The Plant Cell, Vol. 6, 1035–1047, August 1994

RESEARCH ARTICLE

A Novel Extracellular Matrix Protein from Tomato Associated with Lignified Secondary Cell Walls

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A cDNA clone representing a novel cell wall protein was isolated from a tomato cDNA library. The deduced amino acid sequence shows that the encoded protein is very small (88 amino acids), contains an N-terminal hydrophobic signal peptide, and is enriched in lysine and tyrosine. We have designated this protein TLRP for tyrosine- and lysine-rich protein. RNA gel blot hybridization identified TLRP transcripts constitutively present in roots, stems, and leaves from tomato plants. The encoded protein seems to be highly insolubilized in the cell wall, and we present evidence that this protein is specifically localized in the modified secondary cell walls of the xylem and in cells of the sclerenchyma. In addition, the protein is localized in the protective periderm layer of the growing root. The highly localized deposition in cells destined to give support and protection to the plant indicates that this cell wall protein alone and/or in collaboration with other cell wall structural proteins may have a specialized structural function by mechanically strengthening the walls.

INTRODUCTION

The cell wall is a dynamic cell compartment that confers unique and distinctive features to plant cells; it is recognized as having specific functions essential to the entire plant (Cassab and Varner, 1988). The construction and architecture of the cell wall vary in an orderly manner in accordance with the developmental stages of cells (Varner and Lin, 1989). This pliable character of cell walls is a direct consequence of changes in the proportion and degree of assembly of various structural polysaccharides, including cellulose microfibrils, pectin, and hemicellulose polymers (Roberts, 1989; Levy and Staehelin, 1992). Also, differences between cell walls of defined cell types originate as a consequence of deposition of more specific polymers with defined functions, such as lignin (which serves to harden and stiffen the walls of tracheary elements) or cutin (which serves to prevent water loss in epidermal cells and provides a mechanical barrier to prevent pathogen ingress). Plant cell walls also contain structural proteins that participate in the flexible integration of environmental responses within plastic developmental programs. To date, three prominent classes of structural cell wall proteins have been characterized: (1) the hydroxyproline-rich glycoproteins (HRGPs), (2) the proline-rich proteins (PRPs), and (3) the glycine-rich proteins (GRPs). The genes encoding these structural proteins are tightly regulated, and their expression shows cell type specificity and developmental regulation (Keller, 1993; Showalter, 1993).

HRGPs have been found in seed coats deposited in epidermal and hourglass cells (Cassab and Varner, 1987) and in the cambial as well as parenchymous cells of soybean stems (Ye and Varner, 1991). A tobacco HRGP gene is active in the early stages of initiation of lateral roots (Keller and Lamb, 1989). HRGP-like proteins are also expressed in flowers of tobacco plants (Wu et al., 1993) and during early events of maize embryo differentiation (Ruiz-Avila et al., 1991). On the other hand, the bean GRP 1.8 localizes primarily to tracheary elements of the protoxylem (Keller et al., 1989).

Analysis of the bean GRP 1.8 gene has shown that both developmental expression and wound induction are mediated by specific sequences within the GRP 1.8 promoter (Keller and Baumgartner, 1991). Several other plant species express GRP genes abundantly in primary xylem and in the cambium region (Ye and Varner, 1991; Ye et al., 1991). Expression of genes encoding PRPs has also been studied, and they seem to be developmentally regulated in different plant organs (Hong et al., 1989; José-Estanyol et al., 1992; Wyatt et al., 1992; Wang et al., 1993). Collectively, it seems that plants have evolved regulatory mechanisms allowing genes encoding structural cell wall proteins to be expressed specifically and in accord with the different functions exerted by the different tissues.

The expression of genes encoding some of these cell wall proteins can be altered by different cues (e.g., wounding, elicitor treatment, or pathogen attack) (Lamb et al., 1989), and the deposition of the encoded proteins in the cell wall results in alterations of the functional properties of this compartment. Bradley et al. (1992) have reported a very rapid oxidative cross-

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linking mechanism for insolubilization of preexisting cell wall structural proteins upon elicitation that precedes the activation of transcription-dependent processes and that seems to be under developmental control.

Here, we report on the structure, expression analysis, and cell type–specific immunolocalization studies of an encoded protein from tomato plants that seems to represent a novel class of cell wall proteins. The specific deposition of this protein in cells containing lignified secondary cell walls, such as tracheary elements of the xylem and cells of the sclerenchyma, suggests a correspondingly specific function for this protein in the reinforcement of the walls of these cells.

RESULTS

cDNA Cloning and Nucleotide and Predicted Amino Acid Sequences of TLRP

A family of cDNA clones was isolated from a tomato cDNA library using a differential screening approach designed to detect genes up-regulated as a consequence of ethylene treatment (Tornero et al., 1994; Vera et al., 1994). Clones representing genes precociously induced by ethylene but showing moderate levels of expression in nontreated plants were selected for further studies in development. One of them (clone pTE4) had an insert of 512 bp and hybridized to an RNA of approximately the same length (see below).

The nucleotide sequence of this cDNA and its predicted amino acid sequence are presented in Figure 1A. The cDNA contains an open reading frame (ORF) of 264 nucleotides beginning at the ATG initiation codon (at position 48 to 50) and ending at the stop codon TAA (at position 312 to 314). A polyadenylation consensus signal (AATAAA) was found at 81 bp upstream of the polyadenylation site (Figure 1A). The ORF encodes a polypeptide of 88 amino acids with a calculated molecular weight of 9919 and a pI of 8.7. Analysis of the predicted amino acid sequence revealed an N-terminal region with characteristics of a signal peptide (Von Heijne, 1986) and a predicted cleavage site at positions 26 and 27. The absence of an endoplasmic reticulum retention signal (KDEL or HDEL) (Denecke et al., 1992) suggests that the processed protein is exported from the endoplasmic reticulum once it is synthesized. Particularly interesting in the deduced amino acid sequence of this protein is the high degree of homology of the N-terminal region of the preprotein with that of different GRPs, including that of tobacco (van Kan et al., 1988), carrot (EMBL accession number X15706), *Chenopodium* (Kaldenhoff and Richter, 1989), and tomato (Showalter et al., 1991) (Figure 1D). This homology persists even after the predicted cleavage sites of the signal peptides at position 26 to 27 in which the characteristic sequence E/D-L-A/S-E-T is maintained. From amino acid 33 and beyond, no further homology was found with any of these or other proteins existing in data bases.

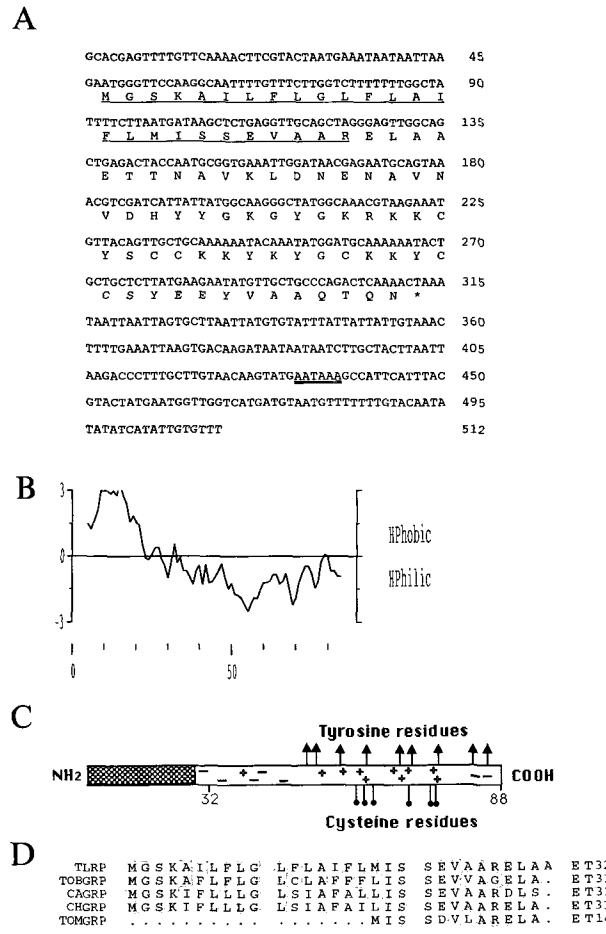

Figure 1. Sequence and Structural Features of the Tomato TLRP.

(A) DNA sequence of the cDNA encoding TLRP and predicted amino acid sequence. The putative signal peptide sequence is underlined. The putative polyadenylation signal is double underlined. The stop codon is marked with an asterisk. DNA bases are numbered at the right. The nucleotide and deduced amino acid sequence have been submitted to EMBL, GenBank, and DDBJ as accession number X77373.

(B) Hydropathic plot (Kyte and Doolittle, 1982) from the deduced amino acid sequence of TLRP. Values above the horizontal line indicate hydrophobic (HPhobic) region, and values below the horizontal line represent hydrophilic (HPhilic) region.

(C) Topological features of the derived amino acid sequence of TLRP are shown. Tyrosine residues are indicated by triangles. Cysteine residues are indicated by dots. Negatively charged N-terminal and C-terminal regions are indicated by minus signs. The positively charged central region is indicated by plus signs. The putative signal peptide at the N terminus is shown by the checkered rectangle.

(D) Sequence similarities of the N terminus of TLRP to those of different GRPs. Residues identical to those of TLRP are dotted. TOBGRP, tobacco GRP; CAGRP, carrot GRP; CHGRP, *Chenopodium* GRP; TOMGRP, tomato GRP.

Hydropathy analysis (Kyte and Doolittle, 1982) (Figure 1B) of the deduced amino acid sequence shows that the hydrophobic signal sequence is followed by a highly hydrophilic region representing the mature protein. Assuming the cleavage site at position 26, the mature protein has 62 amino acid residues with a calculated molecular weight of 7138 and a pI of 8.45. The amino acid sequence of the mature protein contains interesting structural arrangements. Both the N-terminal and C-terminal parts of the mature protein are acidic, whereas the central part of the protein is highly basic. Table 1 illustrates that the mature protein has a high lysine content (10 residues or 16%). These lysine residues are clustered in the basic central part of the polypeptide (Figures 1A and 1C). The C-terminal end and more prominently the N terminus of the mature protein are acidic with two or five aspartate or glutamate residues, respectively. The protein is also highly enriched in tyrosine residues (nine residues or 14.5%) and cysteine residues (six residues or 9.6%) (Table 1), which are also clustered in the central basic region (Figure 1C). Also, a Tyr-Lys-Tyr motif is found in this central region (Figure 1). This short sequence seems to be responsible for the peroxidase/H_2O_2–catalyzed generation of intramolecular isodityrosine linkages in HRGPs (Fry, 1986; Varner and Lin, 1989). From the particular amino acid composition of this protein, we propose to name this protein as TLRP (for tyrosine- and lysine-rich protein).

Table 1. Amino Acid Composition of the Tomato TLRP in Both the Precursor and Mature Form of the Protein

	Mature TLRP		Precursor TLRP	
Residue	Number of Residues	Mol %	Number of Residues	Mol %
Ala	6	9.68	10	11.36
Cys	6	9.68	6	6.82
Asp	2	3.23	2	2.27
Glu	5	8.06	6	6.82
Phe	0	0.00	3	3.41
Gly	4	6.45	6	6.82
His	1	1.61	1	1.34
Ile	0	0.00	3	3.41
Lys	10	16.12	11	12.50
Leu	2	3.23	7	7.95
Met	0	0.00	2	2.27
Asn	5	8.06	5	5.68
Pro	0	0.00	0	0.00
Gln	2	3.23	2	2.27
Arg	1	1.61	2	2.27
Ser	2	3.23	5	5.68
Thr	3	4.84	3	3.41
Val	4	6.45	5	5.68
Trp	0	0.00	0	0.00
Tyr	9	14.52	9	10.23

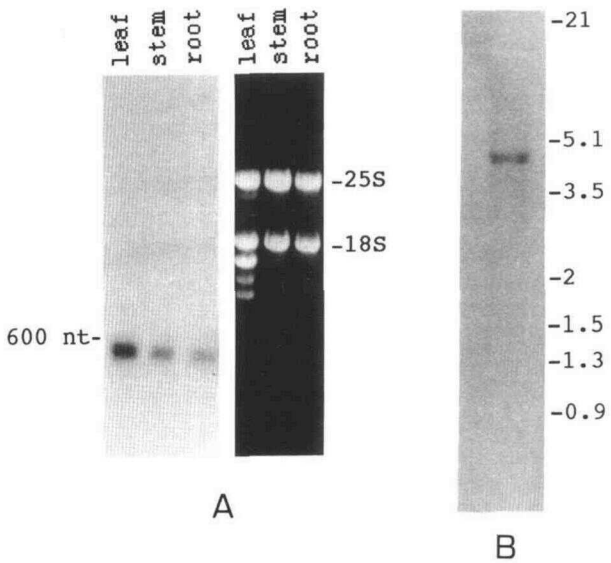

Figure 2. RNA and DNA Gel Blot Hybridization Analysis of TLRP Sequences.

(A) Comparative RNA gel blot analysis of transcripts of TLRP in different plant organs (leaves, stems, and roots) of tomato plants 5 weeks after germination. The blot was hybridized with the TLRP cDNA probe (left). The autoradiogram was exposed for 12 hr. The corresponding ethidium bromide–stained gel is shown at right, and the positions of the rRNAs (18S and 25S) are indicated to the right of the gel. At the left, the position of a 600-nucleotide (nt) RNA molecular marker is indicated.

(B) DNA gel blot of tomato genomic DNA digested with XbaI. The blot was hybridized with the TLRP cDNA probe. Molecular length markers in kilobases are indicated at right.

TLRP Transcripts and DNA-Related Sequences

RNA gel blot was used to check the steady state mRNA level that hybridizes with the TLRP cDNA sequences. Comparative hybridization of the cDNA insert with total cellular RNA extracted from leaves, stems, and roots from tomato plants showed that this mRNA exists in all of the three organs studied (Figure 2A). This RNA gel blot showed that the TLRP mRNA appears to be a single species of less than 600 bases, and thus, the 512-bp cDNA clone represents most of the TLRP mRNA sequence.

The possible existence of other tomato genes with a coding region similar to TLRP was also considered. In an attempt to explore this possibility, we hybridized XbaI-digested genomic DNA with the TLRP cDNA. The DNA gel blot presented in Figure 2B showed only a single hybridizing band of 4 kb that hybridized under moderately stringent conditions (60°C). A single hybridizing band was also observed when DNA was digested with EcoRI or PstI restriction endonucleases and hybridized under the same conditions (data not shown).

TLRP Appears To Be a Highly Insoluble Cell Wall Protein

To generate TLRP-specific antibodies and study the localization of the protein in plant tissues, a TLRP fusion protein was constructed. A polymerase chain reaction (PCR)–generated DNA product corresponding to the complete ORF of the mature TLRP was subcloned into the plasmid pGEX-KG (Guan and Dixon, 1991). The resulting plasmid (pGEX-TLRP), which contains the glutathione-*S*-transferase (GST) fused in frame with the mature TLRP, permitted purification of the fusion protein on a glutathione-agarose column when expressed in *Escherichia coli* (Figure 3A). The purified protein was 7 kD larger than the 26-kD GST protein. This difference in molecular mass was expected as a result of the fusion of GST with the mature TLRP.

Protein gel blot analysis using antibodies raised against the purified GST–TLRP fusion protein and that were arrested from those IgG proteins recognizing GST showed that the antibody recognized only the TLRP part of the GST–TLRP fusion protein (Figure 3B). By using this antibody, we first tried to detect the presence of TLRP in tissue print preparations from stem sections of tomato plants. Although the appropriate controls with antibodies against several other proteins were positive (data not shown), no clear signal could be obtained when the antibodies raised against the fusion protein were used to detect the presence of TLRP in tissue prints. Also, attempts to detect the presence of TLRP by gel blot analysis of protein samples (cytosolic, microsomal, or crude cell wall fraction) from a variety of tissue types, including leaf, stem, and root, during different developmental stages of the plant were negative, because the antibodies were unable to recognize any protein in crude extracts (data not shown). Even when buffers containing different detergents (e.g., SDS, urea, or guanidine salts) were used for making protein extracts, we failed to solubilize the protein. Only when crude cell wall fractions were incubated with anti-TLRP serum and later with a fluorescein isothiocyanate–labeled second antibody was fluorescence retained in cell walls, whereas no fluorescence was retained when similar experiments were performed with control preimmune serum (data not shown).

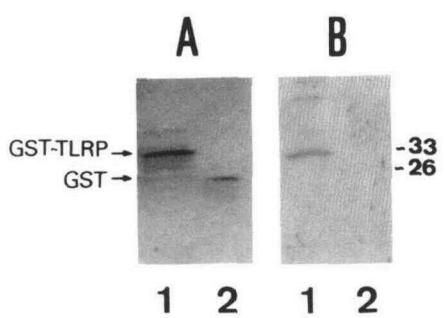

Figure 3. *Protein Gel Blot Analysis of the GST–TLRP Fusion Protein.*

The GST–TLRP fusion protein (lanes 1) or GST alone (lanes 2) was produced in *E. coli* and isolated by affinity binding to glutathione-Sepharose.

(A) SDS–polyacrylamide gel stained with Coomassie Brilliant Blue R 250.

(B) Immunoblot staining of a protein blot corresponding to the gel shown in **(A)** in which the GST-arrested antiserum recognized the TLRP part of the fusion protein.

Estimated molecular masses of GST and GST–TLRP proteins are 26 and 33 kD, respectively, as indicated at right.

These observations together with the high abundance of tyrosine in the TLRP led us to believe that this protein was cross-linked to the cell walls. Insolubilization of plant cell wall proteins rich in tyrosine residues has been well documented (Cooper and Varner, 1984; Fry, 1986; Bradley et al., 1992), and this insolubilization seems to be derived from the intermolecular oxidative coupling of two tyrosine molecules connected through a diphenyl ether bridge known as isodityrosine linkage (Fry, 1982).

Immunolocalization of TLRP

To determine in which cell type(s) TLRP was localized, different tissue sections were treated with anti-TLRP antiserum. Tissue sections of different plant organs (leaves, stems, and roots) from tomato plants showed a highly specific localization for this protein. In cross-sections from the stem (Figures 4A to 4D), TLRP was localized specifically in the xylem vessels and fibers of the central vascular bundle and in those cells of the sclerenchyma (phloem fibers) abutting the external phloem. This localization was more clearly observed in longitudinal tissue sections of the stem where the immunolabeling was retained all along the stele (Figures 4E and 4F). The wall disposition of these cells, with the characteristic spiral-like pattern of lignification, was clearly observed, and in oblique longitudinal sections of the stem the antibody recognition appeared to match the pattern of lignin deposition (Figures 4G and 4H).

Immunohistochemical detection of TLRP in leaves again revealed a highly specific localization in the leaf vasculature. TLRP was localized in the xylem elements of the midrib and also of the secondary and small veinlets of the leaves, which became specifically immunodecorated by the antibody (Figures 5A to 5E). Cross-sections of the leaf lamina, around regions of the midrib and the minor veins distributed in the leaf, showed the presence of TLRP in the xylem, which consists entirely of tracheids. The surrounding inner and outer phloem components of the leaf vasculature or the parenchymatic tissues did not reveal any specific staining with the antibody.

Root sections also showed a characteristic immunodecoration of the xylem elements of the stele when incubated with anti-TLRP serum (Figures 5F to 5J). The immunolabeling was again associated with the cell walls of the xylem elements, and in longitudinal sections it followed a pattern similar to lignin deposition in the cell walls (data not shown). Particularly interesting was the specific localization of TLRP in the outer

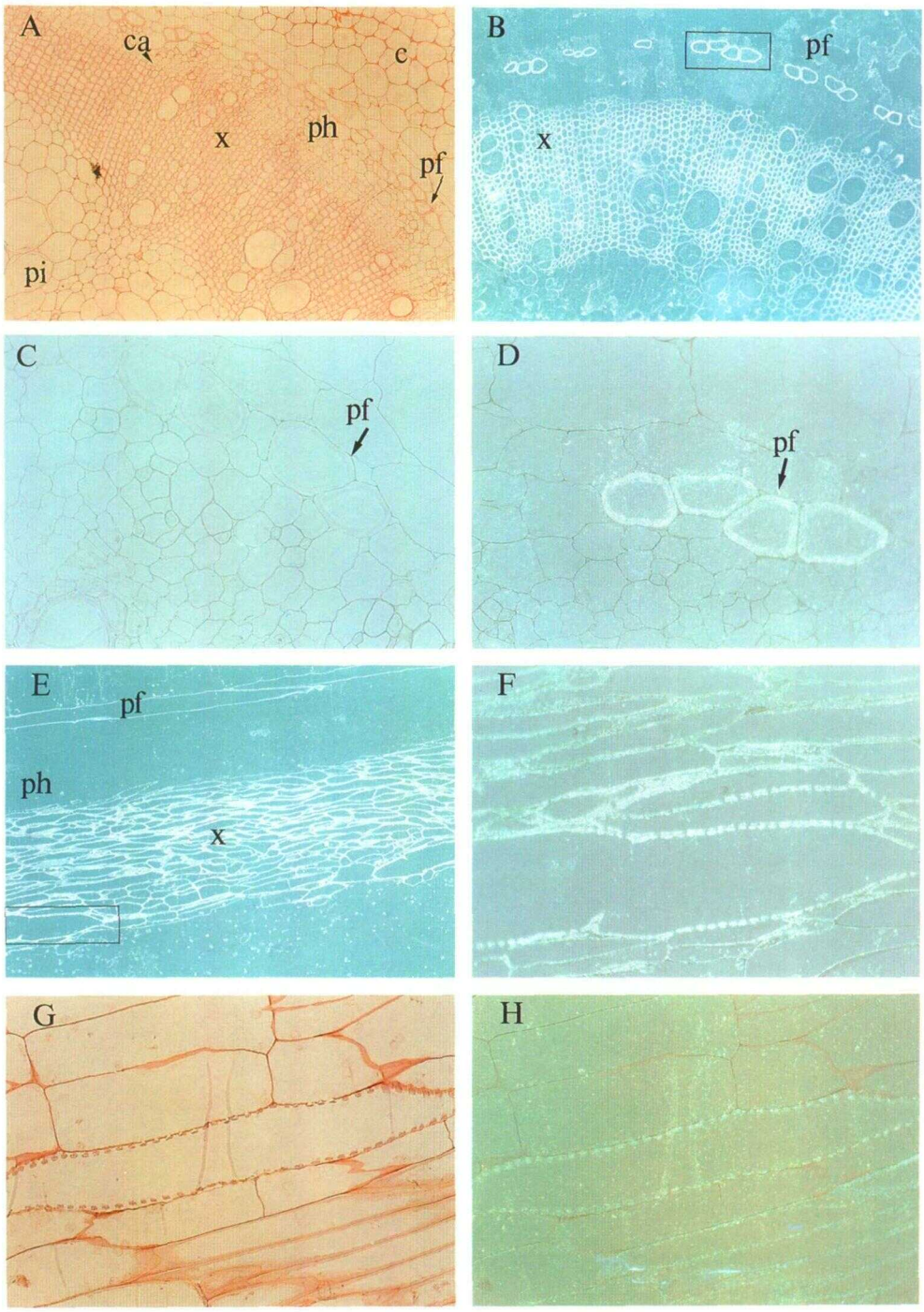

Figure 4. Immunohistochemical Localization of the TLRP in Stem Sections of Tomato Plants.

(A) Safranin O–stained cross-section of a mature tomato internode.
(B) Similar section to that shown in **(A)** incubated with anti-TLRP serum and illuminated under epipolarized light. The boxed region is magnified in **(D)**.
(C) Magnification of a stem section incubated with preimmune serum and illuminated under a combination of bright and epipolarized light.
(D) Magnification of the area indicated in **(B)** around the phloem fibers and illuminated with bright and epipolarized light.
(E) Longitudinal section of the stem incubated with anti-TLRP serum and illuminated with epipolarized light.
(F) Magnification of the area indicated in **(E)** and illuminated under bright and epipolarized light.
(G) Safranin O–stained magnified cross-section showing a xylem element.
(H) Same cross-section as shown in **(G)** after incubation with anti-TLRP serum and illumination under a combination of bright and epipolarized light.
c, cortex; ca, cambium; pf, phloem fiber; ph, phloem; pi, pith parenchyma; x, xylem. Magnification ×12 in **(A)**, **(B)**, and **(E)**; magnification ×48 in **(C)**, **(D)**, **(F)**, **(G)**, and **(H)**.

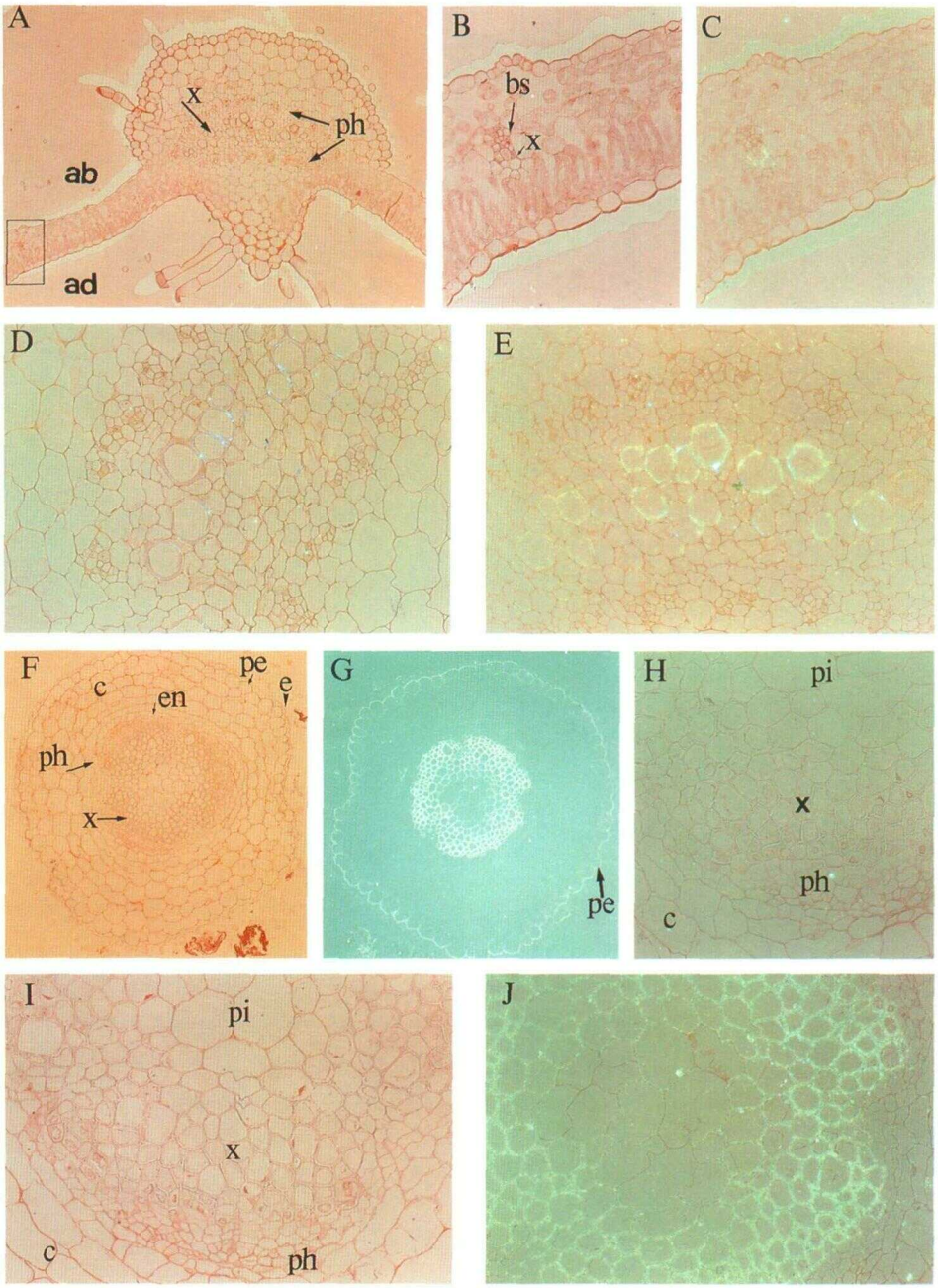

Figure 5. Immunohistochemical Localization of the TLRP in Leaf and Root Sections of Tomato Plants.

(A) Safranin O–stained cross-section of a developed leaf from tomato. Magnification of the boxed area is shown in **(B)**.
(B) Magnification of the area indicated in **(A)** that corresponds to a minor vein.
(C) Same area shown in **(B)** after incubation with TLRP antiserum and illumination under a combination of bright and epipolarized light.
(D) Magnification of a cross-section of the leaf around the midrib and incubated with preimmune serum and illuminated under bright and epipolarized light. The natural birefringence of the lignified cell walls can be observed.
(E) A section similar to that shown in **(D)** but incubated with anti-TLRP serum.
(F) Safranin O–stained cross-section of a tomato root.
(G) Same cross-section shown in **(F)** after incubation with anti-TLRP serum and illumination under epipolarized light.
(H) Cross-section of the root incubated with preimmune serum and illuminated under epipolarized light.
(I) Stained cross-section of the root illuminated under bright light.
(J) Same as the cross-section shown in **(I)** after incubation with anti-TLRP serum and illuminated under a combination of bright and epipolarized light.
ab, abaxial; ad, adaxial; bs, bundle sheath; c, cortex; e, epidermis; en, endodermis; pe, peridermis; ph, phloem; pi, pith parenchyma; x, xylem.
In **(A)**, **(F)**, and **(G)**, magnification ×12; in **(B)**, **(C)**, **(D)**, **(E)**, **(H)**, **(I)**, and **(J)**, magnification ×48.

cell walls of a subepidermal monolayer of cells that seemed to correspond to the exodermis or peridermal layer of the root (Figure 5G). This is a suberized cell layer that is often partially lignified and serves protective functions (Esau, 1965). Control sections incubated with preimmune serum did not show any specific staining in any of the tissues analyzed (Figures 4C, 5D, and 5H).

Ultrastructural Localization of TLRP

To determine more precisely the association of the tomato TLRP with the xylem elements, we examined ultrathin sections of resin-embedded leaf sections by immunoelectron microscopy as described previously for other proteins from tomato plants (Vera et al., 1989a, 1989b). After the samples had been processed with anti-TLRP serum and protein A–gold particles, the antigens were shown to be specifically localized in the cell walls of tracheary elements (Figures 6A, 6B, 6D, and 6F). These cells appeared lignified with the characteristic reticulate thickening of the secondary walls and were devoid of any cytoplasmic organization; these are characteristics of differentiated tracheary elements (Esau, 1965). At higher magnification, we observed that the TLRP-specific immunogold labeling was prominent in the lignified secondary cell wall ingrowths (Figures 6D to 6F). Controls run with preimmune serum showed no specific labeling of the lignified secondary cell walls (Figure 6C). The strong immunolabeling of the secondary walls of the tracheary elements when incubated with anti-TLRP serum contrasted sharply with the nearly absent labeling that could be observed in the cytosol or in different organelles of the surrounding living xylem parenchyma cells that had not yet started to elaborate secondary walls. The intact primary walls of the neighboring parenchymatous cells of the xylem elements as well as those primary walls of the tracheary elements showed weak labeling. However, this labeling was not very significant if it is compared to the gold labeling observed for the secondary cell walls (see Table 2). From these results, we cannot disregard the possibility that very discrete amounts of TLRP are also deposited in primary cell walls.

It is worth mentioning that the localization of the TLRP described here is different from that reported for the closely related GRP 1.8 protein from bean (Ryser and Keller, 1992); the latter is localized in unlignified primary walls of protoxylem cells and not in the secondary thickenings as is the case for TLRP. Whether these different but specific patterns of deposition of TLRP and GRP1.8 are coordinated or represent independent events in the process of xylem formation is not yet known.

Developmental Regulation of TLRP Gene Expression

The TLRP gene described here is expressed in leaves, stems, and roots of fully grown plants. To determine its expression pattern during early developmental stages of growth, 12-day-old seedlings were dissected into roots, hypocotyls, cotyledons, and shoot apex, and total RNA was isolated and probed with a TLRP cDNA probe. Figure 7A shows that the TLRP gene is strongly expressed in all four organs analyzed, and the amount of the corresponding mRNA is roughly the same in all four parts of the seedling. The steady state level of accumulation of the TLRP mRNA is higher (as determined from the intensity of the hybridizing signal) than that found for the different organs of a fully developed plant (Figure 2A).

Vascular differentiation in the plant is a continuous process that is initially established at the seedling stage and subsequently regulated as the plant grows and develops (Aloni, 1987). In tomato plants, a gradient of secondary growth in the stem is already initiated in young plants and gradually extends along the axis of the plant as the stem matures. We wondered if the expression of TLRP along the plant axis also exhibited the same type of developmental regulation. Total RNA was extracted from the first or apical internode near the shoot apex and from the eight subsequent older internodes downward to the root. Expression of TLRP in the node, the main root, and lateral roots was also analyzed to complement this study. Figure 7B shows that TLRP mRNA can be detected in the first apical internode close to the apex. The gene seems to be down-regulated in the next two young internodes downward, and the accumulation of mRNA was again detectable in each of the succeeding seven older internodes, including the oldest one that comprises the stem–root junction. Expression was also detectable in nodes and the main root but not in lateral roots. The same RNA blot was hybridized with a tomato DNA probe representing a lignin peroxidase gene that is constitutively expressed in stems and induced in other parts of tomato plants by pathogen stress (Vera et al., 1994). Transcript accumulation for this lignin peroxidase is similar to that observed for TLRP transcripts in mature internodes, whereas no accumulation was observed in the younger internodes close to the apex and in the lateral roots (Figure 7B).

The early expression observed for the TLRP gene in young seedlings and in the internode close to the apex, where initial differentiation of the vasculature is taking place, suggests that expression of TLRP may be a crucial event for the formation of primary xylem. The recovered expression of the TLRP gene in mature internodes is accompanied by a marked accumulation of lignin (as determined from phloroglucinol staining of the corresponding internode cross-sections; data not shown), and the expression of a lignin-peroxidase gene, which presumably participates in the synthesis of lignin during secondary growth, further substantiates the association of TLRP with secondary xylem formation.

The differentiation of xylem is a complex developmental process governed by an interplay of growth factors. There is evidence that the plant hormone ethylene, either naturally produced or induced as a consequence of auxin action, is involved in the vascular differentiation events that lead to xylem formation (Roberts, 1976; Roberts and Miller, 1982). In fact, Miller and Roberts (1984) have shown that the ethylene releasing agent 2-chloroethylphosphonic acid promoted xylem differen-

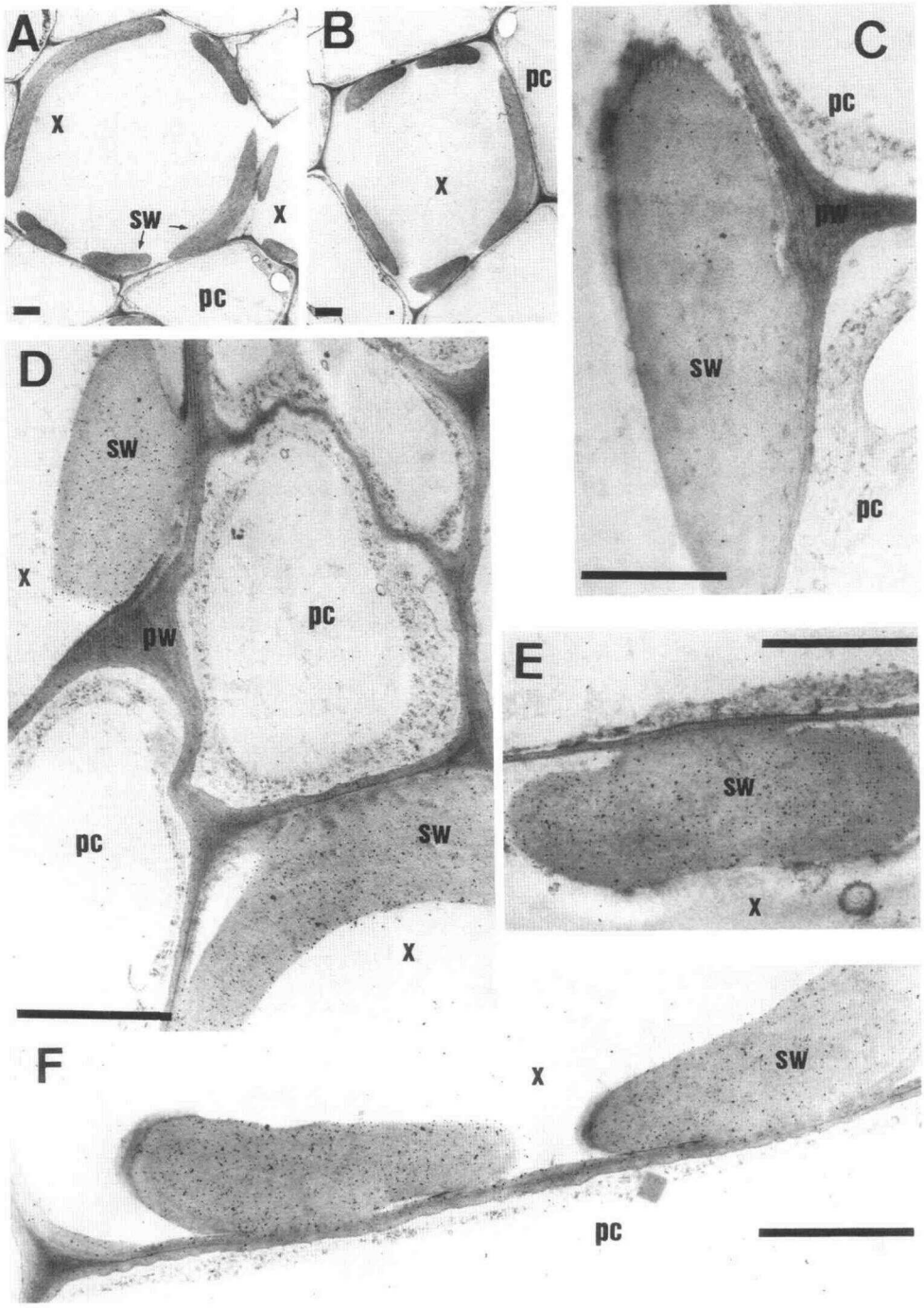

Figure 6. Immunocytochemical Localization of TLRP in the Lignified Secondary Cell Walls of Tracheary Elements.

(A) and **(B)** Low magnification of tissue sections showing an overall view of two different tracheary elements surrounded by parenchyma cells. Bars = 8 μm.

(C) Magnification of a region of the secondary cell wall of a tracheary element similar to that shown in **(A)** incubated with preimmune serum. Bar = 1 μm.

(D) to **(F)** Magnification of different areas of the tracheary elements comprising different modified secondary cell wall fields that are intensively labeled by the anti-TLRP serum. Bars = 1 μm.

pc, parenchyma cell; pw, primary wall; sw, secondary wall; x, xylem cell (tracheary element).

Table 2. Immunolocalization of TLRP in Different Cell Compartments[a]

Cellular Compartment	Labeling Density (gold particles/μm^2 ± SE) Anti-TLRP Serum	Preimmune Serum	Corrected[b]
Intercellular space	4.2 ± 1.3	2.2 ± 1.0	2.0 ± 1.1
Cytosol	4.0 ± 2.0	1.6 ± 0.3	2.4 ± 1.5
Chloroplast	3.2 ± 1.4	2.1 ± 1.3	1.2 ± 0.1
Nucleus	3.3 ± 2.6	2.4 ± 0.6	1.4 ± 0.3
Vacuole	6.2 ± 2.1	2.8 ± 1.4	3.4 ± 1.7
Primary cell wall	15.1 ± 4.2	4.1 ± 1.7	12.0 ± 2.8
Secondary cell wall	143.7 ± 11.9	7.1 ± 3.1	136.6 ± 7.7

[a] For each cell compartment and for each antiserum, gold-labeling density was examined in a number of samples accounting for a total surface exceeding 20 μm^2.

[b] Corrected, (labeling density for TLRP anti-serum) – (labeling density for control preimmune).

tiation. Miller et al. (1985) have suggested that ethylene may play a role in controlling lignification during xylogenesis, resulting in early maturation of tracheary elements.

In an attempt to study the manner of induction of the TLRP gene by ethylene, we treated leaf tissue with buffered 2-chloroethylphosphonic acid solutions and analyzed the accumulation of TLRP mRNA. As shown in Figure 8, application of this ethylene-releasing compound to tomato leaves produced a very rapid increase in the steady state levels of TLRP mRNA. Likewise, lignin peroxidase mRNA was notably induced. A cDNA probe for a tomato PR-Q′ β-1,3-glucanase gene, which is also known to be regulated by ethylene (Domingo et al., 1994), was used as a probe in similar RNA gel blots. It also detected the accumulation of the corresponding transcripts. However, the time of maximal accumulation of each of these mRNAs differed. Increased TLRP and lignin peroxidase mRNAs could first be observed after 6 to 12 hr. Increase in β-1,3-glucanase mRNA could first be observed at 36 hr after treatment, and its accumulation continued to increase sharply even after 60 hr.

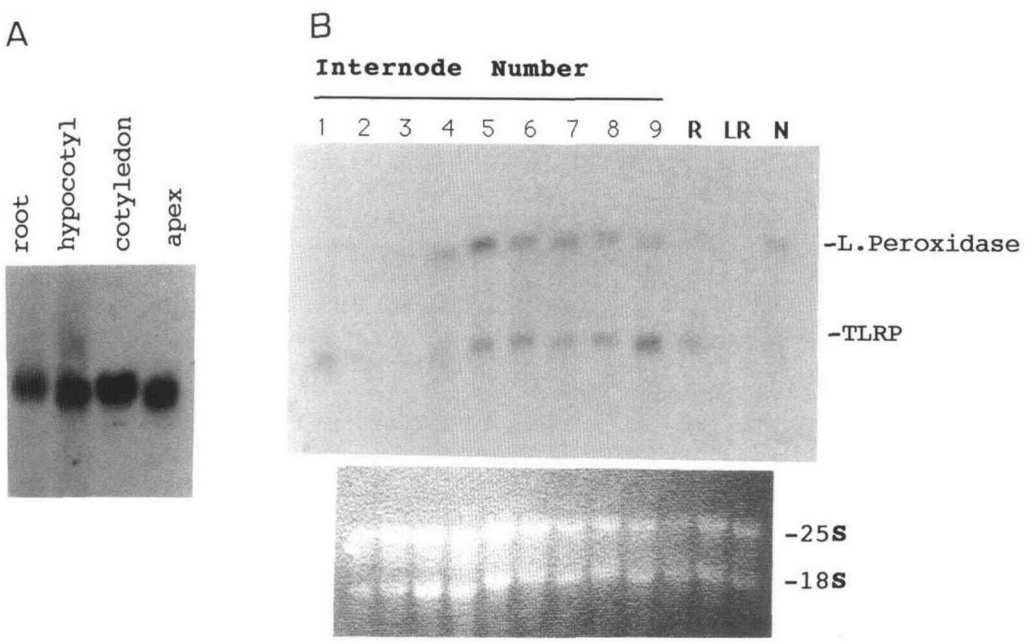

Figure 7. RNA Gel Blot Analysis of Transcripts of TLRP in Seedlings and in Different Internodes of Tomato Plants.

(A) Accumulation of TLRP mRNA in different tissues of tomato seedlings; namely roots, hypocotyls, cotyledons, and shoot apices. The autoradiogram was exposed for 12 hr.

(B) Lanes 1 to 9 correspond to RNA samples obtained from the different internodes of tomato plants. The internodes were numbered from the top (close to the apex, number 1) to the bottom (close to the root, number 9) of the plant. RNA samples from the main root (R), lateral roots (LR), and the node (N) between the fourth and the fifth internodes were also included in this experiment. The blot was hybridized with TLRP and lignin peroxidase (L. peroxidase) DNA probes. The autoradiogram was exposed for 12 hr. An ethidium bromide register is shown at the bottom, and the position of the rRNAs (18S and 25S) are indicated to the right of the gel.

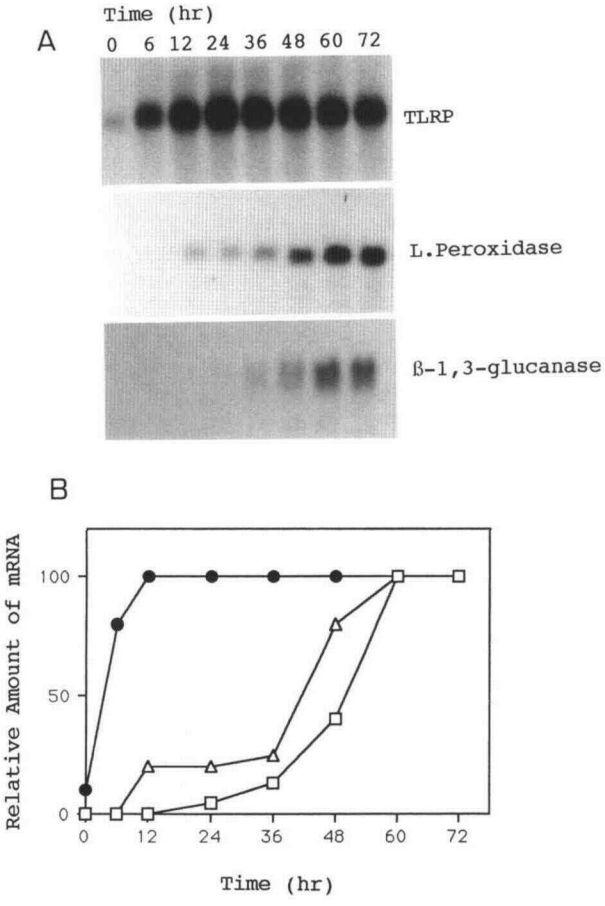

Figure 8. Induction of mRNA Accumulation of TLRP, Lignin Peroxidase, and PR-Q′ β-1,3-Glucanase by 2-Chloroethylphosphonic Acid Treatment on Tomato Leaves.

(A) RNA gel blots. L. Peroxidase, lignin peroxidase.
(B) Densitometric scanning of the autoradiograms shown in **(A)**. (●), TLRP signals; (△), lignin peroxidase signals; (□), PR-Q′ β-1,3-glucanase signals.
Time (in hours) represents the period after treatment of tomato leaves with 1 mM 2-chloroethylphosphonic acid solution.

These different kinetics of induction may reflect divergences in the transduction pathway of the ethylene signal that will lead to activation of genes with different biological roles. These include (1) defensive roles, which the β-1,3-glucanase gene and other defense genes with similar induction kinetics demonstrate (Domingo et al., 1994; Tornero et al., 1994), and (2) developmental roles, which have been described in this report for the TLRP gene.

DISCUSSION

We have isolated a cDNA clone that represents a novel extracellular matrix protein that becomes rapidly cross-linked to the secondary cell walls.

Structurally, the mature TLRP (62 amino acids long) contains nine tyrosine residues; this accounts for more than 14% of the total amino acid residues of the mature protein. High tyrosine content is also a characteristic of different structural cell wall proteins (Cassab and Varner, 1988). This amino acid residue is implicated in the nonreversible intermolecular cross-linking of these proteins through isodityrosine linkages, a process catalyzed by specific cell wall peroxidases under proper oxidative conditions (Fry, 1986).

The tomato TLRP becomes insoluble; it was not detected in tissue prints or protein extracts of different plant organs but was still present when embedded and sectioned material was examined. Intermolecular cross-links in the form of isodityrosine could account for the observed insolubility of this particular protein. Whether this insolubilization is mediated by cross-linking to itself, to other cell wall structural proteins, or to non-protein components is not known. Theoretically, such cross-linking could allow for the formation of an ordered disposition of this protein in the secondary cell walls. Bradley et al. (1992) demonstrated that this insolubilization of cell wall proteins with high tyrosine content can be performed by a fast signal–perception mechanism at the cell wall level, and this rapid mechanism may confer to the plant a capability to rapidly respond to different physiological and developmental demands. Moreover, TLRP contains a characteristic Tyr-X-Tyr sequence. Epstein and Lamport (1984) have shown that the first and third tyrosine's residues in such a Tyr-X-Tyr motif also form a covalent intramolecular isodityrosine cross-link. The latter will not render TLRP insoluble, but we can hypothesize that it will contribute to the general structure of this protein (e.g., by creating a kink in the protein backbone). Further experiments are needed to clarify to what extent isodityrosine cross-linking of TLRP is contributing to the overall process of secondary cell wall formation characteristic of xylem cells.

Interesting to note in the characterization of the tomato TLRP is the presence of a distinct structural domain. In this protein, a negatively charged N terminus is followed by a highly positively charged central region. This positively charged central region, in which 10 of 30 amino acid residues are basic (nine Lys and one Arg) and none is acidic, coincides with the region in which the tyrosine residues are clustered. This organization in the arrangement of positively charged groups in the protein may indicate that TLRP, instead of being entirely embedded in the fibrous wall matrix, might be exposed and then interacts with other charged moieties in the extracellular matrix. In fact, highly charged structural domains were implicated in the biogenesis of the extracellular matrix during *Volvox* embryogenesis (Ertl et al., 1992), and this suggests that similar roles may be performed by highly charged domains of other cell wall proteins.

Another structural characteristic of the tomato TLRP is the high proportion of cysteine residues. It contains six cysteine residues that account for 10% of the total amino acid residues. The cysteine residues should be able to participate in disulfide bond formation within the TLRP, contributing to the structure of the protein, or might participate in the formation of disulfide bonds with other cysteine groups of other proteins

present in the cell wall. Because of reversibility of disulfide bonds, the interactions mediated by these cysteine residues with other cell wall constituents may be functionally versatile and may provide an elastic character to the cell walls of tracheary elements.

The precursor form of TLRP contains a conserved signal peptide sequence homologous to several GRPs. It is interesting to note that this homology is even greater if we consider DNA sequence homology, particularly with the tobacco GRP (van Kan et al., 1988). In this case, the nucleotide sequence identity with TLRP is 90% in the 5′ nontranslated region that precedes the initiation codon (data not shown). This high homology disappears after the signal sequence where an intron exists in the tobacco GRP gene. The homology with this tobacco GRP gene is recovered again in the 3′ nontranslated region preceding the polyadenylation tail of the TLRP sequence. The close evolutionary proximity of tomato and tobacco genomes suggests that recombination phenomena might have taken place in the origin and evolution of the tomato TLRP described here and the tobacco GRP. Similarly, gene duplication and recombination events have been proposed to have participated in the generation of gene families encoding different cell wall proteins (Showalter and Varner, 1989; Showalter and Rumeau, 1990).

The specific localization of TLRP in those cells undergoing lignification, such as fibers and vessels of the xylem tissue and also in the sclerenchyma cells, suggests a correspondingly specific function for this cell wall protein. Because dead tracheary elements of the xylem have to retain their shape despite the pressure of the surrounding cells to function as water conduits, reinforcement of their cell walls is paramount for maintenance of this functional character. Hence, deposition of TLRP in the walls of xylem-associated cells may reinforce them to withstand mechanical forces. The same consideration can be held for the interpretation of the specific localization of TLRP in the lignified cell walls of the sclerenchyma, a tissue exhibiting elastic properties and whose principal function is giving support to the plant (Esau, 1965). Similarly, the specific deposition of TLRP in the outer face of the protective periderm monolayer of the root may help the root to withstand the encountered strong mechanical pressure during its growth through compact soil.

Because these cell functions are critical for the plant to survive, we can expect that the process of differentiation of these cell types expressing the TLRP gene must be tightly regulated and that additional cellular factors participating in the final cell wall architecture must exist. In this regard, a soybean HRGP was shown to be localized in sclerenchyma tissue and in the vascular tissue (Cassab and Varner, 1987). Also, a bean GRP was shown to be localized exclusively in the lignified secondary thickenings of tracheary elements of the protoxylem (Keller et al., 1989). Thus, it could be that TLRP will participate with these colocalized cell wall proteins in mediating the final building and shaping of the lignified secondary cell walls.

Further characterization of the gene described here together with the use of transgenic plants expressing modified versions of this cell wall protein should provide significant insights into the role of cell wall constituents in the process of xylem differentiation.

METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum* cv Rutgers) were grown from seeds in compost pots under standard greenhouse conditions. Treatments of leaves with 1 mM 2-chloroethylphosphonic acid were performed as described previously (Domingo et al., 1994).

cDNA Library Construction and Screening

cDNA library construction, differential screening, and selection of clones was as previously described (Tornero et al., 1994; Vera et al., 1994). One of the selected clones was chosen for further studies (clone designated as pTE4). This λ clone was converted to the pBluescript SK+ form by coinfection with R408 helper phage (Stratagene) to perform DNA sequencing.

RNA and DNA Gel Blot Analyses

RNA was purified from different tomato plant tissues as described previously (Logemann et al., 1987). For RNA gel blot analysis, 15 μg of total RNA was electrophoresed on 1% agarose gels containing formaldehyde and blotted onto Nytran membranes (Schleicher & Schuell) as described previously (Maniatis et al., 1982). Equal loading of RNA was verified by ethidium bromide staining of the gel before transfer to the membrane. RNA hybridizing to the gene-specific probe was quantified by densitometry of the autoradiographs in a transmission densitometer (Advance American Biotechnology, Fullerton, CA). DNA was isolated from leaves as described previously (Rogers and Bendich, 1988). For DNA gel blot analysis, 10 μg of DNA was digested with restriction enzymes, electrophoresed in a 0.7% agarose gel, and blotted onto Nytran membranes as described previously (Maniatis et al., 1982). RNA and DNA gel blots were probed with the entire cDNA insert, which was radiolabeled by random priming using T7 polymerase (Pharmacia). Hybridization and washing conditions of filters were done at high stringency (70°C) as described previously (Church and Gilbert, 1984).

DNA Sequencing

DNA sequence analysis was performed on both strands by the dideoxy chain termination method as described by Hattoni and Sakaki (1987). Nested deletions of the cDNA cloned in pBluescript SK+ were generated using an exonuclease III–S1 nuclease kit (Promega). Sequence searches and analyses were done using FASTA, MAP, and BESTFIT routines of the University of Wisconsin Genetic Computer Group software package (Devereux et al., 1987).

Protein Expression, Purification, and Antibody Production

A synthetic oligonucleotide 5′-GGAATTCTAGAGGTTGCAGCTAGGGAG-3′ and a T7-specific primer were used as primers to generate

a polymerase chain reaction (PCR) product from the pTE4 cDNA clone. This PCR product was digested with EcoRI and XhoI and was ligated in the corresponding restriction sites of pGEX-KG (Guan and Dixon, 1991), a vector that contains the glutathione-*S*-transferase (GST), to yield the plasmid pGEX-TLRP, which was used to produce the GST–tyrosine- and lysine-rich protein (TLRP) fusion protein. The GST-TLRP junction was sequenced to assure that these fragments were in the correct reading frame. Restriction digests and ligation reactions were conducted as described by Maniatis et al. (1982).

For production of the GST-TLRP fusion protein, *Escherichia coli* XL1-Blue (Stratagene) containing pGST-TLRP was grown and induced with 0.3 mM isopropyl β-D-thiogalactopyranoside; the resulting fusion protein was purified on glutathione-Sepharose as described by Guan and Dixon (1991). Fifty micrograms of the purified fusion protein was suspended in Freund's complete adjuvant and subcutaneously injected into a New Zealand white rabbit. Booster injections were done at 2-week intervals for 2 months. The rabbit was then bled and the serum collected. To eliminate any cross-reactivity of the antiserum with GST, purified GST protein was bound to 2 mL of glutathione-Sepharose and was incubated with 5 mL of crude antiserum for several hours. The antiserum was recovered by centrifugation and kept frozen at −20°C for additional studies. The specificity of the GST-arrested antibody to TLRP was checked by protein gel blot analysis after resolving proteins by SDS-PAGE as described previously (Vera et al., 1989a).

Immunohistochemical and Immunocytochemical Methods

The first fully developed leaves from the third internode as well as the fifth internode and roots of full-grown tomato plants were harvested and immediately fixed with 4% paraformaldehyde in 0.2 M PO_4 buffer, pH 7.2, by applying vacuum pressure for 2 min; they were kept at 4°C for 4 hr. The material was then rinsed extensively in the same buffer without paraformaldehyde, dehydrated through a series of ethanol solutions (30, 50, 70, 90, and 98%) for 60 min each, and embedded in hydroxyethyl methacrylate Technovit 7100 according to the protocol of the manufacturer (Reichert-Jung, Heidelberg, Germany). The Technovit blocks were placed in an ultramicrotome (Ultracut E; Reichert), and sections were transferred to glass slides coated with 2% Tespa (3-aminopropyltrimethoxysilane) in acetone. All washes and incubations with antibodies were done at room temperature. The basic medium was PBS containing 5% BSA (blocking) or 1% BSA plus 0.05% Tween 20. Sections were blocked by a 30-min incubation; the sections were then incubated with a 1:200 dilution of the TLRP antiserum and incubated for 2 hr with the sections. Control slides were treated similarly with a preimmune serum diluted 1:200. After three washes of 10 min each, an anti-rabbit IgG gold conjugate (10 nm; Sigma), diluted 1:20, was used as a secondary antibody in combination with a silver enhancer kit (IntenSE), following the protocols of the manufacturer (Amersham International). The sections were stained with 0.5% safrinin O for 30 min, and a Diaphot microscope (Nikon Europe B. V., The Netherlands) with an episcopic fluorescence attachment (IGS filter) was used for sample visualization and photography. To optimize differential visualization of silver-gold particles and tissues, the photographs were taken using a combination of bright and epipolarized light.

Processing of tissue samples, preparation of protein–A gold particles, and protocols for incubation, washes, and staining of tissue samples for electron microscopy were as previously described for other tomato proteins (Vera et al., 1989a, 1989b), except that the tissue samples were embedded in Spurr's epoxy resin instead of Lowicryl K4M, because the former yielded clearer results with a lower background. Sections were incubated with a 1:200 dilution of the anti-TLRP serum. Controls were run with preimmune serum diluted 1:200. Counting of gold particles on prints with the aid of a multipurpose grid and statistical analysis were done as previously described (Vera et al., 1989a).

ACKNOWLEDGMENTS

We thank Dr. Beat Keller for helpful comments on the manuscript. This research was supported by the Ministry of Science and Education (DGICYT Project No. PB90-0746) from Spain. C.D. thanks the Conselleria de Educación y Ciencia de Valencia for her fellowship. M.D.G. thanks the Ministry of Science and Education for her fellowship.

Received March 21, 1994; accepted June 1, 1994.

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