Molecular Cloning and Characterization of cDNAs Associated with Tracheary Element Differentiation in Cultured *Zinnia* Cells[1]

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Mesophyll cells isolated mechanically from leaves of *Zinnia elegans* L. cv Canary bird differentiate into tracheary elements (TE) semisynchronously and at high frequency. Using this system, three cDNA clones, TED2 to TED4, whose corresponding mRNAs were expressed in a close association with tracheary element differentiation, were isolated by differential screening of a λgt11 cDNA library. The library was prepared using poly(A)+ RNA from cells cultured in a TE-induced medium for 48 h prior to morphological changes, including secondary cell-wall thickenings and autolysis. Northern analysis indicated that mRNAs corresponding to the clones were expressed preferentially in cells differentiating into TEs prior to the morphological changes. The expression of the mRNAs was found not to be induced by α-naphthaleneacetic acid or benzyladenine solely and not to be associated directly with cell division. Analysis of the nucleotide sequence of TED4 showed that the cDNA contains an open reading frame of 285 bp, encoding a polypeptide comprising 95 amino acid residues with a predicted molecular mass of 10.0 kD. A homology search of the nucleotide and amino acid sequences of TED4 with several data bases revealed a significant similarity to those of the barley aleurone-specific clone B11E, which was isolated as an aleurone-specific cDNA from 20-d postanthesis grain.

In higher plants differentiated mature cells have the potential to redifferentiate to other types of cells, tissues, organs, and whole plants under appropriate experimental conditions. The first step of such redifferentiation is cytodifferentiation, which occurs in individual cells. Cytodifferentiation from parenchyma cells into TEs, which are major constituents of xylem, has been used as an excellent model for cytodifferentiation in higher plants for several reasons (Roberts et al., 1988; Fukuda, 1989, 1992).

First, the morphological characteristics of TEs, which have annular, spiral, reticulate, or pitted secondary wall thickenings, enable us to distinguish easily between differentiated and undifferentiated cells under a light microscope. Second, the formation of TEs can be induced in tissue and cell cultures of many species by various stimuli, such as the inclusion of exogenous plant hormones, light conditions, and wounding. Third, TE differentiation is irreversible, and nuclei and cell contents are lost at its final step. This enables us to rule out

the possibility of dedifferentiation or redifferentiation to other types of cells after termination or in the progression of TE differentiation. Finally, TE differentiation is closely associated with specific biochemical events, i.e. active synthesis and deposition of secondary wall components such as wall polysaccharides, lignin, and wall proteins, which enable us to find biochemical and molecular markers associated with TE differentiation.

Some enzymes related to the biosynthesis of secondary wall components such as xylan synthase (Suzuki et al., 1991), PAL (Fukuda and Komamine, 1982), 4-coumarate:CoA ligase (Church and Galston, 1988), and a cell-wall-bound peroxidase (Fukuda and Komamine, 1982; Church and Galston, 1988; Sato et al., 1993) have been characterized and are regarded as good biochemical markers specific for TE differentiation. In addition, three kinds of cell wall proteins, GRPs, HRGPs, and PRPs, have been reported to be located in the secondary cell walls of TEs and could be biochemical markers (Ye and Varner, 1991; Fukuda, 1992). Thelen and Northcote (1989) have reported that the appearance of elevated levels of a degradative type of nuclease, acting on single-stranded nucleic acids, was characteristic of the later stages of TE differentiation in *Zinnia* cells. This enzyme, which was purified as a single polypeptide, was also thought to be a good biochemical marker of TE differentiation.

Although, as described above, many biochemical markers specific for TE differentiation have been reported, analyses of the markers at the molecular level are not sufficient. Recently, the expression of the PAL gene in differentiating *Zinnia* cells was studied with a cDNA for bean PAL as a probe (Lin and Northcote, 1990), and it was demonstrated that the increase in PAL activity during TE differentiation was caused by an increased level of PAL mRNA. Using transgenic tobacco plants into which a heterologous PAL sequence was introduced, Elkind et al. (1990) demonstrated a reduction in the lignification of cell walls of TEs concomitant with a reduction of PAL activity. It is known that PAL is encoded by a small family of genes (Ohl et al., 1990). In bean, the PAL2 gene has been shown to be expressed in

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Abbreviations: dT, thymidine; FdU, 5-fluorodeoxyuridine; fb-GRP1.8, French bean GRP 1.8; GRP, glycine-rich protein; GUS, β-glucuronidase; HRGP, hydroxyproline-rich glycoprotein; NAA, α-naphthaleneacetic acid; PAL, phenylalanine ammonia-lyase; PCR, polymerase chain reaction; PhT, 6(5H)-phenanthridinone; PRP, proline-rich protein; TE, tracheary element.

association with lignification during TE differentiation using transgenic tobacco plants (Bevan et al., 1989; Liang et al., 1989). Moreover, analysis of regulatory properties of the bean PAL2 promoter showed that cis elements essential for expression during TE differentiation were located between nucleotides −289 and −74, in which a negative element was suspected to be contained between −135 and −119 (Leyva et al., 1992).

Genes of GRPs isolated from several species have been shown to be expressed highly in differentiating xylem cells (Keller et al., 1989b; Condit et al., 1990; Ye and Varner, 1991). Keller et al. (1989a) have found that a 494-bp promoter fragment of the fbGRP1.8 gene fused to the GUS reporter gene expressed the gene in vascular tissue of transgenic tobacco plants. Further analysis of the fbGRP1.8 promoter has revealed the presence of a negative element (Keller and Baumgartner, 1991). Recent reports have indicated that the genes of PRPs and HGRPs were also expressed in association with TE differentiation (Stiefel et al., 1990; Ye et al., 1991; Ye and Varner, 1991). Thus, genes of PAL, GRPs, PRPs, and HGRPs are considered to be good molecular markers of xylem differentiation.

However, these molecular markers are associated with the secondary wall formation occurring in the later stages of TE differentiation. Thus, to investigate TE differentiation fully on a molecular basis, it is also necessary to obtain genes that are expressed in the earlier stages. Such genes, expressed in a temporally regulated manner, can be isolated only in an efficient experimental system in which TE differentiation is induced synchronously and at high frequency. Fukuda and Komamine (1980, 1982) and Fukuda (1989, 1992) established an experimental system in which single cells isolated mechanically from Zinnia leaves differentiate into TEs semisynchronously and at high frequency. This system is considered most suitable for the isolation of new marker genes of TE differentiation. Fukuda and Komamine (1983) reported that two polypeptides began to be synthesized at 48 h of culture of differentiating Zinnia cells prior to morphological changes. Using the Zinnia system, therefore, we tried to isolate cDNA clones whose corresponding mRNAs are expressed preferentially in cells differentiating into TEs prior to morphological changes by differential screening of a cDNA library constructed from poly(A)$^+$ RNA from differentiating Zinnia cells cultured for 48 h. With this approach three new cDNA clones associated with TE differentiation were isolated.

MATERIALS AND METHODS

Plant Material and Culture Methods

A modified method of Fukuda and Komamine (1980, 1982) was used for preparation and culture of single cells. First leaves of 14-d-old seedlings of Zinnia elegans L. cv Canary bird were surface sterilized in a solution of sodium hypochlorite (approximately 0.05%, v/v) and then macerated with a homoblender in a culture medium. Media shown in Table I were used (Fukuda and Komamine, 1980, 1982; Sugiyama et al., 1990). FdU and PhT were added directly to cell suspensions at a concentration of 10^{-6} M and 10^{-5} M, respectively. To the FdU-treated cells, dT was added at a concentration of 10^{-4} M after 10 h of culture.

Purification of RNA

Total cellular RNA was isolated by a modification of the method of Logemann et al. (1987). Cells cultured for various times were collected by centrifugation. The cells were then mixed with GTC solution (4 M guanidine thiocyanate, 50 mM Mes, pH 7.0, 20 mM EDTA, pH 8.0, and 10% [v/v] mercaptoethanol). The mixture was homogenized by a sonicator (TOMY UD-201) at duty 50 and output 10 for 2 min and supplied with N-lauroylsarcosine sodium salt at a final concentration of 0.5% (w/v). The homogenate was centrifuged at 12,000g for 10 min at 4°C and the supernatant was precipitated with ethanol. The pellet was collected, dried under vacuum, and extracted with phenol:chloroform (1:1, v/v) three times. The final aqueous phase was precipitated with ethanol, washed with 70% (v/v) ethanol, and dried. The pellet was dissolved in distilled water and reprecipitated with 3 M LiCl at 4°C for 16 h. The pellet was collected by centrifugation at 12,000g for 10 min at 4°C, washed with 70% (v/v) ethanol, and dried. The dried pellet was dissolved in distilled water and stored at −20°C. Poly(A)$^+$ RNA was prepared by oligo(dT) cellulose chromatography (Collaborative Research, Inc., Bedford, MA) according to the method of Maniatis et al. (1982) with modifications. The poly(A)$^+$ RNA was precipitated with ethanol and dissolved in distilled water to a concentration of 0.5 mg/mL.

Construction of a cDNA Library

A λgt11 cDNA library was constructed from poly(A)$^+$ RNA isolated from the cells cultured in D medium for 48 h. Double-stranded cDNA was prepared using a cDNA synthesis system plus kit (Amersham International), which is based on the RNase H method of Gubler and Hoffman (1983). The cDNA was ligated to the vector λgt11 and packaged in vitro using a cDNA cloning system λgt11 kit (Amersham International).

Differential Screening

The packaged library was plated at a low plaque density (about 3000 plaques per 140 cm^2 dish) and screened by differential hybridization. Duplicate nylon-membrane (Biodyne A 1.2 μm, Pall Biosupport Division, Glen Cove, NY) replicas were lifted from each of the plates, denatured with denaturation solution (0.5 M sodium hydroxide and 1.5 M sodium chloride), neutralized with neutralizing solution (0.5 M Tris-HCl, pH 7.4, and 1.5 M sodium chloride), and then fixed by baking at 80°C for 1 h. At the first screening, plaques in the nylon membranes were screened by hybridization with ^{32}P-labeled single-stranded cDNA probes synthesized from the poly(A)$^+$ RNA isolated either from cells cultured in D medium or Cp medium for 48 h. Prehybridization was performed in a Southern hybridization solution (100 μg/mL denatured herring sperm DNA, 5× Denhardt's solution [1× Denhardt's solution: 0.02% (w/v) Ficoll, 0.02% (w/v) PVP, and 0.02% (w/v) BSA], 5× SSC [1× SSC: 0.15 M sodium chloride and 15 mM sodium citrate], and 0.1% [w/v] SDS) at 65°C for 1 h and hybridization in the same solution with probes at 65°C for 12 to 20 h according to the instructions from the manufacturer of the nylon membranes.

Posthybridization steps were carried out by washing the

membranes with a washing buffer (0.1–2.0× SSC and 0.1% [w/v] SDS) at room temperature for 1 to 2 h. The phage DNAs that hybridized to cDNA probes were visualized by autoradiography. The plaques that gave positive hybridization signals were selected. The selected plaques were isolated and resuspended in 1 mL of SM buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 7 mM MgSO₄, and 0.01% [w/v] gelatin). Phages in the isolated plaques were replated at a density of about 50 to 100 plaques per 64 cm² dish and rescreened by the same method as the first screening. Subsequently, each of the cDNA inserted in the selected clones was amplified with PCR using λgt11-specific primers, and then RNA blot analysis and cross-hybridization were performed with them.

Preparation of cDNA Probes

³²P-Labeled single-stranded cDNA probes were prepared by reverse transcription of poly(A)⁺ RNAs using oligo(dT) primers. cDNA probes were prepared by labeling with [α-³²P]dCTP using a Multiprime DNA labeling system and a Megaprime DNA labeling system (Amersham International).

PCR

PCR was performed in 50 μL of reaction mixture (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.01% [w/v] gelatin, and 200 μM deoxyribonuceloside 5'-triphosphates mixed solution) in a microtube with λgt11 primers (forward and reverse) (Takara Shuzo Co., Kyoto, Japan) and 1.25 units of Taq polymerase, using 25 cycles of 1 min at 93°C, 1 min at 56°C, and 2 min at 73°C. Amplified cDNAs were purified with a phenol/chloroform extraction and used for cross-hybridization or as cDNA probes for northern blot analysis.

Subcloning

Phage DNAs of the selected clones were isolated by the liquid lysate method (Maniatis et al., 1982). cDNA inserts were excised with EcoRI or KpnI and then constructed into EcoRI or KpnI sites of the plasmid vector, pBluescript KS M13+ (Stratagene, La Jolla, CA), to facilitate further analysis.

RNA Blot Hybridization

Total RNA was denatured at 65°C for 15 min in 10 μL of loading buffer (14% [v/v] formaldehyde, 50% [v/v] formamide, 20 mM Mops, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA). The denatured RNA was separated on 1.2% agarose gels containing 0.66 M formaldehyde. RNAs were transferred to nylon membranes using a VacuGene XL vacuum blotting system (Pharmacia LKB Biotechnology, Uppsala, Sweden) and hybridized with ³²P-labeled cDNA probes. Prehybridization and hybridization were performed at 42°C in a northern hybridization solution (100 μg/mL denatured herring sperm DNA, 50% [v/v] deionized formamide, 5× SSPE [1× SSPE: 0.18 M sodium chloride, 0.01 M sodium phosphate buffer, pH 7.7, and 0.1 mM EDTA], 5× Denhardt's solution, and 0.1% [w/v] SDS) without a cDNA probe for 1 h and with the cDNA probe for 12 to 20 h. Posthybridization steps were performed by the same method of differential hybridization. The fragments of cDNA that hybridized to

corresponding RNAs were visualized by autoradiography or the image analyzing system (Fuji Photo Film Co., Tokyo, Japan).

Nucleotide Sequencing

Deletion derivatives of clones were generated using a double-stranded nested deletion kit (Pharmacia LKB Biotechnology). Nucleotide sequences were determined by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using a Multiwell microtiter plate DNA sequencing system T7 DNA polymerase kit (Amersham International). The determined nucleotide sequences were compared with those contained in the European Molecular Biology Laboratory nucleotide sequence data base (release 27.0). Polypeptide sequences deduced from the determined nucleotide sequences were compared with those contained in National Biomedical Research Foundation protein sequence data base (release 28.0).

RESULTS AND DISCUSSION

Cell Culture

Zinnia mesophyll cells were cultured in various culture media (Table I). In either D medium or mD medium, 30 to 40% of the cell population differentiated semisynchronously into TEs between 52 and 72 h. Approximately 30% divided in D medium and approximately 10% divided in mD medium between 36 and 72 h. The rate of cell division in Cp medium was close to that in D medium, but TE formation was rarely induced (less than 1% in the late stages of culture). In Co medium, mCo medium, CN medium, and CB medium, neither TE formation nor cell division was observed.

Construction and Screening of the cDNA Library

Poly(A)⁺ RNA was isolated from Zinnia mesophyll cells cultured in D medium for 48 h and a λgt11 library was

Table I. *Concentration of plant hormones and addition of inhibitors in various media, and occurrence of TE differentiation and cell division*

Cultures were initiated in the medium containing indicated concentrations of plant hormones. P and F medium were supplemented with 10⁻⁶ M PhT or 10⁻⁵ M FdU, respectively, at the initiation of cultures. To the F medium, dT was added at a concentration of 10⁻⁴ M after 10 h of culture.

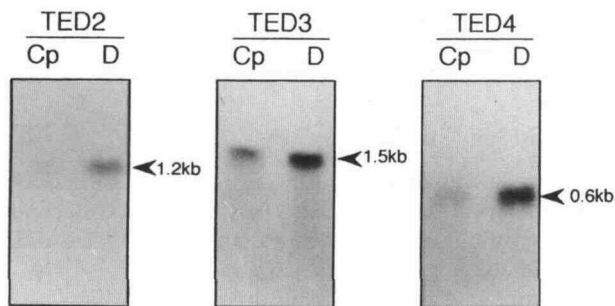| Medium	NAA	BA	Inhibitors	Differentiation	Cell Division
	mg/L	mg/L			
D	0.1	1.0	None	+	+
mD	0.1	1.0	None	+	+
Cp	0.1	0.001	None	(+)	+
CN	0.1	0	None	−	−
CB	0	1.0	None	−	−
Co	0	0	None	−	−
mCo	0	0	None	−	−
P	0.1	1.0	PhT	−	+
F	0.1	1.0	FdU	−	−
T	0.1	1.0	FdU + dT	+	+

Figure 1. Differential expression of TED2, TED3, and TED4 mRNAs between TE-induced and control cultures. Total RNA was extracted from cells cultured for 48 h in D or Cp medium. Samples (10 μg/lane) were subjected to electrophoresis on a 1.2% agarose/formaldehyde gel, transferred to nylon membranes, and then hybridized with ^{32}P-labeled cDNA inserts of TED2, TED3, and TED4.

constructed from double-stranded cDNA prepared from this, which contained approximately 1.4×10^6 recombinants. Differential plaque hybridization was performed with the cDNA library for screening genes that were preferentially expressed in cells cultured in D medium. ^{32}P-labeled single-stranded cDNA was prepared from poly(A)$^+$ RNA isolated from cells cultured for 48 h in D medium and used as a positive probe. ^{32}P-labeled single-stranded cDNA from cells cultured in Cp medium was used as a negative probe. This is because cells cultured in Cp medium rarely differentiate into TEs but divide at a similar rate in D medium, which enables us to isolate genes that are expressed in association with TE differentiation but not with cell division.

Plaques that gave hybridization signals with the positive probe but not with the negative probe were selected and rescreened. Of approximately 45,000 plaques screened in this manner, 102 plaques were selected as differentially hybridizing plaques and rescreened. Finally, three clones were selected and designated as TED2, TED3, and TED4, respectively. To confirm that mRNAs corresponding to the clones were expressed preferentially in the TE-induced culture, the cDNA inserts of the three clones that were amplified with PCR techniques and labeled with [α-^{32}P]dCTP were hybridized to RNA from the cells cultured for 48 h in D medium or Cp medium.

The TED2 to TED4 cDNA inserts preferentially hybridized to RNA species of approximately 1.2, 1.5, and 0.6 kb, respectively, from cells cultured in D medium (Fig. 1). The expression of the TED3 transcript in cells cultured for 48 h in Cp medium was temporal and was not observed in cells cultured for more than 48 h in Cp medium, although high expression continued in cells cultured for more than 48 h in D medium at that time (data not shown). The isolated cDNA inserts were subcloned into pBluescript KS M13+. Restriction maps of these cDNA clones are shown in Figure 2.

Kinetics of the Appearance of mRNAs Corresponding to TED cDNA Clones in TE-Induced Cultures

To examine the changes in the levels of mRNAs corresponding to TED cDNA clones during TE differentiation,

total RNA was isolated from *Zinnia* mesophyll cells cultured for various times in mD medium or mCo medium, and the RNA was analyzed by northern blot hybridization (Fig. 3). The TED2 mRNA was not detected in cells cultured for shorter than 24 h in mD medium. Its level increased rapidly between 24 and 48 h of culture and decreased thereafter. The TED3 mRNA was first detected in cells cultured for 24 h in mD medium and increased until 72 h. The TED4 mRNA appeared at 48 h of culture and increased until 72 h in mD medium. On the other hand, the TED2 to TED4 mRNAs were present at undetectable levels in control cells cultured in mCo medium. This result suggests that the TED genes are expressed preferentially in cells differentiating into TEs and that the expression started prior to the morphological changes that started from around 52 or 60 h.

Hormonal Effects on the Level of TED mRNAs

The hormonal effects on the level of TED mRNAs were analyzed in the cells cultured for 48 h in Co, CB, CN, Cp, and D media (Fig. 4). In cells cultured in D medium, the TED2 to TED4 mRNAs were present at high levels. In Co, CB, and CN media, in contrast, the mRNAs were found to be undetectable. The three mRNAs were expressed at low levels in cells cultured in Cp medium. Therefore, these results indicate that the expression of TED mRNAs is not induced by a phytohormone such as NAA and BA, but is induced in association with TE differentiation.

Effects of FdU and PhT on the Levels of TED mRNAs

To clarify that the expression of TED mRNA is associated with TE differentiation but not cell division, we examined the levels of TED mRNAs in the cells cultured for 48 h in D medium with inhibitors of DNA synthesis. It has been indicated that repair-type synthesis of nuclear DNA in cultured mesophyll cells of *Zinnia* is required for TE differentiation (Sugiyama and Komamine, 1990; Fukuda, 1992). Sugiyama and Komamine (1987) showed that *m*-aminobenzamide and nicotinamide, inhibitors of ADP-ribosyltransferase, which is associated with DNA excision repair, inhibited TE differentiation without affecting cell division of isolated *Zinnia* me-
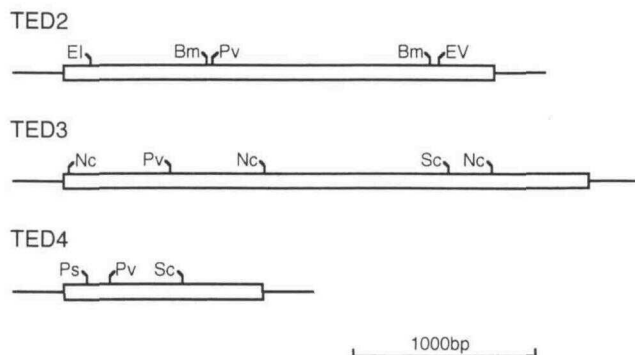

Figure 2. Restriction maps of TED2, TED3, and TED4 cDNA inserts. Open boxes indicate cDNA inserts of TED2, TED3, and TED4. Restriction sites: Bm, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; Nc, *Nco*I; Ps, *Pst*I; Pv, *Pvu*II.
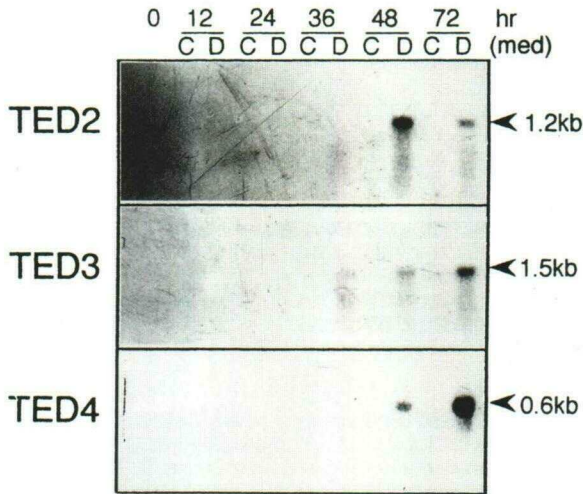
Figure 3. Changes in the expression of TED2, TED3, and TED4 mRNAs during culture. Total RNA was extracted from cells cultured in mD (D) or mCo (C) medium at the indicated times. The RNA was hybridized as described in the legend to Figure 1.

sophyll cells. Recently, PhT has been reported to specifically inhibit repair-type DNA synthesis (Banasik et al., 1992). With this reagent, we cultured *Zinnia* mesophyll cells, inhibiting TE differentiation without affecting cell division (Y. Shoji, personal communication). Cells cultured for 48 h in the presence of PhT had lower levels of expression of each TED
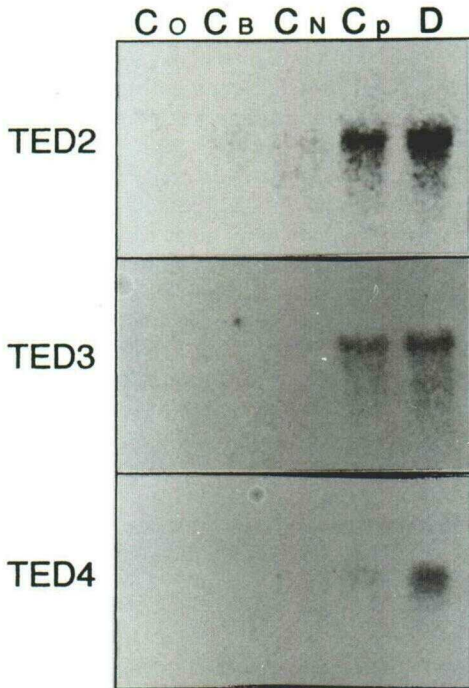

Figure 5. Northern blot analysis of the effects of DNA synthesis inhibitors, FdU and PhT on the expression of TED2, TED3, and TED4 mRNAs. Total RNA was extracted from cells cultured for 48 h in D medium in the presence of 10^{-6} M FdU (lane F), 10^{-6} M FdU followed by the addition of 10^{-4} M dT at 10 h (lane T), 10^{-5} M PhT (lane P), and without any inhibitor (lane D). The RNA was hybridized as described in the legend to Figure 1.

Figure 4. Northern blot analysis of hormonal effects of the expression of TED2, TED3, and TED4 mRNA. Total RNA was extracted from cells cultured for 48 h in Co, CB, CN, Cp, and D media. The RNA was hybridized as described in the legend to Figure 1.
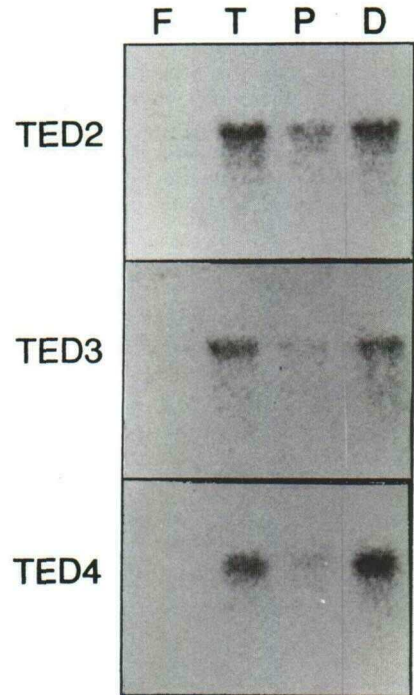

Figure 6. Nucleotide sequence of TED4 and amino acid sequence of deduced TED4 polypeptide. ***, Putative stop codon.

Figure 7. Alignment of nucleotide sequences of coding regions of TED4 and aleurone-specific cDNA clone B11E, and alignment of deduced amino acid sequences of TED4 (above TED4 rows) and B11E (below B11E rows). Asterisks indicate identical nucleotides. Highlighted and shaded amino acids indicate identical and biochemically similar amino acids, respectively.

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                                               10                        20
                        MetArgSerIleThrTyrThrSerLeuTyrAlaAlaAlaIleLeuLeuMetMetIleLeuAlaHisSerGln
TED4                    ATGAGGTCAATTACATACACATCACTGTATGCTGCAGCAATCCTGATGATGATCTTAGCCGGATCGCAA
                        *  *          *                 *  **  ** *** * *      ** **  ** *
B11E   ATGGCGATGGCGATGGGGATGGCGATGAGGAAGGAGGCAGCGGTGGCCGTGATGATGGTGATGGTGGTGACGCTGGCGGCGGGTGCGGAC
       MetAlaMetAlaMetGlyMetAlaMetArgLysGluAlaAlaValAlaValMetMetValMetValValThrLeuAlaAlaGlyAlaAsp
                                               10                        20                        30


                              30                        40                        50
               ThrThrMetAlaValThrValSerGlnValThrGlnLeuGlnAlaProValSerAlaSerAlaIleSerSerSerSerSerProThrSerSerGlnValSer
TED4           ACCACTATGGCAGTGACATGCCAGGTTACACAGCTGGCGCCATGTGCGTCTGCCATCTCATCATCGTCGCCACCATCATCGCAGTGCTGC
               *       ** ** * * *** **       * ********       ** ***** ** ***      * **  **        **:*****
B11E           GCGGGAGCGGGAGCGGCGTGCGAGCCCGGCGCAGCTGGCGGTGTGCGCGTCGGCGATCCTGGGCGGGACGAAGCCGAGCGGCGAGTGCTGC
               AlaGlyAlaGlyAlaAlaValSerGluProAlaGlnLeuAlaValValSerAlaSerAlaIleLeuLeuGlyGlyThrLysProSerGlyGluValSer
                              ↑     40            ↑         50                      ↗ 60


                        60                        70                        80
           AlaLysIleLysGluValLeuLysProValLeuValSerIleValMetValSerAsnProSerLeuLysAlaValValSerSerProAsnAlaLysLys
TED4       GCTAAGATAAAAGAGCAGAAACCATGTCTATGCCAGTACATGAAGAACCCAAGTCTAAAGGCGTATGTTTCGTCTCCAAATGCCAAGAAG
           *  **  *      * ****  *      **  * ********  * *** ****  *      *** ** **      *** * **      *
B11E       GGGAACCTGCGGGCGCAGCAGGGGTGCTTGTGCCAGTACGTCAAGGACCCCAACTACGGGCACTACGTGAGCAGCCCACACGGCGCGGAC
           GlyAsnLeuArgAlaGlnGlnGlyCysLeuCysGlnTyrValLysAspProAsnTyrGlyHisTyrValSerSerProHisGlyAlaArgAsp
                        ↑ 70                        80                        90


                        90          95
           ValAlaAsnAlaCysGlyValThrIleProLysValSer
TED4       GTTGCCAATGCTTGTGGGGTTCCGATACCGAAATGCTAG
           ***      ** **  * **  ***** * ******
B11E       ACCCTCAACTTGTGCGGCATACCCGTACCGCACTGCTAG
           ThrLeuAsnLeuCysGlyIleProValProHisGlyVal
                        100   102
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mRNA compared with control (Fig. 5, lane P). FdU, which significantly inhibits both the formation of TE and cell division in isolated *Zinnia* mesophyll cells (Sugiyama et al., 1990), prevented the expression of TED2 to TED4 mRNAs (Fig. 5, lane F). This inhibitory effect was restored by addition of dT, which recovered cell division and TE differentiation (Fig. 5, lane T). These results suggest that TED2 to TED4 mRNAs are expressed in association with TE differentiation but not with cell division.

Determination of Nucleotide Sequence of TED4 and Homology Analysis

Nucleotide and deduced amino acid sequences of TED4 were determined (Fig. 6). The TED4 cDNA contained 28 bp of the 5′ untranslated region, and an open reading frame of 285 bp followed by 221 bp of 3′ untranslated region containing 9 bp of poly(A) tail. The typical consensus sequence for plant translation start sites was not observed in the vicinity of the start codon. The perfect consensus polyadenylation sequence was also not observed in the 3′ untranslated region. The deduced polypeptide comprised 95 amino acid residues with a predicted molecular mass of 10.0 kD. Eleven hydrophobic amino acid sequences were contained in the amino-terminal region, which may be a transit peptide (von Heijne, 1983).

A homology search of the nucleotide and amino acid sequences of TED4 with several data bases revealed no homology with previously characterized sequences, but it did reveal a significant similarity to the sequences of the barley aleurone-specific clone B11E, which was isolated as an aleurone-specific cDNA by differential screening of an aleurone

cDNA library from 20-d postanthesis grain (Jakobsen et al., 1989). A high degree of similarity between TED4 and B11E polypeptides was observed, especially in the C-terminal half of the polypeptides (Fig. 7).

Jakobsen et al. (1989) have reported that B11E encodes a 10.4-kD protein with a putative signal sequence and a possible metal-binding "finger" composed of four Cys residues. The deduced TED4 polypeptide also contained a putative signal sequence and some Cys residues coincident with the "finger" (Fig. 7, arrows). The B11E transcripts are known to be expressed in a temporally and spatially regulated manner. At present it is unknown why such similar genes are expressed in a highly ordered manner in different tissues of different species of plants. Further analysis of the function of TED4 and B11E is needed to elucidate this question.

The main purpose of the present work was to isolate new genes that can be used as molecular markers of TE differentiation in early stages prior to morphological changes. To isolate such genes expressed in a temporally regulated manner, it is necessary to use an efficient experimental system in which TE differentiation can be induced synchronously and at high frequency. The *Zinnia* culture system that has been established by Fukuda and Komamine (1980) satisfies these requirements best at present (Fukuda, 1989, 1992; Sugiyama and Komamine, 1990). By a differential screening of the cDNA library prepared from the *Zinnia* culture, three clones were isolated whose corresponding mRNAs were expressed preferentially in the TE-induced culture prior to morphological changes, and these were designated as TED2 to TED4 (Figs. 1 and 3).

Hormonal analysis indicated that the expression of the genes corresponding to the TED clones was not induced by

NAA or BA solely (Fig. 4). Analysis with inhibitors of DNA synthesis revealed that the expression was not associated with cell division (Fig. 5). These results suggested that the genes corresponding to the TED clones were expressed in close association with TE differentiation. These genes appear to be expressed earlier than genes of PAL (Lin and Northcote, 1990) and cell wall proteins (Ye and Varner, 1991); therefore, TED clones may be used as molecular markers for the earlier stages of TE differentiation. Although the functions of the gene products have not been identified, it is likely that they play important roles in the initiation or progression of morphological changes of TE differentiation. At present, in situ hybridization of the TED genes with intact plant tissue is under way to confirm that they are expressed specifically in differentiating TEs in intact *Zinnia* plants.

ACKNOWLEDGMENTS

LITERATURE CITED

Banasik M, Komura H, Shimoyame M, Ueda K (1992) Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl) transferase. J Biol Chem 267: 1569–1575

Bevan M, Shufflebottom D, Edwards K, Jefferson R, Schuch W (1989) Tissue and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. EMBO J 8: 1899–1906

Church DL, Galston AW (1988) 4-Coumarate:coenzyme A ligase and isoperoxidase expression in *Zinnia* mesophyll cells induced to differentiation into tracheary elements. Plant Physiol 88: 679–684

Condit CM, Mclean BG, Meagher RB (1990) Characterization of the expression of the petunia glycine-rich protein-1 gene product. Plant Physiol 93: 596–602

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ (1990) Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc Natl Acad Sci USA 87: 9057–9061

Fukuda H (1989) Cytodifferentiation in isolated single cells. Bot Mag Tokyo 102: 491–501

Fukuda H (1992) Tracheary element formation as a model system of cell differentiation. Int Rev Cytol 136: 289–332

Fukuda H, 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, Komamine A (1982) Lignin synthesis and its related enzymes as markers of tracheary element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. Planta 155: 423–430

Fukuda H, Komamine A (1983) Changes in the synthesis of RNA and protein during tracheary element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. Plant Cell Physiol 24: 603–614

Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25: 263–269

Jakobsen K, Klemsdal SS, Allen RB, Bosnes M, Alexander D, Olsen O-A (1989) Barley aleurone cell development: molecular cloning of aleurone-specific cDNAs from immature grains. Plant Mol Biol 12: 285–293

Keller B, Baumgartner C (1991) Vascular-specific expression of the bean GRP 1.8 gene is negatively regulated. Plant Cell 3: 1051–1061

Keller B, Schmid J, Lamb CJ (1989a) Vascular expression of a bean cell wall glycine-rich protein-β-glucuronidase gene fusion in transgenic tobacco. EMBO J 8: 1309–1314

Keller B, Templeton MD, Lamb CJ (1989b) Specific localization of a plant cell wall glycine-rich protein in protoxylem cells of the vascular system. Proc Natl Acad Sci USA 86: 1529–1533

Leyva A, Liang X, Pintor-Toro JA, Dixon RA, Lamb CJ (1992) cis-Element combinations determine phenylalanine ammonia-lyase gene tissue-specific expression patterns. Plant Cell 4: 263–271

Liang X, Dron M, Schmid J, Dixon RA, Lamb CJ (1989) Developmental and environmental regulation of a phenylalanine ammonia-lyase-β-glucuronidase gene fusion in transgenic tobacco plants. Proc Natl Acad Sci USA 86: 9284–9288

Lin Q, Northcote DH (1990) Expression of phenylalanine ammonia-lyase gene during tracheary-element differentiation from cultured mesophyll cells of *Zinnia elegans* L. Planta 182: 591–598

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163: 16–20

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Ohl S, Herrick SA, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2: 837–848

Roberts LW, Gahan PB, Aloni R (1988) Vascular Differentiation and Plant Growth Regulators. Springer-Verlag, Berlin

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Sato Y, Sugiyama M, Górecki R, Fukuda H, Komamine A (1993) Interrelationship between lignin deposition and the activities of peroxidase isoenzymes in differentiating tracheary elements of *Zinnia*: analysis using L-α-aminooxy-β-phenylpropionic acid and 2-aminoindan-2-phosphonic acid. Planta 189: 584–589

Stiefel V, Ruiz-Avila L, Raz R, Vallés MP, Gómez J, Pagés M, Martínez-Izquierdo JA, Ludevid MD, Langdale JA, Nelson T, Puigdomènech P (1990) Expression of a maize cell wall hydroxyproline-rich glycoprotein gene in early leaf and root vascular differentiation. Plant Cell 2: 785–793

Sugiyama M, Fukuda H, Komamine A (1990) Characteristics of the inhibitory effect of 5-fluorodeoxyuridine on cytodifferentiation into tracheary elements of isolated mesophyll cells of *Zinnia elegans*. Plant Cell Physiol 31: 61–67

Sugiyama M, Komamine A (1987) Effect of inhibitors of ADP-ribosyltransferase on the differentiation of tracheary elements from isolated mesophyll cells of *Zinnia elegans*. Plant Cell Physiol 28: 541–544

Sugiyama M, Komamine A (1990) Transdifferentiation of quiescent parenchymatous cells into tracheary elements. Cell Differ Dev 31: 77–87

Suzuki K, Ingold E, Sugiyama M, Komamine A (1991) Xylan synthase activity in isolated mesophyll cells of *Zinnia elegans* during differentiation to tracheary elements. Plant Cell Physiol 32: 303–306

Thelen MP, Northcote DH (1989) Identification and purification of a nuclease from *Zinnia elegans* L.: a potential molecular marker for xylogenesis. Planta 179: 181–195

von Heijne G (1983) Patterns of amino acids near single-sequence cleavage sites. Eur J Biochem 133: 17–21

Ye Z-H, Song Y-R, Marcus A, Varner JE (1991) Comparative localization of three classes of cell wall proteins. Plant J 1: 175–183

Ye Z-H, Varner JE (1991) Tissue-specific expression of cell wall proteins in developing soybean tissues. Plant Cell 3: 23–37