

Subcellular Localization of Expansin mRNA in Xylem Cells^{1,2}

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Terminal differentiation of many vascular cells involves cell wall changes. Cells first elongate their primary wall, then lay down a lignified secondary wall, which is often followed by digestion of the primary wall. Expansins are wall proteins that regulate wall changes, but little is known about the specific functions of the many individual expansin isoforms. An in vitro cell culture of synchronously differentiating tracheary elements was used to identify three new expansins and to compare their expression kinetics with the timing of wall changes. The genes encoding these expansins from zinnia (*Zinnia elegans*), designated *ZeExp1*, *ZeExp2*, *ZeExp3*, are expressed during cell elongation. *ZeExp1* and *ZeExp2* mRNA decrease at the early stage of secondary wall formation, whereas *ZeExp3* does not. In planta, all three *ZeExp* mRNAs are found predominantly in a single flank of cells adjacent to protoxylem and metaxylem vessels and in cells roughly at the radial position of the fascicular and interfascicular cambium. Furthermore, within these cells, *Exp* mRNA is localized exclusively either to the apical or basipetal end of cells depending on the expansin gene and organ, providing the first evidence for polar localization of mRNA in plant cells. *ZeExp1* and *ZeExp3* mRNA are localized at the apical tip, whereas *ZeExp2* mRNA is found in the basal tip. These observations indicate that these three expansins are xylem cell specific and possibly involved in the intrusive growth of the primary walls of differentiating xylem cells.

Plant cells expand by controlling slippage of the wall network of microfibrils and adhering matrix polysaccharides and proteins (Cosgrove, 1993). A family of cell wall proteins called α -expansins are wall-loosening factors that can promote extension of isolated cell wall, probably by disrupting noncovalent bonds between the cellulose microfibrils and matrix polymers (McQueen-Mason and Cosgrove, 1994; McQueen-Mason, 1995; Cosgrove, 1998). The α -expansins are represented by a large gene family (Cosgrove, 1998, 1999), suggesting that they serve various functions throughout development. A ripening-related expansin in tomato fruit may enhance accessibility of noncovalently bound polymers to endogenous enzyme action during ripening (Rose et al., 1997). It has also been proposed that α -expansins are involved in leaf organogenesis (Fleming et al., 1997; Reinhardt et al., 1998) and vascular cell differentiation (Cho and Kende, 1998), but otherwise little is known about the specific role of the individual expansins.

Tracheary elements (TEs) are functional cell corpses that form the water-conducting vessels in plant xylem. During normal stem development, primary TEs develop from meristematic cells of procam-

bial cells, then later by fascicular and interfascicular cambial cells (Shininger, 1979). Prior to secondary wall formation, procambium cells first expand primarily along the longitudinal axis by intrusive growth (Bannan, 1956; Roberts and Uhnak, 1998). In this type of cell extension found among different xylem cell types, the wall matrix in the apical regions of the cell shears. Since these apical regions do not attenuate during growth, wall precursors and growth-limiting proteins must also be directed to the cell wall-growing zone, probably by longitudinal microtubules.

RESULTS

Three α -expansin cDNA clones (*ZeExp1*, *ZeExp2*, and *ZeExp3*) were isolated from a cDNA library constructed from zinnia (*Zinnia elegans*) cell culture induced for TEs for 48 h (Ye and Varner, 1993) using a mix of Arabidopsis *Exp1* and *Exp9* probes. The three cDNA sequences were compared with the Arabidopsis *Exp1* sequence using the CLUSTAL W algorithm (Thompson et al., 1994). The 949-bp *ZeExp1* cDNA represents a partial sequence lacking a signal peptide of presumably 25 to 28 amino-terminal peptides (Fig. 1), but contained a 337-bp 3'-untranslated region (UTR). The 1,088-bp *ZeExp2* contained a 5'-UTR of 15 bp, an open reading frame of 738 bp, and a 3'-UTR of 335 bp. The 1,054-bp *ZeExp3* contained a 5'-UTR of 23 bp, an open reading frame of 729 bp, and a 3'-UTR of 302 bp. The deduced polypeptides of *ZeExp2* and *ZeExp3* were 242 and 245 amino acids, respectively,

¹ This work was supported by the Department of Energy (grant no. DE-FG02-93ER13179 to D.J.C.), by the National Science Foundation (grant no. IBN-9807801 to A.M.J.), and by the U.S. Department of Agriculture.

² This paper is dedicated to and in memory of Prof. William C. Dickison.

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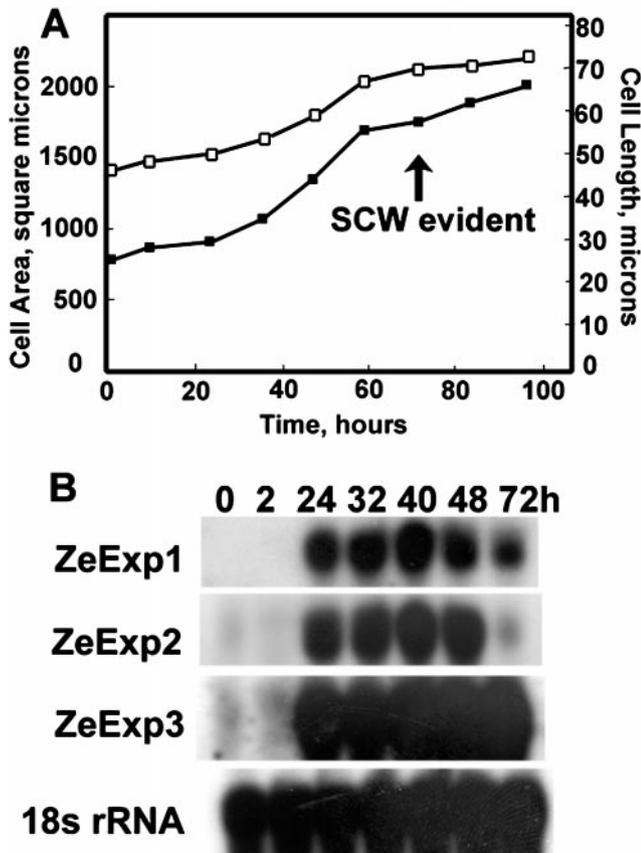


Figure 3. Expression of three expansin genes during TE induction in zinnia cell culture compared with cell growth. A, Zinnia cells induced to differentiate into TEs were photographed at the indicated times and average lengths (□) and areas (■) determined. The average SE of the mean for cell length and area were 1.8 μm and 57 μm², respectively. SCW, Secondary cell wall. B, Total RNAs were extracted from cells cultured in TE inductive medium at the indicated times. Total RNA (10 μg) was loaded in each lane and hybridized with gene-specific probes. Numbers above blots indicate the duration of mesophyll cells in inductive medium. The same blot was hybridized with Arabidopsis 18S rRNA probe to check for loading variability.

Expression of the expansin genes was correlated with primary wall expansion and elongation during TE development in vitro. The expression level of all three expansin genes increased after 24 h of inductive levels of hormones; however, the decay patterns were different among the three genes (Fig. 3B). The *ZeExp1* expression level did not change from 24 h until 48 h, and decreased moderately at 72 h, a time when secondary cell wall thickenings are detectable by microscopy (Groover et al., 1997; Fig. 3A). *ZeExp2* showed a similar expression pattern to that of *ZeExp1* until 48 h, however, the mRNA level decreased to nearly the basal level after 72 h of induction. *ZeExp3* mRNA remained constant.

Expression of Expansin Genes in Planta

Northern analyses revealed that three expansin mRNAs approximately 1,200 nt in length were ex-

pressed in stem tissue and barely detectable in cotyledon and leaf tissues (Fig. 4B). A significant level of *ZeExp1* but not *ZeExp2* or *ZeExp3* mRNA was also detected in roots (Fig. 4B).

Cell-Specific Expression and Intracellular Localization of Expansin Transcripts

In situ hybridization was used to identify expansin-expressing cells in stem tissues (Figs. 5 and 6). Transverse sections probed with sense control RNAs (Fig. 5B) lacked hybridization signal; however, all three expansin mRNAs were detected at the same subtissue location by antisense probes (Fig. 5A, data for *ZeExp2* and *ZeExp3* not shown). Expansin mRNA signals were confined to the developing primary xylem cells, predominantly adjacent to vessel members, and in cells at the future position of the interfascicular cambium. Surprisingly, the hybridization signals routinely appeared punctate (arrows in Fig. 5A). Serial transverse sections revealed that these punctate signals were at most 16 μm in depth (data not shown), suggesting that these expansin mRNAs are highly localized at the specific sites. In situ hybridization of longitudinal sections confirmed the size and punctate shape of the hybridization signals. The single flank of elongated cells adjacent to protoxylem or metaxylem vessels showed strong hybridization of the *ZeExp* riboprobes (Fig. 5, C and E), but occasionally nonadjacent cells located 50 to 100 μm from vessel members also showed clear hybridization.

In all cases, hybridization signals were exclusively located at the distal ends of cells (Fig. 5, C and E), but the apical versus basal location was dependent on the riboprobe used and the tissue. Medial sections

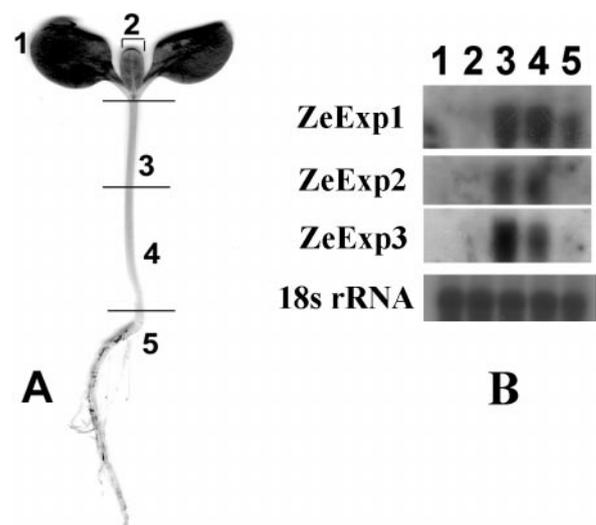


Figure 4. Expression of expansin genes in various organs in zinnia seedlings. A, Ten-day-old zinnia seedling showing the regions used for northern analysis shown in B. 1, Cotyledons without petioles; 2, leaves; 3, upper part of stems; 4, lower part of stems; 5, roots. B, *ZeExp1*, *ZeExp2*, and *ZeExp3* northern blots showing tissue-specific expression.

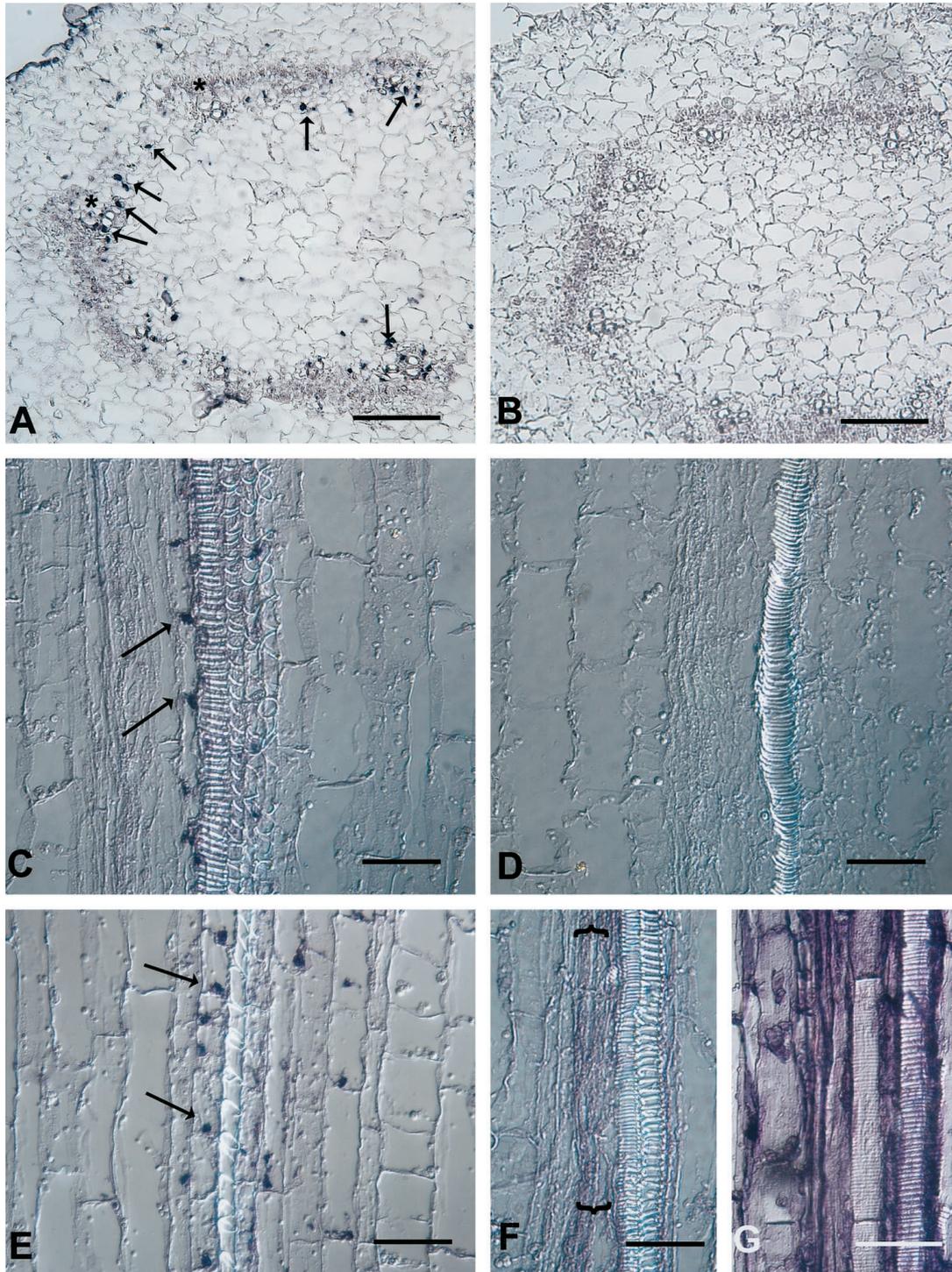


Figure 5. In situ localization of *ZeExp1* and *ZeExp2* mRNA in zinnia stem tissues. A, Cross-section of the upper stem probed with the antisense (A) and sense (B) *ZeExp1* 3'-UTR RNA. Arrows indicate some of the punctate patterned hybridization signals. Note that the signals are found exclusively in the vascular bundles (two bundles are highlighted by asterisks) and in cells near where the interfascicular cambium forms. Longitudinal sections of the upper stem were probed with the antisense (C) and sense (D) *ZeExp1* 3'-UTR RNA and the antisense *ZeExp2* 3'-UTR RNA (E). Arrows indicate the apical (C) and basal (E) location of the punctate hybridization signals. Note that the indicated flank of cells in (C) and (E) appear to begin tip growth from the apical corners. Longitudinal sections probed with the zinnia *Ted3* (F) and 18S rRNA (G) antisense probes, respectively, indicate an even hybridization signal in cells associated with the protoxylem (F) or all cells (G). A and B, Bars = 100 μm ; C to G, bars = 50 μm .

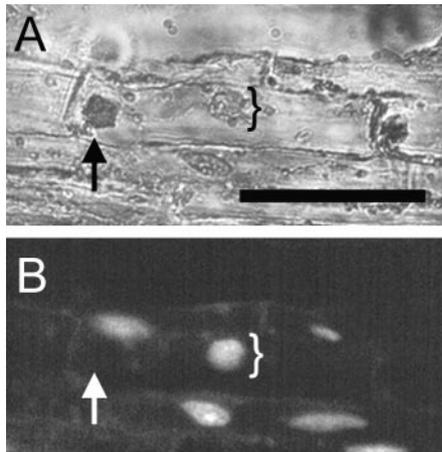


Figure 6. Relative location of expansin mRNA signal and the nucleus in a single cell. Longitudinal section in situ hybridized with the *ZeExp3* antisense probe was stained with DAPI to visualize the nucleus under UV light (B). The section was observed under a combination of white and UV light (A) and UV light only (B). Note that the position of the localized expansin transcript indicated by arrows does not colocalize with the position of the nucleus indicated by brackets. Bar = 50 μ m.

through the base of the cotyledonary petiole revealed *ZeExp1*, *ZeExp2*, and *ZeExp3* mRNA at the cell base. In contrast, at a position just below the apical meristem where protoxylem differentiates, *ZeExp1* and *ZeExp3* mRNAs were found in the apical cytoplasm, while *ZeExp2* mRNA was found in the basal cytoplasm (Table I; Fig. 5, C and E). More precisely, hybridization signal was found in a distal corner of the cells flanking the vessels (Fig. 5E), in each case where intrusive growth appeared to occur (note carefully the elongated corners of the cells in the indicated flanks in Fig. 5, C and E).

Several lines of evidence showed that the punctate pattern of *ZeExp* mRNA hybridization is not an artifact but, rather, is evidence of the cytoplasmic location of the *ZeExp* message. First, only xylem cells hybridized with *ZeExp* antisense probes (Fig. 5, A–E), so the pattern was not due to probe aggregation. Second, an 18S rRNA revealed a relatively diffuse cytoplasmic staining even in the xylem cells, where *ZeExp* probes hybridize in the punctate pattern, indicating that fixation artifacts did not cause cytoplasmic aggregation in these particular cells (Fig. 5G). Third, a TED3 probe, previously shown to hybridize

to xylem cells (Demura and Fukuda, 1994), had a hybridization signal that was evenly distributed throughout the cytoplasm (Fig. 5F) in the same cell types in which expansin mRNA hybridization was shown to be punctate. Fourth, the punctate pattern was not due to nuclear sequestration of the *ZeExp* message (Fig. 6).

The strongest evidence that the punctate hybridization signals are not due to fixation artifacts comes from the observation that the *ZeExp1* and *ZeExp3* hybridization patterns were apical, whereas for *ZeExp2* they were basal in the same cell types from the same fixed tissue (Fig. 5, C versus E). Clearly, fixation or hybridization artifacts cannot explain the sequence specificity of this opposite localization.

DISCUSSION

We introduce three new members of a large gene family of expansins, and indirectly show that they play a specific developmental role in shoot histogenesis. These expansin genes are expressed in developing TEs in vitro, with kinetics and expression patterns linking the function to the regulation of primary wall expansion of xylem-specific cells. In planta, expression of these genes is limited to a specific set of xylem cells.

Several xylem cell types elongate by intrusive growth (Bannan, 1956; Roberts and Uhnak, 1998), whereby wall matrix slips and wall precursors are delivered to these localized regions of the wall by direct secretion through the endomembrane system. However, loosening of the existing wall at the localized site must precede or accompany wall deposition before intrusive growth can ensue. Given what is known about the biochemical properties of expansins, it is reasonable to presume that this loosening is accomplished by the action of expansins. In situ hybridization clearly indicates that expansin mRNA is located exclusively at the end of the cytoplasm in a cell type that appears poised to elongate by intrusive growth. We propose that these expansin mRNAs are subcellularly localized to the site of future growth so as to provide restricted wall loosening by expansins produced there.

The identity and fate of the flanking cells showing localized expansin mRNA is not known with certainty. While their morphology points to their being

Table I. Subcellular location of expansin mRNA in xylem cells

Expansin	Cotyledon Petiole			Hypocotyl		
	Apical	Basal	Unclear	Apical	Basal	Unclear
	no. ^a					
<i>ZeExp1</i>	27 (54%)	0	23	42 (84%)	0	8
<i>ZeExp2</i>	37 (74%)	0	13	0	40 (80%)	10
<i>ZeExp3</i>	36 (72%)	0	14	41 (82%)	0	9

^a No. of cells scored having the indicated location in this organ. Nos. in parentheses are the percent of the total cells scored. Unclear means it was not possible to locate the end wall.

fully differentiated xylem parenchyma and not precursors to vessel members, their tissue location and signs of intrusive growth (Fig. 5E) suggest they may transdifferentiate into fibers.

Asymmetric RNA localization is well documented in animal cells and invertebrates (Bashirullah et al., 1998), where this mechanism accounts for asymmetric distribution of proteins in polarized somatic cells and embryos. Fewer cases are found in lower organisms, although there is one example each for localized mRNA in yeast (Takizawa et al., 1997) and a brown algae (Bouget et al., 1996). However, the work here presents the first evidence, to our knowledge, for mRNA localization in land plants (Embryophyta).

RNA localization requires an intact cytoskeleton (Oleynikov and Singer, 1998), and 3'-UTR of localized mRNA plays an important role in RNA localization (Kimberly et al., 1992; Goldspink et al., 1997). It has been reported that almost one-tenth of randomly collected *Drosophila* ovarian mRNA shows asymmetric localization (Dubowy and Macdonald, 1998). The function of RNA localization may prevent random movement of RNA within the cytoplasm and bring it into contact with regulatory factors (Bassell and Singer, 1997). In the case of mRNA encoding co-translated proteins such as the expansins described here, targeting message to specific microdomains on the endoplasmic reticulum may be involved in directed trafficking of the secreted protein (Weeks and Melton, 1987).

Longitudinally oriented microtubules may direct expansin mRNA to a specific intracellular location, where expansin mRNA is translated and secreted to the growing or neighboring cell wall to cause localized cell wall loosening, although expansin protein was not localized in the present study. A previous notion was that microtubules direct transverse deposition of cellulose microfibrils (Fukuda, 1996), but this is not supported by the recent evidence that cortical microtubules are actually oriented parallel to the long axis of elongating cells during TE differentiation, and that overall cell elongation is caused by localized intrusive growth of cells (Roberts and Uhnak, 1998). Parallel orientation of microtubules in these cells is consistent with a role of apical trafficking of mRNA and wall precursors. In light of the current evidence for tip-localized expansin mRNA and the known role for microtubules in mRNA trafficking (Bassell and Singer, 1997), we propose that the seemingly paradoxical longitudinal distribution of microtubules in elongating TE occurs to direct growth-limiting expansins to the cell tip rather than to direct cellulose deposition.

MATERIALS AND METHODS

Plant Material and Culture Media

Zinnia (*Zinnia elegans* L. cv Envy) was purchased from Stokes Seed (Buffalo, NY). Plants were grown in a growth

chamber at 25°C with 14 h of light (110 $\mu\text{mol photons m}^{-2} \text{s}^{-2}$) and 60% relative humidity. Mesophyll cells of 8- to 16-d-old seedlings were isolated and cultured as described in Groover et al. (1997). Cells were cultured in inductive medium as described by Fukuda and Komamine (1980) with 0.1 mg/L α -naphthalene-acetic acid and 0.2 mg/L 6-benzylamino-purine.

Isolation and Sequencing of cDNA Clones

The zinnia cDNA library, provided by Dr. Z-H Ye (University of Georgia), was constructed from leaf mesophyll cells cultured for 48 h in TE induction medium (Ye and Varner, 1993). The library was screened with a mixture of ^{32}P -labeled full-length *AtExp1* and *AtExp9* cDNAs at 60°C, followed by two 60°C $1\times$ SSC washes. Three independent cDNA phage clones were subcloned into pBluescript II KS+ phagemid vectors (Stratagene, La Jolla, CA), and sequenced by the Automated DNA Sequencing Facility at The University of North Carolina at Chapel Hill.

Genomic DNA Extraction and Southern-Blot Analyses

Genomic DNA was isolated from 10-d-old seedlings as described previously (Dellaporta et al., 1983). Ten micrograms of genomic DNA was digested with *EcoRI* and *HindIII* and fractionated in a 0.6% (w/v) agarose gel, followed by a Southern transfer to a Nytran membrane (Schleicher & Schuell, Keene, NH). The blots were hybridized with a mixture of full-length *ZeExp2* and *ZeExp3* cDNA probes in 50 mM PIPES (1,4-piperazinediethanesulfonic acid) (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, and 5% (w/v) SDS solution for 16 h at 65°C, and then washed. The first two washes in $5\times$ SSC/5% (w/v) SDS at 65°C were followed by two more washes in $2\times$ SSC/5% (w/v) SDS for low stringency and $0.2\times$ SSC/5% (w/v) SDS for high stringency, respectively.

Northern Analyses

Ten-day-old green seedlings were dissected into cotyledon, leaf, upper stem half, lower stem half, and root (Fig. 4A). Total RNA was isolated from each organ and cells in culture as described previously (Wadsworth et al., 1988). RNA (10 μg) was fractionated on 1.2% (w/v) agarose gels in the presence of 3% (w/v) formaldehyde and transferred to Nytran membrane. Hybridization and washing were as described in Southern-blot analyses, with final washes in $0.2\times$ SSC/5% (w/v) SDS. Gene-specific DNA fragments were prepared by PCR amplification from cDNA clones. For the *ZeExp1*-specific probe, the 340 bp of 3'-UTR was PCR amplified using oligonucleotides 5'CCCGGGAATTC-GAGTGTA AAAACATGAT3' and 5'TTTTTTTGGATCCAA-AATCAATCCATT3' as forward and reverse primers, respectively. For the *ZeExp2*-specific probe, the 293-bp 3' end was subcloned into *EcoRI* site of pBS KS phagemid and subsequently PCR amplified using SK and KS primers. For the *ZeExp3*-specific probe, the 293-bp 3' region was PCR amplified with forward primer (5'AAGCACATCTATGT-

TCT3') and reverse primer (SK). Probes were prepared by ³²P labeling using a random primer kit (Boehringer Mannheim/Roche, Basel).

In Situ Hybridization

In situ hybridization was performed as described previously (Cho and Kende, 1998) except higher concentrations of RNaseA (5–10 µg/mL) were used. Hybridization of the riboprobes was detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase, and visualized by color development according to the manufacturer's instructions (Boehringer Mannheim/Roche). Sense and antisense *ZeExp1*- and *ZeExp2*-specific riboprobes were generated by in vitro transcription of the subcloned 3'-UTR using a digoxigenin labeling kit (Boehringer Mannheim/Roche). The antisense *ZeExp3*-specific, 300-bp riboprobe was prepared by transcription of a *Nar1* restriction of the full-length *ZeExp3* clone. All probes were prepared from 3'-UTR except the *ZeExp3* sense probe, which was generated from the full-length cDNA. Probes were hydrolyzed to 100 nt by alkaline hydrolysis prior to hybridization. Each hybridization with a specific sense or antisense probe was performed at least four times, with the same results. To stain nuclei, 4', 6-diamidino-2-phenyl-indole diacetate (DAPI) was dissolved in water at 1 mg/mL and used at a working concentration of 0.05 mg/mL.

ACKNOWLEDGMENTS

We thank Sabine Hardenack for helpful discussion on in situ hybridization, Jheng-Hua Ye for the cDNA library, Andrew Groover for technical assistance, W. Soh, W.C. Dickson, and J. Romberger for discussions on primary xylem, and Susan Whitfield for help with figures. Daniel M. Durachko assisted with preliminary experiments.

Received November 17, 1999; accepted February 11, 2000.

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