cells in medium containing 1.0 mg/L auxin but no cytokinin for 72 and 96 h (lanes 8 and 9), and from cells in medium containing 1.0 mg/L cytokinin but no auxin for 72 and 96 h (lanes 10 and 11). The corresponding Zinnia accession numbers are presented at the end of Methods.

**(B)** cDNA-AFLP expression patterns of *Ze469*, *Ze126*, and *Ze86*. The Zinnia cells were cultured with auxin and cytokinin for the times indicated above the gels (in hours) before harvesting.

*Ze620*, *Ze92*, and *Ze235* were similar to those of known auxin-induced genes such as the *IAA/AUX* and *GH3* gene families and the NAC domain-containing proteins. The *Cm-NACP* gene is expressed in vascular tissues and is involved in shoot-to-meristem signaling (Ruiz-Medrano et al., 1999). Several lines of evidence indicate that *NAC1*, a transcription activator, mediates auxin signaling to promote lateral root development. An increase of *NAC1* transcript level was detected after only 30 min of exposure of *Arabidopsis* wild-type root cultures to auxin (Xie et al., 2000). These 68 TDFs provide the opportunity for further studies needed to delineate the relationship between the auxin- and/or cytokinin-regulated signaling networks and the functions of both the known and the novel genes that are expressed differentially during the first stages of the developmental pathway of TE formation. A next step is to distinguish genes that are regulated directly by auxin and/or cytokinin from those that are secondary targets. However, additional signals, including ethylene, jasmonate, brassinosteroids, gibberellic acid, and  $\text{Ca}^{2+}$ , are required to complete TE formation (Fukuda, 1996). Cell commitment triggered by auxin and cytokinin is rapid, but the final restriction to TE fate depends on responses to a network of other signal pathways.

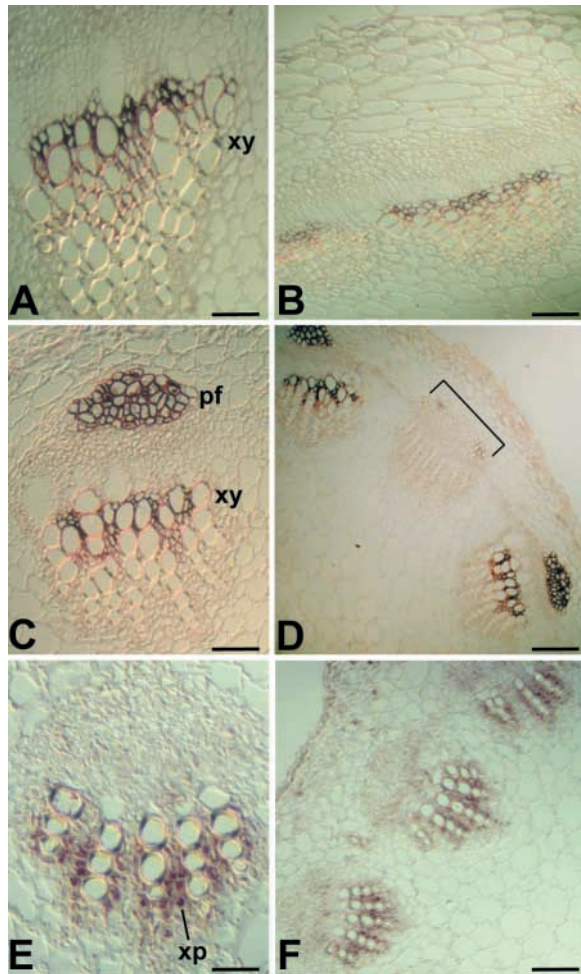
Many plant cells with primary cell walls are capable of growth and division and are pluripotent, which means that some already differentiated cells may be respecified and transdifferentiate to a new cell fate. However, the formation of tracheids is a process of progressive restriction to a single cell fate and is intrinsically irreversible. It is not clear whether the response to auxin and cytokinin is sufficient to commit the cell to a TE fate or if there is a series of intermediate cell fate options, with the final commitment to a TE fate potentially occurring as late as cell autolysis. The idea of the commitment to a TE fate being a multistep process is based on the expression patterns of three *TED* transcripts (Demura

and Fukuda, 1994). It was proposed that these genes are expressed in a temporal order during in vitro culture that corresponds to a spatial distribution in the living plant associated with the progressive restriction of cell fate. Although we did not find these particular genes expressed in the same temporal order in our more synchronous system, either by RNA gel blot analysis (Miloni et al., 2001) or by cDNA-AFLP pattern, this remains an attractive hypothesis. There are two possibilities. The first is that these intermediate stages are simply stages in TE formation that the cell, once committed, must transit. The second is that fate is restricted progressively—for example, to vascular fate, then to xylem, and finally to TE, each step depending on further signaling inputs. If the latter possibility is the case, then it should be possible to provide other signals that would divert the cell to a different developmental pathway (e.g., to induce transdifferentiation to phloem sieve elements). Because TE formation is not cell autonomous until a late stage in the process, it seems more likely that the cells are not committed solely to a TE cell fate by the action of auxin and cytokinin.

From our results, two lines of evidence provide strong support for the idea that the Zinnia system is a robust model for normal plant vascular development rather than for a special pathway of wound-induced vascular connectivity. The first is the presence in the cDNA-AFLP screen of several genes (e.g., *Ze203* [*MONOPTEROS*-like]) that are involved in the establishment of normal vasculature. The second is the in situ results, which confirm that three genes of unknown function from the screen are expressed in plants in different cell types within the vascular bundle, not simply those forming TEs. The patterns of gene expression, when the formation of new vascular strands is taking place, is consistent with the auxin regulation of *Ze126*. It was shown that *Ze469*, a gene whose expression is regulated by both auxin and cytokinin in culture, is localized to young xylem



tissue. The combination of auxin and cytokinin increases its expression, suggesting that they act in a synergistic manner. *Ze86* transcripts are localized conspicuously to the xylem parenchyma cells of vascular bundles, yet they are accumulated late in culture. These patterns of accumulation suggest that each induced cell may adopt one of at least three different cell fates: TEs, xylem parenchyma, and phloem fibers



**Figure 5.** Transverse Sections of the Inflorescence Stems of 4-Week-Old *Zinnia* Plants Probed with Partial Sequences of *Ze469*, *Ze126*, and *Ze86* by in Situ Hybridization.

The sections were probed with antisense digoxigenin-labeled *Ze469* RNA ([A] and [B]), *Ze126* RNA ([C] and [D]), and *Ze86* RNA ([E] and [F]). The *Ze469* transcripts were localized to young xylem tissue (xy). The *Ze126* transcript strongly accumulated in the phloem fibers (pf) and in young xylem tissue (xy) of alternate vascular bundles. No signal was detected in vascular bundles (bracket in [D]) going to the leaves. The *Ze86* transcripts were detected mainly in older xylem parenchyma cells (xp). Shown are photographs of bright-field images using Nomarski optics. Bars = 70  $\mu$ m in (A), (C), and (E) and 120  $\mu$ m in (B), (D), and (F).

(McCann et al., 2001). The final determination of which fate each cell will adopt probably is the result of complex cell-to-cell signaling interactions. This notion is consistent with progressive cell fate restriction following from an initial commitment to a more general vascular cell fate. It also raises the possibility that other intermediate cell states may be present in culture and that these might be identified by some of the new molecular markers described here.

Probably of greatest significance for understanding the molecular basis of xylogenesis is the fact that 40% of the sequences, including those that accumulate within 30 min, are unknowns: they are either hypothetical proteins in the Arabidopsis database or not represented in any of the plant or animal databases. We have demonstrated that a gene of previously unknown function, *Ze469*, is expressed within 30 min and is a marker of vascular cell fate in the stem, confirming that the *Zinnia* system is a useful engine of gene discovery with respect to the very earliest steps in the process of xylogenesis.

## METHODS

### Preparation of Cell Cultures

Mesophyll cells were isolated and cultured as described by Domingo et al. (1998) using medium with no growth factors, 1.0 mg/L benzylaminopurine and 1.0 mg/L naphthalene acetic acid (inductive medium), 1.0 mg/L benzylaminopurine alone, or 1.0 mg/L naphthalene acetic acid alone. Cells were transferred from noninductive to inductive media at 48 h from the wells in six-well plates into 30-mL screw-cap tubes (Bibby Sterilin, Stone, UK); the tubes were centrifuged at 800 rpm for 2 min, and the cells were gently suspended in new medium at a density of  $10^6$  cells/mL.

### cDNA-Amplified Fragment Length Polymorphism

Total RNA was prepared from *Zinnia elegans* cells at different stages of in vitro tracheary element formation using the RNeasy Plant Kit (Qiagen, Valencia, CA). Poly(A<sup>+</sup>) RNA was isolated using magnetic oligo(dT)25 Dynabeads according to the manufacturer's instructions (DynaL Biotech, Wirral, UK). Synthesis of cDNA was performed with Expand Reverse Transcriptase, RNase H, and DNA Polymerase I (Roche Diagnostics, Lewes, UK). cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis was performed according to Bachem et al. (1996) and Durrant et al. (2000). Double-stranded cDNA was digested with the restriction enzymes *ApoI* and *MseI*. The adaptor primers 5'-CTCGTAGACTGCGTACC-3' and 3'-CATCTG-ACGCATGGTTAA-5' (*ApoI*) and 5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5' (*MseI*) were ligated to the restriction fragments, and a PCR preamplification step was performed using the adaptor primers without selective nucleotides. The selective PCR amplification step was performed using the primers 5'-GACTGC-GTACCAATT(C/T)NN-3' (*ApoI*) and 5'-GATGAGTCCTGAGTAANN-3' (*MseI*) (N represents G, C, A, or T). All AFLP reactions were performed as described by Durrant et al. (2000). The bands of interest were cut from the gel with a surgical blade, eluted, and reamplified

with the same primer set used for the initial amplification. The reamplified cDNAs were subcloned using the pGEM-T vector system (Promega) and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with fluorescent sequencing AmpliTaq DNA polymerase (Perkin-Elmer). Database searches were performed at the NCBI World Wide Web server using the Basic Local Alignment Search Tool (BLAST) network service (Altschul et al., 1997). Each transcript-derived fragment sequence was compared against all sequences in the nonredundant database using the BLASTX program. Sequences that returned with no significant homology were compared again against the EST databases using the BLASTN program. Program default parameters were used for all analyses.

### Reverse Transcriptase-PCR Analysis

Total and poly(A<sup>+</sup>) RNA were prepared as described above. First-strand cDNA synthesis was performed using Expand Reverse Transcriptase (Roche Diagnostics). The following primers were used for the reverse transcriptase (RT)-PCR experiments: for *Ze203* (*MONOPTEROS*-like), 5'-CAATGCAACCTCCAACTCAAGAGCTTC-ATGT-3' and 5'-AGATCGCCGTAATCCCATCGCCGTAATCCCA-3'; for *Ze414* (*CSL*-like), 5'-CTATCTTGGTGACCTTCAGGTGAAAAGT-3' and 5'-ATCTCCATCACCATTTCCTAAACAAATT-3'; for *Ze519* (*ZCAD*-like), 5'-CCAAGTTTAATGTAGGGGATTGCGTGGGAGTT-3' and 5'-CTCTCAACATCAGTGCTGAGGAGGTAATCATC-3'; for *Ze349* (*PIN1*-like), 5'-GACGGGAACTTCATGTTACGGTTCGGAAATC-3' and 5'-GGTTGAAACTAGACCTCTAGGTGTTGGGTTTC-3'; for *Ze622* (*REVOLUTA/IFL*-like), 5'-ACATCGCCCGAAGCAGTAACCTAGCTCAAT-3' and 5'-GGCAAATCTGGAACCAACGCCTTTCGTCCA-3'; for *Ze293* (*ZeEXP1*-like), 5'-ACTTGTACAGTCAAGGTACGGGGTGAACACT-3' and 5'-GGATGTGCGTCCGTCAGTGGCTGTGACTCTAA-3'; for *Ze370* (*ZePEL1*-like), 5'-CGTCTTGCTGACTGTGCCATTGGGTTTGGAAA-3' and 5'-TGTAATTGTTAGAAATAGTGATGGCAGTAGA-3'; for *Ze572* (*TED3*-like), 5'-AGTTTGAGTAGCCATTCCATTGTTGGAAT-3' and 5'-CAATAACATCCTCCTCGCCATCGTAGCAC-3'; for *Ze374* (*TED4*-like), 5'-ATGAGGTCAATTACATACACATCACTGTATG-3' and 5'-GCATTCGGTATCGGAACCCACAGCATTGG-3'; for *Ze68* (*TED2*-like), 5'-ATGGTCAAAGCAATTGCAATTCGAATTTG-3' and 5'-TGCATGTTGTGACTTCAGGACCAACTGCTATT-3'; for *Ze452* (*CESA*-like), 5'-CCATTGCTACCCGTTACTTCAATCCCT-3' and 5'-ACCTTGCAAGCATGTGGTTCC-TTGCTCTT-3'; for *Ze94* (*LACCASE*-like), 5'-CCCTGCCAAGTTCCATTGACTATTGATCA-3' and 5'-AACACTAATTGAACGGTTGAGTTGTACTTTA-3'; for *Ze126*, 5'-CAGATATTTGTGGCTTCGTGCGCCTTTTCTG-3' and 5'-GATGATACTTTTGTCTCTCAAATGCAAGATTG-3'; for *Ze469*, 5'-ACCAAGCTTATTTCCATTATACAAAGCAAAG-3' and 5'-GGCTTGGCTTATTTTCTCCGGTGATCAGGTTA-3'; for *Ze86*, 5'-CACCATATGTTATGAAGCTCAAGCTAGGCTTCG-3' and 5'-GAAGATCCTGTGTTCTGCCTCTGTAGAAGCT-3'; for *Ze191* (*CLPC*-like), 5'-CGGCTTATGGCTTTGACTGCTTCATCTTGA-3' and 5'-CACAACTCCGCTACACCGATGATGCACTGGT-3'; for *Ze186* (*SNF2*-like), 5'-AGTAGTACTGACGCACACAAAGCATGCGAAT-3' and 5'-AAGGTGAGATGACCATGTCTGGAGATTCACAT-3'; for *Ze144* (*Inositol 1,3,4-Trisphosphate 5/6 kinase*-like), 5'-TCTGTCAAGCAGCACTACATAACCCGGCATCT-3' and 5'-AGTTTACATGGTTCGTTGTCATTTTCGCAGGT-3'; for *Ze396* (*COMT*-like), 5'-TGGCTCCTGTGTGCAAGTTTTGATCAAGAAT-3' and 5'-GTATCCACTTCATAAATAAGCATCTCCT-3'; for *Ze91* (*SYBL1*-like), 5'-GCAAGGTTCTCTCTATCAATGGCCTT-3' and 5'-GGCAACTTCTCTGCAATT-

GCAACTCAGTGCTCTT-3'; and for *Ze10* (*CELA2*-like), 5'-CATCATCGGCTGCTTTTGACGTGACAGTGA-3' and 5'-ACCTTGCAAGCATGTGGTTCCTTGCTCTT-3'.

For the analysis of differential expression, two rounds of RT-PCR were conducted with two independently isolated total RNA samples. RT-PCR was performed for 15, 20, 24, 28, 32, and 35 cycles to determine the linearity of the PCR. The thermal cycling parameters used for RT-PCR for all genes were as follows: 94°C for 15 s, 62°C for 30 s, and 72°C for 25 s for a total of 25 cycles. *Ze203* was amplified under the same conditions for a total of 33 cycles. As a positive control, a 200-bp 26S rRNA fragment was amplified under the same RT-PCR conditions for a total of 18 cycles using the primer pair 5'-GCACCGATGAAGAAGAATCCATTGAAGA-3' and 5'-AAAGGATTC-TACCAGTCGCTTGATGGGA-3'. The gene specificity of RT-PCR products was confirmed by sequencing.

### RNA in Situ Hybridization

Fragments from the *Ze469*, *Ze126*, and *Ze86* cDNAs (0.5, 0.57, and 0.6 kb, respectively) were cloned into the pGEM-T vector. These fragments were amplified by PCR using the M13-40 and M13 reverse primers. Digoxigenin-labeled sense and antisense RNA probes for *Ze469*, *Ze126*, and *Ze86* clones were synthesized using SP6 and T7 RNA polymerases (Roche Diagnostics). Tissue preparation and in situ hybridization were performed as described by Shu et al. (2000).

### RNA Gel Blot Analysis

Total RNA was prepared from Zinnia cells at different stages of in vitro tracheary element formation, as described above. The RNAs (10 µg per slot) were electrophoresed on 1.4% denaturing formaldehyde-agarose gels and transferred onto Hybond N<sup>+</sup> nylon membranes (Amersham). After immobilization by UV cross-linking, the blots were hybridized with the digoxigenin-labeled AFLP fragments *Ze121*, *Ze198*, *Ze48*, *Ze138*, and *Ze266*. Probe labeling was performed as described above. Hybridization was performed according to the protocol of the DIG Northern Starter Kit (Roche Diagnostics).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for non-commercial research purposes.

### Accession Numbers

The accession numbers for the genes mentioned in this article are as follows: *MONOPTEROS* (At1g19850), *PIN1* (At1g73590), *REVOLUTA/IFL1* (At5g60690), *CESA* (At5g05170), *CSL* (At5g22740), *TED2* (D30800), *TED3* (D30801), *TED4* (D30801), *ZePEL1* (Y09541), *ZeEXP1* (AAF35900), *ZCAD1* (BAA19487), *LACCASE*-like (Y13769), *PG1* (At1g10640), ripening-related protein-like gene from Arabidopsis (At5g62350), Arabidopsis putative endo-β-1,4-glucanase (At1g64390) and putative peroxidase (At5g05340), cotton *CELA2* (U58284), poplar *CEL1* (AF081534), *Ze203* (*MONOPTEROS*-like; AJ316129), *Ze519* (*ZCAD1*-like; AJ316135), *Ze414* (*CSL*-like; AJ409100), *Ze349* (*PIN1*-like; AJ316127), *Ze622* (*REVOLUTA/IFL1*-like; AJ316133), *Ze572* (*TED3*-like; AJ316128), *Ze370* (*PEL1*-like; AJ316131), *Ze293* (*EXP1*-like; AJ316132), *Ze452* (*CESA*-like; AJ409101),

*Ze374* (*TED4*-like; AJ316139), *Ze68* (*TED2*-like; AJ316136), *Ze94* (*LACCASE*-like; AJ488929), *Ze469* (AJ316118), *Ze126* (AJ316137), and *Ze86* (AJ316138).

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