

Characterization of an apoplastic basic thaumatin-like protein from recalcitrant chestnut seeds

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Mature chestnut seeds, with one of the highest moisture contents described to date, accumulate certain defensive proteins at unusually elevated levels. In this work a major 23-kDa thaumatin-like protein, termed CsTL1, has been purified from mature chestnut (*Castanea sativa*) cotyledons. Amino acid sequencing and characterization of its full-length cDNA indicate that CsTL1 is synthesized as a preprotein with a signal peptide 22 amino acids in length. The mature protein contains 16 conserved cysteine residues presumably involved in disulfide bonding and has a high isoelectric point (ca. 9). Unlike most basic pathogenesis-related (PR) proteins, mature CsTL1 is localized to the extracellular matrix, as revealed by immunoelectron microscopy studies of cotyledonary cells. The

isolated protein has *in vitro* antifungal activity against *Trichoderma viride* and *Fusarium oxysporum* and shows strong synergistic effects with CsCh1, the most abundant chestnut cotyledon endochitinase. Moreover, both CsTL1 and CsCh1 appear to be regulated in the same manner during seed development and germination. These observations, along with the recent finding of endoglucanase activity for some TL proteins, support the notion that CsTL1 and CsCh1 are part of a complex seed defensive system against microbial growth. Another possibility is that these, and probably other seed PR proteins, have antifreeze activity. Both functions would be particularly relevant for chestnut seeds given their remarkable moisture content at maturity.

Introduction

Pathogenesis-related (PR) proteins are a structurally diverse group of ubiquitous proteins associated with plant defensive responses (see Yun et al. 1997 for a recent review). Based on their primary structure, enzymatic properties, and biological activity most plant PR proteins have been grouped into 5 major families, designated PR-1 through PR-5 (Van Loon et al. 1994). All members of the PR-5 group, also referred to as thaumatin-like (TL) proteins (Cornelissen et al. 1986), are homologous to thaumatin, a sweet-tasting protein isolated from *Thaumatococcus daniellii* fruits (Van der Wel and Loeve 1972). Independent lines of research have shown that whereas some TL proteins accumulate normally in seeds, fruits or flower tissues in response to developmental cues (Neale et al. 1990, Roberts and Selitrennikoff 1990, Tattersall et al. 1997), specific components become major proteins in vegetative tissues upon osmotic challenge ('osmotins', Singh et al. 1987) or pathogen attack (Cornelissen et al.

1986). Additional stimuli that induce TL gene expression include drought, wounding, UV-light, and treatments with abscisic acid (ABA), salicylic acid or jasmonate-ethylene combinations (Kitajima and Sato 1999). Recently, induction of a tobacco TL gene by different stimuli has been shown to occur in a tissue-specific manner (Raghothama et al. 1997).

Purified TL proteins from several plant species possess antifungal activity *in vitro*, although the exact roles of individual components in disease resistance remain to be determined. Besides inhibiting hyphal growth and spore germination, some TL proteins are known to cause rapid permeabilization of fungal plasma membranes (Woloshuck et al. 1991, Terras et al. 1992). It has been reported recently that osmotin subverts signal transduction to enhance fungal cell susceptibility (Yun et al. 1998). These studies have shown that TL proteins are not only effective against a broad range of fungal species, including several plant patho-

Abbreviations – ABA, abscisic acid; PMSF, phenylmethylsulfonyl fluoride; PR, pathogenesis-related; SA, salicylic acid; TL protein, thaumatin-like protein.

gens, but also exhibit specificity to certain fungi (Yun et al. 1997). Direct evidence of a defensive role was first obtained by Liu et al. (1993), who demonstrated that a constitutively overexpressed TL protein reduces fungal disease symptoms in transgenic potato plants.

Besides TL proteins, it is well established that non-stressed seeds accumulate other defensive proteins as part of their developmental program. Many of these proteins, including chitinases, glucanases, thionins, defensins, lipid-transfer proteins, ribosome-inactivating proteins, and α -amylase inhibitors are active against insects or microorganisms (Bol et al. 1990, Garcia-Olmedo et al. 1992, Yun et al. 1997). The abundance and multiplicity of defensive proteins in seeds and other storage organs is probably explained by the fact that their storage tissues are good substrates for a wide range of heterotrophic organisms. In this respect, it seems reasonable to assume that seeds having higher water contents at maturity are more sensitive to microbial attack. Consistent with this hypothesis, recalcitrant chestnut (*Castanea sativa* Mill.) seeds, with one of the highest moisture contents known at shedding (ca. 50% by weight), have been shown to accumulate chitinases at levels comparable to those of major storage proteins (Collada et al. 1992, Allona et al. 1996, Garcia-Casado et al. 1998). A similar explanation may underlie the recent identification of abundant chitinases and/or TL proteins in fruit pulp from cherry, banana, apple, grape berry, or avocado (Clendennen and May 1997, Tattersall et al. 1997, Diaz-Perales et al. 1998).

We report here the purification and cDNA cloning of a TL protein abundant in chestnut cotyledons. The relative abundance and accumulation pattern of this protein, termed CsTL1, as well as its antifungal properties in combination with chestnut endochitinase CsCh1, are supportive of the existence of a developmentally-regulated defence system involving seed-specific PR proteins with antifungal activity. Since many seed defensive proteins have stress-inducible counterparts in vegetative tissues, we also studied the accumulation of homologous transcripts and proteins in chestnut plantlets exposed to different types of stress.

Materials and methods

Plant materials and treatments

For cDNA library construction and immuno-electron microscopy, seeds of *Castanea sativa* Mill. (European chestnut) were harvested in Zarzalejo, a village NW of Madrid (Spain) at late-mature stage, i.e. 4–6 weeks before fruits fall to the ground (shedding). Mature seeds were germinated and chestnut plantlets were grown in a controlled environment growth chamber as previously described (Soto et al. 1999). For salt-stress experiments, plantlets were watered profusely once with 200 mM NaCl and then held for up to 72 h. Salicylic acid (SA) dissolved in 96% ethanol was applied exogenously (sprayed) at a concentration of 1 mM. For gene expression studies during maturation and germination, seeds were either collected 90, 110 and 140 days after anthesis or germinated in growth chambers and collected 0, 4, 8 and 12 days after the root apex became visible.

Purification of CsTL1

Flour from chestnut cotyledons was partially defatted with diethyl ether (1 $\times$ 10 v/w, 1 h, 4°C) and proteins extracted at 4°C for 1 h with 50 mM Tris-HCl, pH 8.2, 0.1 M NaCl, 1 mM PMSF (10:1, v/w). After centrifugation at 20000 g for 30 min, proteins in the supernatant were precipitated with ammonium sulfate (65% saturation) and resuspended in the same buffer. The pH was then adjusted to 2.5 with 1 M HCl and insoluble proteins were eliminated by centrifugation at 22000 g. After dialyzing the supernatant against water, CsTL1 was purified to apparent homogeneity by two sequential ion-exchange chromatography steps on Econo-Pac S (linear gradient of NaCl in 0.01 M acetate buffer, pH 5.4) and Econo-Pac Q columns (linear gradient of NaCl in 0.02 M glycine-NaOH buffer, pH 9.5), following the supplier's instructions (Bio-Rad, Hercules, CA, USA).

Protein electrophoresis and immunodetection

One- and two-dimensional electrophoresis, as well as protein immunoblotting on PVDF (polyvinylidene difluoride) membranes were carried out as described previously (Collada et al. 1992). For the latter experiments we used a 1:5000 dilution of monospecific polyclonal antibodies against purified CsTL1 prepared as in Collada et al. (1992).

cDNA cloning

A cDNA library in λ Uni-ZAP XR (Stratagene, La Jolla, CA, USA) was made from late-mature chestnut cotyledon polyA⁺ RNA as described by Soto et al. (1999). Immunoscreeing of this library was performed with a 1:300 dilution of monospecific antibodies against CsTL1. Positive cDNA clones were isolated and subjected to in vivo excision into *Escherichia coli* SOLR cells to render recombinant pBlue-script SK(–) phagemids. For each insert, both strands were completely sequenced using an automated ABI 373 DNA Sequencer. No positive clones were found upon screening of this library with the coding sequence for tobacco osmotin (pOG clone, Nelson et al. 1992), kindly provided by Dr R. A. Bressan, which is probably due to the low similarity between chestnut and tobacco TL proteins (see below).

DNA and RNA blots

Total genomic DNA was isolated from chestnut cotyledons by the method of Dellaporta et al. (1983). DNA (25 μ g) was digested with restriction enzymes and electrophoresed on 0.8% (w/v) agarose gels. Blots were hybridized and washed at low stringency following the supplier's instructions (nylon Magna membranes, MSI, Westborough, MA, USA). Briefly, membranes were hybridized overnight at 65°C and then washed twice in 2 $\times$ SSC (1 $\times$ is 0.15 M NaCl and 15 mM sodium citrate, pH 7) and 0.1% (w/v) SDS at room temperature for 15 min, twice in 1 $\times$ SSC and 0.1% SDS for 15 min, and twice in 0.2 $\times$ SSC and 0.1% SDS for 15 min. Total RNA was extracted and hybridized as in Soto et al. (1999). Labeling of the probes with [α -³²P]dATP was performed with Boehringer Mannheim's Random Primed DNA Labeling kit (Germany).

Immuno-electron microscopy

Chestnut seeds for immuno-EM experiments were collected ~6 weeks before shedding. After low temperature embedding, ultrathin sections were treated with monospecific antibodies against CsTL1 (see above), colloidal gold-labeled (10 nm particles), and stained with uranyl acetate and lead citrate as previously described (Rodriguez-Cerezo et al. 1997).

Fungal growth inhibition assays

Trichoderma viride, *Fusarium oxysporum* f. sp. *lycopersici* and *Botrytis cinerea* spores were collected from cultures on potato dextrose agar (Collada et al. 1992). For growth inhibition assays, fungal spore suspensions were incubated in potato dextrose broth at 25°C for 2 h, diluted 40-fold with sterile water and then transferred to microtiter well plates (1500 spores per well in 90 µl). Proteins to be tested were added in 10 µl of 0.02 M sodium acetate, pH 6.0, supplemented with 32 mM NaCl and plates were kept at 25°C for 4 h. Heat-denatured CsTL1 and CsCh1, as well as bovine serum albumin were included as controls. Finally, 25 µl of (5 ×) potato dextrose broth were added to each well and plates were further incubated at 25°C for 14–20 h. Fungal biomass was estimated from measurements of culture absorbance at 490 nm (Leah et al. 1991). Plots of A_{490} versus concentration of protein added permitted the calculation of the inhibitory activity of each sample. Data from at least 4 independent assays (each with two sample replicas) were used for calculations.

Results

Purification of CsTL1

A salt-soluble protein preparation from *C. sativa* cotyledons was enriched in TL-protein by salting out with ammonium sulfate, followed by differential precipitation at acidic pH. CsTL1 was subsequently purified to apparent homogeneity by two batch ion-exchange chromatography steps (Fig. 1A).

The rabbit polyclonal serum raised against purified CsTL1 recognized a single protein band when a replica of the gel in Fig. 1A was immunoblotted (Fig. 1B). Likewise, only one immunoreactive form was identified in isoelectric focusing × SDS-PAGE blots probed with the same serum (not shown). The isolated protein appeared in Coomassie-stained two-dimensional gels as a single spot with an apparent molecular mass of 22 kDa and pI close to 9 (Fig. 1C). The mobility of CsTL1 in SDS-PAGE decreased under reducing conditions (compare lanes a and b in Fig. 1B), consistent with the cleavage of its 8 disulfide bonds (see below). Similar shifts in electrophoretic behavior have been reported previously for other TL proteins (Vigers et al. 1992). A single amino acid sequence was obtained when the isolated protein was subjected to automated N-terminal sequencing (AKITFTNNCPRTIWPGLT; see Fig. 2). Database comparisons revealed that this sequence had the highest similarity to members of the TL protein family.

Characterization of a cDNA encoding CsTL1 and phylogenetic relationships among TL-proteins

To isolate cDNA clones encoding CsTL1, an expression library from late-mature chestnut cotyledons (1.1×10^6 plaque-forming units) was immunoscreened with antibodies against the purified protein. A total of 6 positive clones (out of 45) were randomly selected and their inserts sequenced. All inserts corresponded to a single nucleotide sequence, which includes a 728-bp open reading frame flanked by 5'- and 3'-noncoding sequences (Fig. 2). The 3'-untranslated region has two putative polyadenylation signals upstream of the poly(A) tail. Comparison of the deduced amino acid sequence, 243 residues long, with the N-terminal sequence determined for purified CsTL1, indicated that the mature protein is synthesized as a precursor with a signal peptide 22 amino acids in length. The cleavage site (HS↓AK) is in agreement with the rules of von Heijne (1986), suggesting that no further processing occurs at the N-terminus of the protein. Analysis of the deduced sequence with PSORT (Nakai and Kanehisa 1992) predicts that CsTL1 follows the vesicular pathway for secretion, in agreement with our

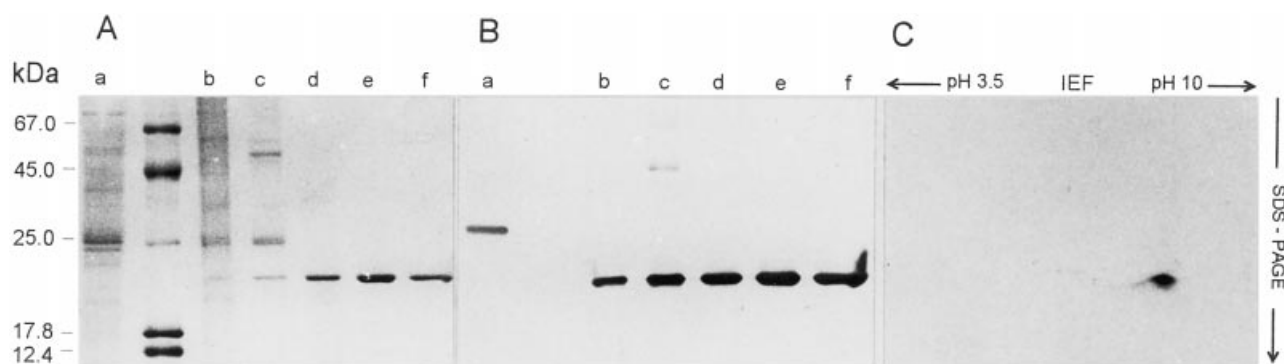


Fig. 1. Purification of CsTL1 from chestnut seeds. A, SDS-PAGE of the following samples: salt-soluble proteins from chestnut cotyledons treated (lane a) or not (lane b) with 2-mercaptoethanol; fraction obtained by salting-out the crude extract with ammonium sulfate followed by precipitation at pH 3.5 (lane c); fraction obtained after differential precipitation at pH 2.5 (lane d); CsTL1-enriched preparation after chromatography on Econo-Pac S (lane e); repurified CsTL1 after chromatography on Econo-Pac Q (lane f). Molecular weight markers are shown between lanes a and b. B, Immunoblot of a replica gel probed with rabbit polyclonal antibodies against CsTL1. C, Coomassie-stained two-dimensional electrophoresis (isoelectric focusing × SDS-PAGE) of purified CsTL1.

accaaacccaagtcc -43
 atcgttaatacataaatactagttaccaccaatttaagcaaca -1
 ATGATGAAAACCTGGCACTCTACGGCCTTACCTTGGCTCTC 42
 M M K T L A L Y G L T L A L 14
 TTTTCTTATCTGGTGGCAGCTCTGCTAAAATAACTTTTACA 84
 F F L S G A H S A K I T F T 28
 AACAACTGTCCAAGAACCATCTGGCCAGGAACCTTAACCTCA 126
N N C P R T I W P G T L T S 42
 GATCAAAAACCTCAACTACCGAACACTGGATTGTGTAGCA 168
 D Q K P Q L P N T G F V L A 56
 TCCAAAGCATCTTTAAGACTGGGTGTTCAAGCTCCATGGAAA 210
 S K A S L T L G V Q A P W K 70
 GGCCGGTCTGGGCGGCAACCCGATGCACCACTCAAGCA 252
 G R F W A R T R C T T N S G 84
 AAGTTCACTTGCAGACTGTGTATTGTAGCACCAGGTCAGGTT 294
 K F T C E T A D C S T G Q V 98
 GCATGCAACGGTAATGGTGCAATCCACGATCTTTAGTA 336
 A C N G N G A I P P A S L V 112
 GAAATCAACATAGCAGCAATCGCGGGATGGACTTTTACGAT 378
 E I N I A A N R G M D F Y D 126
 GTTAGCCTGTATGGCTACAACCTTGCCTGTTTCTGTAGCC 420
 V S L V D G Y N L P V S V A 140
 ACCAGAGGCGGCACAGGTGATTGCAAGGCCACAAGCTGTGCA 462
 T R G G T G D C K A T S C R 154
 GCCAACGTTGAACGGTTTGGCCAGCGAATGCAAGTGAAA 504
 A N V N A V C P A E L Q V K 168
 GGGTCTGATGCGAGCGTGTCTGCTTGAAGAGCGCTTGTACT 546
 G S D A S V L A C K S A C T 182
 GCTTTCAATCAACCAACTACTGTGCTGCTGCTGCTGCTGCTG 588
 A F N Q P Q Y C C T G A F D 196
 ACCGCGAGAACATGTCCAGCCACAAATATCTCGCATCTTT 630
 T A R T C P A T K Y S R I F 210
 AAGCAACAATGCTCCTCAAGCTTATAGCTATGCTTATGATG 672
 K Q Q C P Q A Y S Y A Y D D 224
 TCTACCAGACCTTTACCTGCTCTGGTGCACCCGACTATGTT 714
 S T S T F T C S G A P D Y V 238
 ATCACTTTTGTTCATGAattatagagaggaattatgcgttc 756
 I T F C P 243
 ccaatttggaataaagaactgatattgtttcaagtactaagg 798
 cacatgtatgtacgtgtcaacgaaatagttatttgaataaaa 840
 catagaccaaataataacttcaaaattttacaaaaaa 882
 aaaaaaa

Fig. 2. Nucleotide and predicted amino acid sequence of the *CsTL1* cDNA clone. The N-terminal sequence determined for the mature protein is shown in boldface and the putative signal peptide in italics. Two consensus 3' polyadenylation signals are underlined. The nucleotide sequence has been submitted to the EMBL/EBI Data Bank with accession number AJ242828.

immunolocalization findings (see below). Moreover, no putative vacuolar sorting signals were identified in the deduced sequence. The predicted Mr (23467) and pI (8.5) values are similar to those experimentally determined for CsTL1.

Database searches with the complete deduced sequence confirmed that CsTL1 is a member of the TL protein family. In particular, 16 cysteine residues known to participate in disulfide bonding within this family (Ogata et al. 1992) are strictly conserved in the chestnut protein. While antifungal activity has been demonstrated for many TL proteins, it seems likely that at least some members have additional functions, as suggested by their accumulation patterns during plant development or in response to environmental cues such as salt stress and low temperatures. To analyze the evolutionary origin of putative functional counterparts, a phylogenetic tree was built using the complete sequences of CsTL1 together with 28 additional TL proteins expressed either during normal seed, flower or fruit development, or in

response to hormonal and environmental signals. The topology of the resulting phylogram (Fig. 3) is not supportive of a monophyletic origin for developmentally-regulated TL proteins, and the same is true for those components induced in response to environmental cues. Standard bootstrap analysis further strengthened these conclusions (see Fig. 3). Similar results were obtained when parsimony analysis was performed (Felsenstein 1989).

CsTL1 probably belongs to a multigene family

To determine whether *CsTL1*-related genes are present in the genome of *C. sativa*, total genomic DNA was digested and subjected to Southern blot analysis using the coding

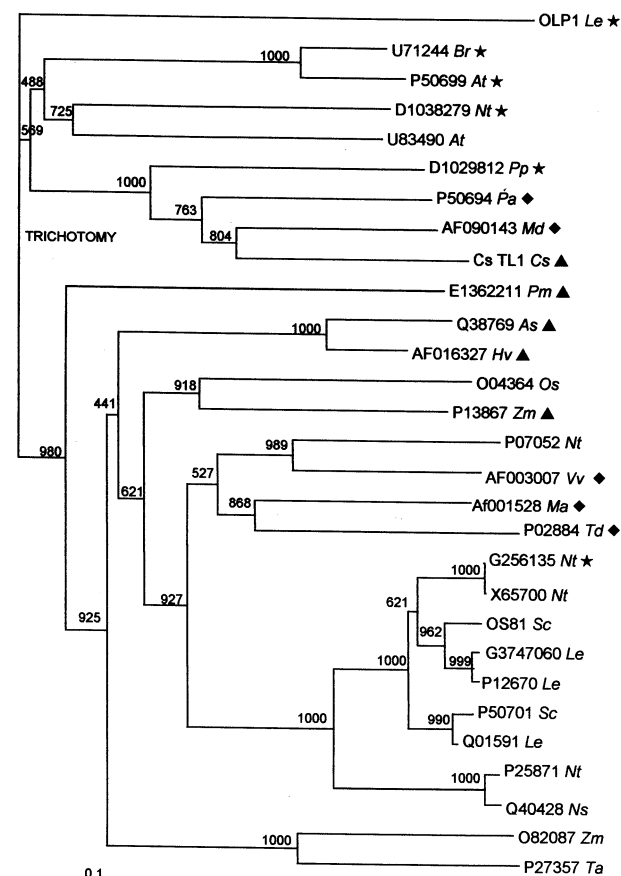


Fig. 3. Phylogram of plant TL proteins. The phylogenetic tree was constructed using the neighbor-joining method of CLUSTAL W (Thompson et al., 1994). For each protein the accession number and a species identifier are listed: At, *Arabidopsis thaliana*; As, *Avena sativa*; Br, *Brassica rapa*; Cs, *Castanea sativa*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; Ma, *Musa acuminata*; Md, *Malus domestica*; Ns, *Nicotiana sylvestris*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pa, *Prunus avium*; Pm, *Pseudotsuga menziesii*; Pp, *Pyrus pyrifolia*; Sc, *Solanum commersonii*; Ta, *Triticum aestivum*; Td, *Thaumatococcus daniellii*; Vv, *Vitis vinifera*; Zm, *Zea mays*. Symbols ★, ◆ and ▲ indicate that the protein is expressed in flowers, fruits or seeds, respectively. When the protein is induced by pathogens, hormonal treatments or abiotic stress factors, no symbols are used. The figure at each branching point shows the number of bootstrap replicas (out of 1000) giving rise to the depicted topology. Horizontal branch length reflects the distance between different TL-proteins.

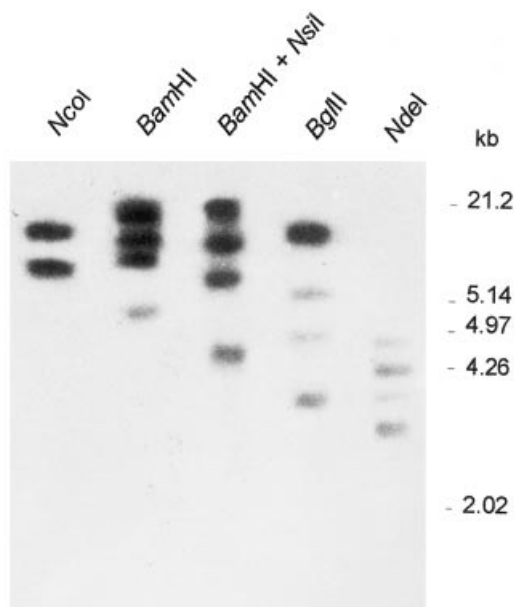


Fig. 4. Genomic Southern blot analysis. Total genomic DNA (25 µg per lane) was digested with the indicated restriction endonucleases (*Nco*I, *Bam*HI, *Bam*HI + *Nsi*I, *Bgl*II or *Nde*I), electrophoresed on 0.8% agarose gels, and blotted to nylon membranes. Blots were then probed at low stringency with the complete *CsTL1* cDNA (*Eco*RI-*Xho*I fragment) as described in Materials and methods. Only *Nco*I cuts the cDNA (single cut at position +202). The size of molecular markers (in kb) is indicated.

region of the isolated cDNA as a probe. Depending on the restriction enzyme used, two to 4 hybridizing fragments could be detected under low stringent hybridization conditions (Fig. 4). These results suggest that several genes encoding *CsTL1*-related proteins exist within the chestnut

genome, in line with previous findings for other plant species (Zhu et al. 1995).

Subcellular location of *CsTL1*

Because good sections are difficult to obtain from chestnut mature seed tissue, cotyledonary cells of late-mature stage were subjected to immuno-EM analysis to determine the subcellular location of *CsTL1*. A staining procedure was chosen that maximizes antigenicity (Rodriguez-Cerezo et al. 1997) and has proven useful for immuno-EM studies of chestnut cotyledonary tissue (Soto et al. 1999). As shown in Fig. 5, the *CsTL1*-specific label was localized both in the cytoplasm and the extracellular matrix. By contrast, no significant labeling was found when similar ultrathin sections were treated with preimmune serum (not shown). The observed aggregation of specific label, along with protein and DNA sequencing data, are both compatible with *CsTL1* being packed into transport vesicles that eventually fuse with the plasma membrane. Once secreted, the protein appears to remain in an aggregated state. Such structures were not observed when *CsHSP17.5*, a small HSP highly abundant in chestnut cotyledons, was immunolocalized by the same procedure (Soto et al. 1999). By contrast, similar aggregates to those shown in Fig. 5 have been reported for a vacuolar TL protein using different fixation and staining methods (Rodrigo et al. 1993).

Gene expression studies during seed maturation and germination

Like their vegetative counterparts, seed PR proteins are thought to be involved in defensive mechanisms. For this

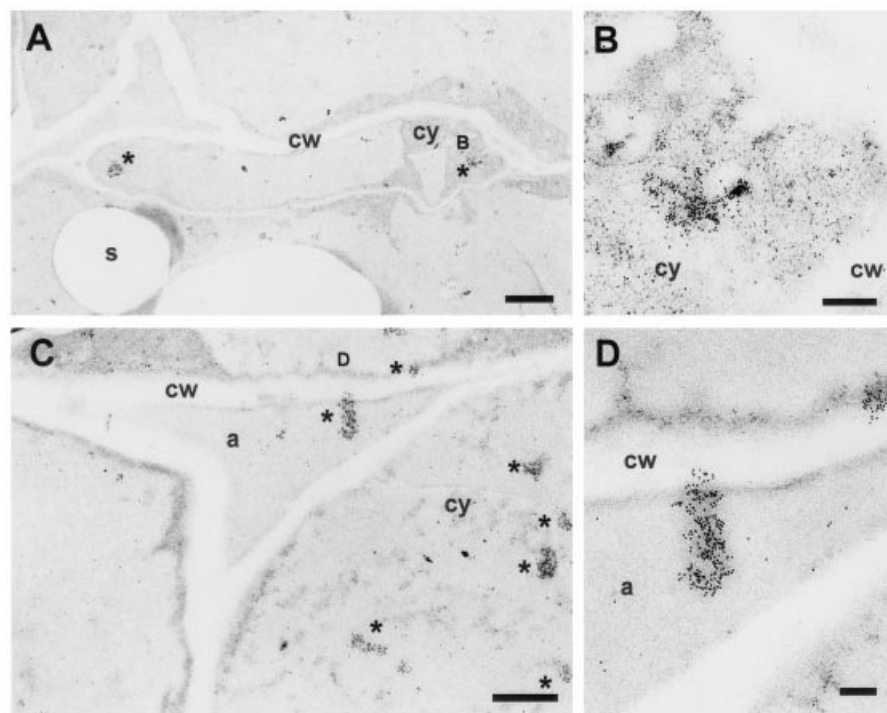


Fig. 5. Immunocytochemical localization of *CsTL1* in late-mature chestnut cotyledonary cells. Monospecific polyclonal antibodies against *CsTL1* were used to determine the subcellular localization of *CsTL1*. The low magnification views show a typical cell (A) and the apoplastic space (C) at this stage of development. B and D, high magnification views of selected binding zones from A and C (indicated by the corresponding letter). The gold label (10-nm particles) was found in the cytosol and extracellular matrix, mostly in aggregated structures (asterisks). a, Apoplastic space; cw, cell wall; cy, cytoplasm; s, starch granules. Bars correspond to 2 (A), 0.3 (B), 1 (C) and 0.2 µm (D).

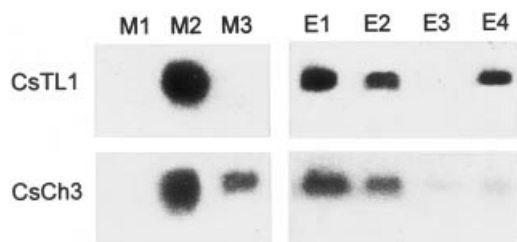


Fig. 6. Northern analysis of *CsTL1*- and *CsCh3*-hybridizing transcripts during seed maturation and germination. RNA was extracted and analyzed using as probes the full-length cDNA for *CsTL1* or the complete coding region for seed endochitinase *CsCh3* (Allona et al. 1996). Approximately 15 µg of RNA was loaded per lane. M1, M2 and M3, maturation stages corresponding to 90, 110 and 140 days after anthesis. E1, E2, E3 and E4, germination stages corresponding to 0, 4, 8 and 12 days after the root apex became visible. Even loading of the different RNA samples was verified by ethidium-bromide staining (not shown).

reason we analyzed the expression patterns of both *CsTL1* and chestnut seed endochitinases, for which antifungal activity has been already demonstrated (Collada et al. 1992, Allona et al. 1996, Garcia-Casado et al. 1998). Concerning seed maturation, northern blot analyses revealed that the highest accumulation of transcripts hybridizing with the *CsTL1* cDNA takes place at the mid-mature stage (Fig. 6). Comparable results were obtained when the same filters were hybridized with the cDNA for chestnut seed endochitinase *CsCh3* (Allona et al. 1996; Fig. 6). High-level expression of thaumatin and chitinase genes was also observed during early stages of seed germination, followed by a gradual decrease in transcript levels. Interestingly, a new induction was observed at late germination (stage E4) for *CsTL1* but not for chitinases.

Stress-induced expression in vegetative tissues

Chestnut plants (14- to 20-week old) kept in growth chambers were treated with SA or subjected to salt stress. Specific mRNA induction was then analyzed separately in roots, stems, and leaves. As revealed by the presence of *CsTL1*-hybridizing transcripts, TL gene(s) appear to be constitutively expressed in roots and, at significantly lower levels, in stems of control plants (Fig. 7). Increased transcript abundance was observed in all organs analyzed of salt-stressed plants, with the highest induction occurring in roots after 72 h (Fig. 7A). The hybridizing fragments appeared slightly smaller in stems than in leaves or roots. By contrast, under our experimental conditions SA-induced *CsTL1* homologs were detected only in roots, with maximal induction 24 h after SA application (Fig. 7B). Since the abundance of mRNA does not always correlate with TL protein synthesis (LaRosa et al. 1992, Zhu et al. 1995), we studied the presence of *CsTL1*-like proteins in the same plants by Western blotting. Proteins cross-reactive with anti-*CsTL1* antibodies were detected in all organs of salt-stressed plants after 48 h, whereas no signal was found in SA-treated plants (up to 40 µg of total protein per lane; not shown). Nor did we find increased levels of *CsTL1* homologs upon infection with the fungal pathogen *B. cinerea*. A significant induction

of cystatin mRNA occurred nevertheless in the latter plants (Pernas et al. 1998; G. Salcedo, personal communication).

Antifungal activity of *CsTL1*

The antifungal properties of *CsTL1* were investigated in growth inhibition tests using *T. viride* and *F. oxysporum* f. sp. *lycopersici*. The concentration of *CsTL1* required to inhibit fungal growth by 50% was approximately 10 µg ml⁻¹ in both cases (Fig. 8). The inhibitory activity of purified *CsCh1*, the most abundant endochitinase in chestnut cotyledons (Collada et al. 1992), was not significantly different from that of *CsTL1*. In assays with mixtures of the two proteins, clear synergistic effects were observed against both fungal species, with the strongest effect being found in the case of *F. oxysporum*. By contrast, neither *CsTL1* nor *CsCh1* showed inhibitory activity towards *B. cinerea*, even when assayed together using up to 5 µg ml⁻¹ of each protein in the mixture.

Discussion

The recalcitrant seeds of chestnut have one of the highest water contents known at shedding. During their normal development, these seeds accumulate significant amounts of proteins that are known to be involved in tolerance to different types of stress, both biotic and abiotic (Collada et al. 1992, Allona et al. 1996, Collada et al. 1997, Garcia-Casado et al. 1998, Pernas et al. 1998, Soto et al. 1999). In this paper we report on the isolation and cDNA cloning of *CsTL1*, a basic TL protein from chestnut cotyledons that shows antifungal activity. Its predicted amino acid sequence (243 residues) includes the N-terminal sequence determined for mature seed *CsTL1* plus a putative signal peptide 22 residues in length. The highest sequence identity in databank searches was found with plant TL proteins, including 16

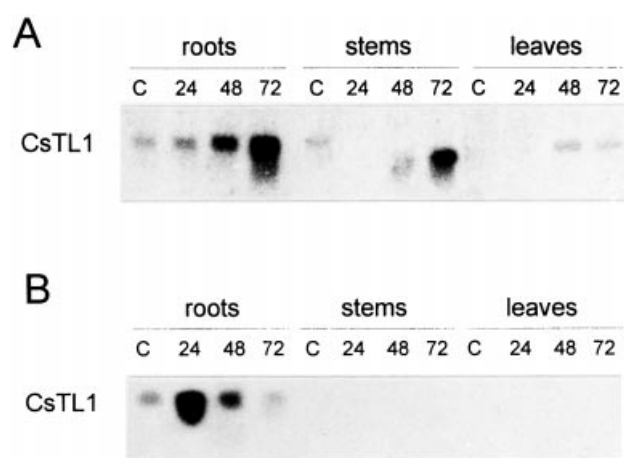


Fig. 7. Induction of *CsTL1* homologs in chestnut plantlets subjected to salt-stress (A) or SA treatments (B). RNA (15 µg) was analyzed as in Fig. 6. For salt-stress experiments plantlets were profusely watered once with 200 mM NaCl. mRNA induction in response to SA (1 mM) was tested for up to 72 h after a single hormone application. C, control plants.

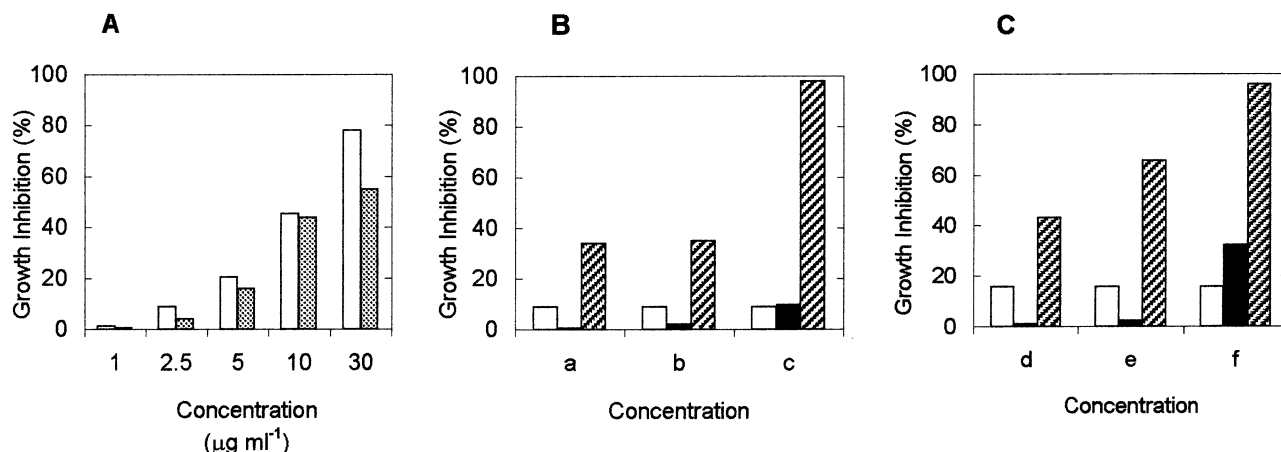


Fig. 8. Antifungal activity of CsTL1 and endochitinase CsCh1. Growth inhibition assays were carried out in microtiter well plates as described in 'Materials and methods'. A, Inhibition of *F. oxysporum* (white) and *T. viride* (gray) growth by purified CsTL1. B, Inhibition of *F. oxysporum* growth by CsTL1 alone (white), CsCh1 alone (black), and mixtures of CsTL1 and CsCh1 (dashed). CsCh1 was assayed at 0.5 (a), 1.0 (b) and 2.5 $\mu\text{g ml}^{-1}$ (c), whereas CsTL1 was assayed at 2.5 $\mu\text{g ml}^{-1}$ in all cases. C, Inhibition of *T. viride* growth by the same proteins as in B. CsCh1 was assayed at 0.5 (a), 1.0 (b) and 5 $\mu\text{g ml}^{-1}$ (c), and CsTL1 was assayed at 5 $\mu\text{g ml}^{-1}$. Each plotted value represents the average of at least 4 independent assays (each with two replicas) relative to the average value for controls. Relative standard deviations were less than 10%.

characteristic cysteine residues involved in disulfide bonding (Ogata et al. 1992).

Whereas the basic forms of PR proteins such as chitinases, β -1,3-glucanases and TL proteins are typically localized to the vacuole (Bol et al. 1990, Kitajima and Sato 1999), immuno-EM studies of chestnut cotyledonary cells revealed that at least some CsTL1 molecules (pI ca. 9) are secreted to the extracellular matrix (Fig. 5). Congruent with this finding, the deduced amino acid sequence of CsTL1 lacks known vacuolar sorting information, including a characteristic C-terminal propeptide necessary for targeting vacuolar PR proteins (Liu et al. 1996). Moreover, two basic TL-proteins of 16 and 19 kDa have been recently purified from the intercellular fluid of barley leaves (Trudel et al. 1998). After being secreted, CsTL1 molecules appear to remain in an aggregated state. Tomato P23, a vacuolar TL protein induced upon viroid infection, has also been shown to form aggregated structures of unknown nature inside the vacuole (Rodrigo et al. 1993). In neither case do these structures prevent the proteins from being readily extracted with saline buffers of low ionic strength.

In Southern blotting experiments, the genome of *C. sativa* appears to contain several genes probably encoding CsTL1-related proteins. Using the *CsTL1* cDNA as a probe we analyzed the induction of TL genes in vegetative organs of stressed chestnut plantlets (Fig. 7). Upon high-salt treatments, induction of homologous transcripts was observed in roots, stems, and leaves. Western blot analyses using antibodies against CsTL1 showed that such transcript induction is correlated with the accumulation of immunoreactive proteins in the 3 organs. By contrast, TL gene induction in response to SA treatments occurs in an organ-specific manner and is not paralleled by accumulation of the corresponding protein. Similar results, also supportive of a post-transcriptional control of TL proteins, have been reported previously in tobacco and potato (LaRosa et al. 1992, Zhu et al. 1995, Raghothama et al. 1997).

Because of their elevated water content, chestnut seeds should be more sensitive to microbial attack and other types of environmental stress, such as extreme temperatures. The antifungal properties of seed TL proteins and endochitinases might thus be related to the existence of a complex defensive system that protects the nutrient-rich seeds from microbial growth. Consistent with this hypothesis, there is a coordinated synthesis of CsTL1 and endochitinases during both seed development and germination (Fig. 6). Moreover, both proteins show synergistic effects in vitro against fungal growth (Fig. 8), as previously reported for homologous barley seed proteins (Hejgaard et al. 1991). Such synergism may be related to the recent finding that some TL proteins can bind β -1,3-glucans (Trudel et al. 1998), or even possess endoglucanase activity (Grenier et al. 1999). A different possibility suggested by recent results is that both types of proteins play a role in tolerance to low temperature stress. Several PR proteins, including TL proteins and chitinases, are expressed during cold acclimation (Zhu et al. 1993). In addition, some of these cold-induced PR proteins have antifreeze activity (Hon et al. 1995, Dave and Mitra 1998). Interestingly, the antifreeze proteins from winter rye leaves accumulate in the apoplastic space (Antikainen et al. 1996) and the same appears to be true for both CsTL1 (Fig. 5) and chestnut chitinases (authors' unpublished results). On the other hand, it has been recently shown that certain fungal-induced PR proteins from winter rye leaves lack antifreeze activity, in contrast to their cold-induced homologs (Hiilovaara-Teijo et al. 1999).

Although many genes have been identified for plant TL proteins, either induced by different stimuli or in response to developmental signals (e.g. Yun et al. 1997, Kitajima and Sato 1999), it is remarkable that presumed functional counterparts do not appear to have a monophyletic origin (Fig. 3). This finding might reflect the fundamental role played by the three-dimensional structure of TL proteins, regardless of their specific functions. Recent structural studies have

demonstrated that TL proteins share a common spatial fold (Ogata et al. 1992, Batalia et al. 1996, Koiwa et al. 1999). It would thus make sense from an evolutionary point of view that functional divergences within this family take place through quasi-punctual sequence alterations, which implies a smaller genetic load. This hypothesis is also supported by the finding that only certain TL proteins with antifungal activity also have antifreeze properties (Hon et al. 1995, Hiilovaara-Teijo et al. 1999).

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