

Characterization and Expression of an Antifungal Zeamatin-like Protein (Zlp) Gene from *Zea mays*

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A cDNA clone encoding a basic thaumatin-like protein of *Zea mays* was recovered from a mid-development seed cDNA library. The gene, *Zlp*, encoded a protein that was nearly identical with maize zeamatin and α -amylase/trypsin inhibitor. Expression of *Zlp* mRNA was highest in the endosperm tissue of seed 4 weeks after pollination. Expression of zeamatin-like (ZLP) protein correlated with mRNA; also, a low basal level of ZLP expression in leaf was not appreciably induced by abiotic stresses. ZLP was expressed with its own signal peptide in insect cells and in transgenic *Arabidopsis* and tomato plants. ZLP was secreted in all three systems, with correct processing of the signal peptide. ZLP expressed in transgenic tomato was found to be partially subjected to a proteolytic cleavage after residue 180, by an unknown mechanism, to give a "nicked" isoform of ZLP. Purified ZLP from all three sources, as well as purified "nicked" ZLP from tomato, demonstrated fungal inhibition against *Candida albicans* and *Trichoderma reesei*, with marginal inhibition observed against *Alternaria solani* and *Neurospora crassa*.

PR proteins are a subset of plant defense proteins that were first described in the hypersensitive response of tobacco to tobacco mosaic virus (Van Loon and Van Kammen, 1970). PR proteins share biochemical characteristics including selective extractability at low pH, resistance to proteolysis, and intercellular localization (Fritig et al., 1989). PR protein expression has also been shown to correlate with the development throughout a plant of systemic acquired resistance (Tuzun et al., 1989; Ward et al., 1991; Uknes et al., 1992), a generally disseminated defense response to a variety of biotic and abiotic agents (Tuzun et al., 1989; Brederode et al., 1991; Ward et al., 1991). The nomenclature of PR proteins was initially based on their resolution in native acrylamide gels, and five categories were initially assigned; however, more sophisticated characterization has identified additional species, some of which co-migrate upon native gel electrophoresis (Van Loon et al., 1987). Although it was initially theorized that the action of PR proteins served to inhibit viral spread, more broad-based defensive mechanisms have come to light. PR3-class proteins are chitinases (Legrand et al., 1987), and PR2-class proteins are β -1,3-glucanases (Kaufmann et al., 1987); these hydrolases have been shown to inhibit fungal growth in vitro (Mauch et al., 1988).

Basic homologs to the acidic PR proteins have also been

described. These differ from the acidic PR proteins in their subcellular localization and their response to stress signals and developmental signals (Memelink et al., 1990). For example, basic chitinases and β -1,3-glucanases are expressed in healthy plant roots, floral organs, and seed tissues in response to hormonal and developmental signals (Neale et al., 1990; Leah et al., 1991; Linthorst, 1991). The function of these hydrolases in normal plant or seed physiology, beyond any potential defensive function, is unclear.

Proteins of the PR5 class are highly homologous to the sweet-tasting protein thaumatin (Edens et al., 1982). Like other classes of PR proteins, TLPs are expressed in response to a variety of infections, stress, and developmental signals. As yet they have no known enzymatic function, and their role in normal plant physiology is unknown. Although a *Zea mays* TLP has been reported to be a trypsin/ α -amylase inhibitor (Richardson et al., 1987; Richardson, 1991), such inhibition has not been reported for any other TLP. The TLP superfamily now contains more than a dozen members from a wide variety of monocotyledonous and dicotyledonous species. In addition, a new class of related but substantially smaller acidic TLPs has been identified in several monocotyledonous species (Bryngelsson and Green, 1989; Rebmann et al., 1991; Frendo et al., 1992).

Although no enzymatic function has been ascribed to TLPs, a direct defensive function has been demonstrated for some members of this protein superfamily. Tobacco osmotin has been shown to inhibit *Phytophthora infestans* (Woloshuk et al., 1991). Various seed TLPs have also been purified as inhibitors of *Candida albicans*, *Neurospora crassa*, and *Trichoderma reesei* (Roberts and Selitrennikoff, 1990), *Alternaria solani* and *Fusarium oxysporum* (Huynh et al., 1992a), and *Trichoderma viridae* (Bryngelsson and Green, 1989).

We have undertaken the molecular cloning and heterologous expression of the basic TLP of *Z. mays* seed. Here we report the cloning of a cDNA that encodes a protein nearly identical with the previously reported maize trypsin/ α -amylase inhibitor. The gene is expressed primarily in seeds, although also to a limited extent in stressed leaf tissue. Despite a marked codon bias, we have overexpressed this gene in insect cells and two transgenic plant species and shown that the heterologously expressed protein retains antifungal activity.

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Abbreviations: CaMV, cauliflower mosaic virus; IWF, intercellular wash fluid; MAI, maize amylase inhibitor; PR, pathogenesis related; TLP, thaumatin-like protein; ZLP, zeamatin-like protein.

MATERIALS AND METHODS

Plant Tissue Preparation

Tassels of 8-week-old *Zea mays* cv B73 plants were self-pollinated within 24 h of anthesis. Tassels were removed 24 h later, and bags were placed over the ears for the remainder of seed development. For seed dissection, developing kernels were excised from cob tissue and quickly separated into embryo and nonembryo tissue (e.g. pericarp, aleurone, endosperm) and frozen in liquid nitrogen. Every time point of seed development was collected from a different plant. Root, leaf, and husk tissue were also collected from 8-week-old plants and frozen in liquid nitrogen. For protein extraction, tissue was homogenized in 20 mM sodium acetate, pH 5.2 (see below), with an overhead stirrer (Brinkmann, Westbury, NY) and clarified by microcentrifugation.

Foliar treatment experiments were performed on 2-week-old seedlings grown in Metromix-350 (Grace-Sierra Co., Malpitas, CA) at 22°C with a 12-h photoperiod. Mercuric chloride (0.2%), potassium salicylate (1 mM), ethephon (2-chloroethylphosphonic acid; 1 mM), or water was sprayed to runoff on plants, which were then bagged separately for overnight incubation. Bags were removed and plants were grown an additional 2 d prior to leaf harvest, at which time necrosis from the mercuric chloride treatment was evident. Leaf tissue was frozen in liquid nitrogen. For protein extraction, tissue was homogenized in 20 mM sodium acetate, pH 5.2, using an overhead stirrer and clarified by microcentrifugation.

Analysis of RNA

Total RNA was prepared from plant tissue as described previously (Rochester et al., 1986). Polyadenylated RNA was enriched from total RNA by adsorption to oligo-deoxythymidine cellulose (Sigma). RNA samples were electrophoresed in agarose gels in the presence of formaldehyde according to standard procedures (Sambrook et al., 1989). Northern blots were prepared by upward capillary transfer onto Hybond-C extra (Amersham) according to manufacturer's directions.

Hybridization of northern blots with a homologous *Arabidopsis thaliana* TLP gene (D.E. Malehorn and D.M. Shah, unpublished data) was performed using a DNA restriction fragment bearing the carboxy-terminal half of that gene. The DNA was radiolabeled with [³²P]dCTP (Amersham) using a random-priming kit (Stratagene, La Jolla, CA). Hybridization was performed in 6× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7), 10× Denhardt's solution, 100 µg/mL yeast tRNA at 42°C. Filters were rinsed twice at room temperature for 15 min with 6× SSC, 0.05% SDS.

Northern blots were later reprobed using the 0.7-kb *Sall* fragment containing the entire *Zlp* coding sequence. Filters were hybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.1% SDS, 100 µg/mL yeast tRNA at 42°C. Filters were sequentially washed at increasing stringencies (0.1× SSC at 60, 65, and 70°C) and autoradiographed after each wash.

Seed cDNA Library Construction

Five micrograms of poly(A)-enriched mRNA from mid-development whole seed was used in a cDNA synthesis kit

(Amersham) with the exception that avian reverse transcriptase (Boehringer-Mannheim, Indianapolis, IN) was used. Double-stranded cDNA was methylated using a methylation kit (Promega, Madison, WI), ligated with *EcoRI* linkers (New England Biolabs, Beverly, MA), and exhaustively digested with *EcoRI* (Boehringer-Mannheim). DNA fragments between 0.5 and 2.0 kb were purified and cloned into *EcoRI*-cut λgt10 arms (Stratagene). DNA was packaged into phage using a packaging kit (Stratagene).

Approximately 1×10^5 plaque-forming units were plated on *Escherichia coli* C600 hfl- and transferred to nitrocellulose filters for in situ plaque screening according to standard methods (Sambrook et al., 1989). The probe was nearly the entire open reading frame of the homologous *A. thaliana* TLP gene. Twelve hybridizing clones were recovered and plaque purified through an additional round of in situ screening. The cDNA inserts of recombinant phage were subcloned into pUC118 (Viera and Messing, 1987). Several λ clones yielded identical cDNA inserts, a representative one of which was designated pMON22568.

DNA Sequencing

Plasmid DNA isolated on CsCl gradients was denatured with alkali (Hattori and Sakaki, 1986) and sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977) using the Sequenase, version 2.0, kit (United States Biochemical). Universal and reverse sequencing primers were used for appropriate plasmid subclones. Oligonucleotide primers (Midland Certified Reagent Co., Midland, TX) were utilized to complete sequencing of both strands. Formamide (40%) was incorporated into urea/acrylamide sequencing gels to relieve sequencing compressions. DNA sequence analysis was facilitated by using the Genetics Computer Group Sequence Analysis software package (Devereux et al., 1984).

Transgenic Plant Engineering

A "sense" oligonucleotide primer (5'-CCCAGATCT-ATGGCAGGTTCCGTGG-3') and an "antisense" primer (5'-CCCAGATCTCACGGGCAGAAGACGACC-3') were used to engineer the coding region of the *Zlp* gene as a *BglII* cassette. These oligonucleotides were used in a PCR using the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT). The template for the reaction was single-stranded phagemid DNA (the antisense strand of pMON22568) prepared by helper phage (M13KO7; Stratagene) infection. The reaction included a 3:1 ratio of 7C-deaza-dGTP (Boehringer-Mannheim) to dGTP. Reaction conditions included 1-min denaturing at 94°C, 2-min annealing at 45°C, 3-min extension at 72°C, repeated for 30 cycles, followed by 15-min extension at 72°C. Reaction product was blunted with deoxyribonucleoside triphosphates and Klenow polymerase (Promega), digested with *BglII*, and cloned into the *BglII* site of pMON7258 to give pMON22569, with the Met codon proximal to the *HindIII* site of the multiple cloning site. The product of the PCR reaction was completely verified by DNA sequencing.

The *BglII* insert of pMON22569 was subcloned into the *BglII* site of plant binary expression vector pMON977 to give pMON8936. The direction of insertion placed the Met codon

of the *Zlp* gene proximal to the CaMV 35S promoter and distal to the E9 polyadenylation region. The vector pMON977 also contains the T-DNA right border sequence, spectinomycin resistance marker, and *NptII* gene driven by the CaMV 35S promoter for selection of transformed plants. The binary vector was transferred from *E. coli* JM101 to *Agrobacterium tumefaciens* ABI by triparental mating (Horsch and Klee, 1986). The ABI strain is *A. tumefaciens* A208 carrying the disarmed pTiC58 plasmid pMP90RK (Koncz and Schell, 1986).

A. thaliana ecotype RLD was transformed by the root explant method as described previously (Valvekens et al., 1988). Seeds from primary transformants were germinated and plants were maintained as described previously (Samac and Shah, 1991). Tomato (*Lycopersicon esculentum* cv UC82b) was transformed by the leaf disc method as described previously (McCormick et al., 1986).

Genomic Southern Hybridization

Large-scale preparation of genomic DNA was performed on leaf tissue of 3-week-old *Z. mays* cv B73 seedlings (Shure et al., 1983), followed by two sequential CsCl gradient purifications. For the isolation of genomic DNA from transgenic plant samples, the supernatant from the first LiCl precipitation in the RNA isolation (see above) was adjusted to 0.3 M sodium acetate, and the DNA was recovered by ethanol precipitation and resuspended in water.

Small-scale genomic DNA isolated from transgenic plants was digested overnight with restriction endonucleases, electrophoresed in 0.8% agarose gels, and transferred to nylon membrane (Hybond N-plus, Amersham) by upward capillary transfer (Sambrook et al., 1989). Filters were hybridized with the *BglIII* insert of pMON22569.

ZLP Purifications

Total protein was extracted from transgenic plant leaves by grinding under liquid nitrogen, followed by homogenization in 500 mM sodium acetate, pH 5, using a Polytron PTA 20S probe (Brinkmann). Extracts were clarified by centrifugation, followed by filtration through Miracloth (Calbiochem, La Jolla, CA). Proteins were precipitated by adding ammonium sulfate to 75% saturation. Proteins were resuspended in 100 mM Tris, pH 7.5, insolubles were removed by centrifugation, and the sample was desalted using PD-10 columns (Pharmacia, Piscataway, NJ) to 10 mM Tris, pH 7.5, immediately prior to Mono-S cation-exchange chromatography. Chromatography was conducted by fast protein liquid chromatography on a Mono-S HR 5/5 column (Pharmacia) in 10 mM Tris, pH 7.5, with elution by NaCl gradient. ZLP purified from transgenic tomato at pH 7.5 was desalted to 20 mM sodium acetate, pH 5.6, concentrated by ultrafiltration using Centricon-3 units (Amicon, Beverly, MA), and reapplied to the Mono-S column equilibrated to the new buffer. Resolution of the full-length and truncated ZLP forms was achieved by using step isocratic elution, with an increase of 1 mM NaCl every 2 min between 40 and 50 mM NaCl.

IWF was obtained from transgenic plants by vacuum infiltration of fresh leaves with 20 mM sodium acetate, pH 5.6,

20 mM NaCl. Infiltrated tomato leaves were rolled and inserted petiole downward into empty syringes, and IWF was recovered by centrifugation of the syringes in collection tubes for 10 min at 700g. Infiltrated *A. thaliana* leaves were not oriented prior to centrifugation. IWF was concentrated by ultrafiltration in Centriprep-10 units (Amicon) and desalted using a PD-10 column (Pharmacia) to 10 mM sodium acetate, pH 5.6, immediately prior to Mono-S cation-exchange chromatography.

Proteins were quantitated using the assay of Bradford (1976) using a commercial kit (Bio-Rad) and analyzed by SDS-PAGE (Laemmli, 1970) in 15% acrylamide gels. Proteins were visualized by silver staining (Enprotech, Hyde Park, MA). Western blots were prepared by electrophoretic transfer onto nitrocellulose (Hybond C-extra, Amersham). Immunovisualization of ZLP and other TLPs on western blots was performed using IgG purified from antiserum raised in rabbit against purified *Z. mays* 22-kD protein (Huynh et al., 1992a). Immunocomplexes on western blots were visualized using enhanced chemiluminescence with donkey anti-rabbit: horseradish peroxidase conjugate as secondary antibody (donkey:horseradish peroxidase antibody and enhanced chemiluminescence kit from Amersham).

Glycosylation of ZLP was assessed using a commercially available kit (First CHOice; Boehringer-Mannheim). Glc oxidase from *Aspergillus niger* (Sigma) was used as a control glycosylated protein, and creatinase (provided with the kit) was used as a nonglycosylated control. Proteins were modified by the incorporation of digoxigenin either in solution prior to SDS-PAGE or on nitrocellulose after western blotting.

Purified ZLP was tested for chitinase and β -1,3-glucanase activity. Glucanase enzyme activity was assessed by detection of reducing sugar (Glc equivalents) released upon incubation with laminarin (Sigma) using the neocuproine colorimetric assay (Nelson, 1957). Chitinase activity was determined using the colorimetric procedure described by Boller and Mauch (1988) with minor modifications. Control hydrolases in these assays were detectable in amounts less than 10 ng. ZLP purified from transgenic plants was tested in protracted incubations in amounts as large as 1 μ g, but no activity was detected (data not shown).

Baculovirus Expression of ZLP

For baculovirus expression, the *BglIII* insert was purified from pMON22569, blunted with Klenow DNA polymerase and deoxyribonucleoside triphosphates, ligated to *NheI* linkers (New England Biolabs), and then cloned into the unique *NheI* site of p β L1 ("BlueBac," a color-selectable baculovirus expression shuttle vector; Invitrogen, San Diego, CA) with the Met codon of the ZLP gene proximal to the polyhedron promoter, to give pMON22570. Co-transfection of sf9 insect cells with pMON22570 and wild-type AcNPV DNA was performed using the MaxBac, version 1, kit (Invitrogen) according to the manufacturer's directions. Purification of recombinant (ZLP-producing and β -galactosidase-producing) virus was achieved by serial limiting dilution. Large-scale culture supernatants were harvested 4 to 7 d after infection, and total proteins were precipitated directly from serum-free culture medium (EXCEL-401; JRH Biochemicals, Lenexa, KS)

by adjusting to 75% saturation with ammonium sulfate. Precipitated proteins were resolubilized in 100 mM Tris, pH 7.5, desalted using PD-10 (Pharmacia) columns to 10 mM Tris, pH 7.5, concentrated by ultrafiltration in Centrprep-10 units, and applied to a Mono-S column (Pharmacia) for cation-exchange chromatography.

Antifungal Assays

The antifungal activity of ZLP on *Candida albicans*, *Neurospora crassa*, and *Trichoderma reesei* was tested as previously described (Vigers et al., 1991). Optimal results for the *C. albicans* inhibition assay were obtained by incorporating 2×10^4 cells/mL and 0.5 mg of nikkomycin (Calbiochem) per mL into Sabourad's dextrose agar. Inhibition of *Alternaria solani* was performed as previously described (Huynh et al., 1992a, 1992b). Commercially available thaumatin (Sigma) was used as an additional control for antifungal testing. Thaumatin was resuspended in 10 mM sodium acetate, pH 5.6, desalted by gel filtration to this same buffer, concentrated by ultrafiltration, and then subjected to ion-exchange liquid chromatography using a Mono-S HR5/5 column (Pharmacia). The principal species recovered was identified as thaumatin by SDS-PAGE and western blot cross-reaction with antiserum to the maize seed 22-kD protein (Huynh et al., 1992a). Thaumatin was desalted by gel filtration to 10 mM sodium acetate, pH 5.2, and concentrated by ultrafiltration prior to antifungal testing.

RESULTS

cDNA Cloning and Sequence Analysis

Northern blots of RNA from various maize vegetative and seed tissues were hybridized with a heterologous TLP gene from *A. thaliana* (D.E. Malehorn and D.M. Shah, unpublished data). A potential message was observed in poly(A⁺) RNA but not in nonpolyadenylated RNA from developing seed (data not shown). Poly(A⁺) mRNA was isolated from whole seed 27 d after pollination and used to generate a cDNA library in λ gt10. Insert cDNA was size selected by preparative electrophoresis to between 0.5 and 2 kb. This library was screened with the *Arabidopsis* TLP gene as a probe. Twelve independent strongly hybridizing isolates were plaque purified. The *Eco*RI inserts of 11 isolates were subcloned into pUC118. Six isolates had inserts of 550 to 700 bp, and five had inserts of approximately 900 bp. Seven isolates were sequenced from both ends, and all were derived from the same sequence. Because the sequence encodes a protein distinct from the previously characterized maize TLPs, the gene has been designated *Zlp* for "zeamatin-like protein."

One representative clone was completely sequenced in both strands. The 957-bp insert contained 28 bp of 5' non-translated leader (including the *Eco*RI linker), a 681-bp open reading frame, followed by a single stop codon, and 245 bp of 3' nontranslated sequence (including a 24-residue polyadenylate "tail" and the *Eco*RI linker). The coding region of the *Zlp* gene is 71% G+C overall, but the G+C content in the third (or wobble) position of degenerate codons is 96%; 13 amino acids are encoded solely by codons with G+C in the wobble position.

The cDNA encodes a 227-amino acid preprotein of 23.998 kD. The presence of a 21-amino acid signal peptide is inferred by alignment to other TLPs (Fig. 1). The mature protein has a predicted molecular mass of 22.1 kD. The calculated isoelectric point is 9.1. The *Zlp* cDNA encodes a protein nearly identical with, but distinct from, the maize bifunctional inhibitor MAI (Richardson et al., 1987), zeamatin (Roberts and Selitrennikoff, 1990), and maize seed 22-kD antifungal protein (Huynh et al., 1992a). The encoded protein is hereinafter referred to as ZLP.

The ZLP amino acid sequence differs from MAI at six residues; similar homology is expected to zeamatin itself, which has been reported to be identical with MAI through the 60 amino-terminal residues (Vigers et al., 1991). Three of the disparities between ZLP and MAI (Leu⁹¹→Val; Leu¹²¹→Ile; Ile¹²²→Leu) are conservative and maintain hydrophobic-

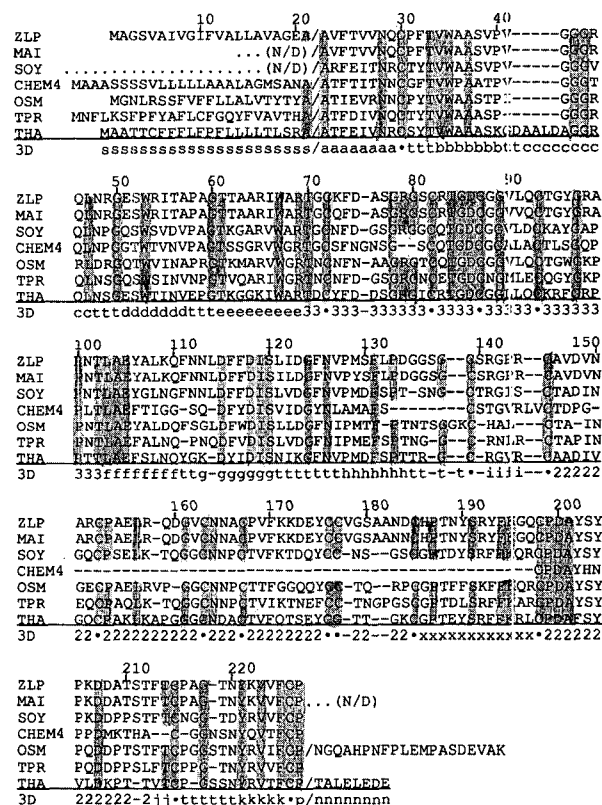


Figure 1. Comparison of the amino acid sequence of ZLP deduced from the nucleotide sequence of *Zlp* cDNA clone (accession number U06831) with representative TLPs. ZLP, maize seed zeamatin-like protein; MAI, maize seed α -amylase/trypsin inhibitor (Richardson et al., 1987); SOY, soybean leaf P21 (Graham et al., 1992); CHEM4, maize leaf stress protein (Frendo et al., 1992); OSM, tobacco osmotin (Singh et al., 1989); TPR, tobacco leaf PR5 protein (van Kan et al., 1989); THA, thaumatin (Edens et al., 1982). The bottom line ("3D") relates structural elements and their approximate position from the tertiary structure of thaumatin, determined by x-ray crystallography (De Vos et al., 1985; Ogata et al., 1992). s, signal peptide; a, b, c, d, e, f, g, h, i, j, k, l, β strands of the β barrel; t, turns; •, Cys residues; x, α helical region; p, carboxy terminus; n, carboxy-terminal extension; N/D, not determined (i.e. no preprotein sequence available).

ity of these regions. Three disparities between ZLP and MAI are nonconservative: Lys⁷³→Gln; Met¹²⁹→Tyr; and Asp¹⁸⁴→Asn. Met at position 129 is a strong consensus for TLPs. Lys at position 73 and aspartate at position 184 of ZLP are not consistent with other TLPs, but these fall within regions highly diverged among TLPs.

Homology of ZLP peptide sequence with several representative TLPs is illustrated in Figure 1. Except MAI, ZLP is most homologous (67% identity/81% similarity) to soybean P21 (Graham et al., 1992). Slightly less similarity is shown (61% identical/76% similar) to both acidic and basic TLPs from tobacco (van Kan et al., 1989). Homology with acidic, truncated TLPs from *Z. mays* (Frendo et al., 1992) is only 54% identity/67% similarity. Such nucleic acid divergence between TLP homologs from the same plant species is not uncommon: in tobacco, osmotin (Singh et al., 1989) and PR5 (van Kan et al., 1989) are 63% identical; in *A. thaliana*, PR5 (Uknes et al., 1992) and an acidic homolog (D.E. Malehorn and D.M. Shah, unpublished data) are 54% identical.

The ZLP peptide sequence is 58% identical with, and 73% similar to, thaumatin I, for which the tertiary structure has been determined (De Vos et al., 1985; Ogata et al., 1992). All 16 Cys residues, which participate in eight disulfide bonds to stabilize the external loops of thaumatin, are conserved in ZLP as they are in all full-length TLPs.

The ZLP sequence predicts an Asn-linked glycosylation site at residue 189; however, the ZLP expressed and purified from all heterologous systems and repurified from *Z. mays* was found not to be glycosylated (data not shown).

Southern Analysis of *Z. mays* Genomic DNA

Southern blots of *Z. mays* B73 genomic DNA were probed with the entire *Zlp* cDNA (Fig. 2). Several restriction digests gave multiple hybridizing fragments under low-stringency rinse conditions (1× SSPE [0.15 M sodium chloride, 0.01 M

sodium phosphate, 0.001 M EDTA, pH 7.4], 60°C). Upon higher stringency of rinsing, a single restriction fragment became predominant. The smallest unique restriction fragment to which the *Zlp* gene could be ascribed in this manner was a 2.6-kb *Xba*I fragment. Weakly hybridizing fragments may harbor genes encoding other related TLPs, such as the *Chem4* gene encoding a small acidic TLP (Frendo et al., 1992), which shares only 57% nucleic acid homology to *Zlp* gene.

Expression of *Zlp* mRNA in *Z. mays*

Expression of *Zlp* mRNA in *Z. mays* was examined using the entire *Zlp* cDNA as a probe. At low stringency of rinsing (1× SSPE, 60°C) mRNA was detected in a variety of tissues, including root, leaf, and various tissues of developing, desiccated, and germinated seeds (Fig. 3). When the stringency of rinses was increased, the signal from developing seed tissues and from leaf remained stable, but the signals from root and seed scutellum that had undergone imbibition were noticeably diminished. Authentic *Zlp* mRNA expression was predominant in nonembryo tissues of developing seed. Expression was detected by 2 weeks after pollination but was strongly induced by 3 weeks and peaked at 4 weeks after pollination. The *Zlp* mRNA level declined slightly through the final 4 weeks of seed development (data not shown), and full-length *Zlp* mRNA could still be detected in desiccated seeds stored at 4°C for several years. Larger 2.4- and 3.8-kilobase transcripts detected in poly(A⁺) mRNA from nonembryo tissues of developing seed have not yet been characterized; it is unknown whether this reflects the accumulation of unspliced *Zlp* mRNA precursors during seed desiccation.

Western Analysis of ZLP Expression in *Z. mays*

To correlate observed seed expression of *Zlp* mRNA with protein expression, whole seeds at various developmental ages were ground in low-strength salt buffer and homogenates were analyzed by SDS-PAGE (Fig. 4A). Western blots were analyzed using antiserum against the maize seed 22-kD TLP (Huynh et al., 1992a) (Fig. 4B). Expression of ZLP becomes detectable at 3 weeks postpollination, approximately 1 week after the earliest *Zlp* mRNA expression (Fig. 3). Dissection of 27-d-old seed into embryo and nonembryo tissue indicates partitioning of ZLP with the nonembryo (endosperm, aleurone, pericarp) tissues, which correlates with northern blot data. Although northern blots indicate a maximal steady-state level of *Zlp* mRNA at 4 weeks postpollination, ZLP continued to accumulate throughout 6 weeks of development and was maximal in fully desiccated seeds. Germination of seed did not appreciably increase this level. The ZLP constitutes a readily detectable proportion of total protein in desiccated seed and was quantitated to represent approximately 1% of total protein extracted (data not shown).

Expression of ZLP in leaves was only faintly detectable by western blot, and expression was slightly induced by salicylate and ethephon. A cross-reactive protein of approximately 25 kD was induced by mercuric chloride treatment of seedling leaves; salicylate and ethephon treatments induced this species to a very limited extent. A smaller cross-reactive TLP

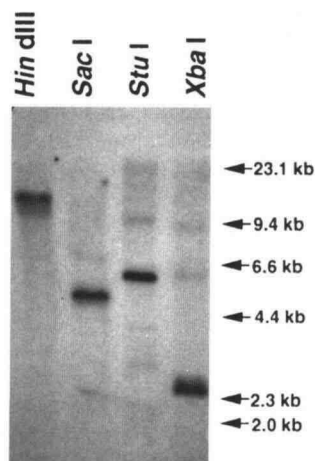


Figure 2. Southern blot of maize genomic DNA for the *Zlp* gene. Genomic DNA (10 µg/lane) digested with *Hind*III, *Sac*I, *Stu*I, or *Xba*I was blotted onto a nylon membrane and hybridized with the entire *Zlp* open reading frame as described in "Materials and Methods." Autoradiographic exposure was for 2 d with intensifying screens. The migration of molecular size markers is indicated by arrows.

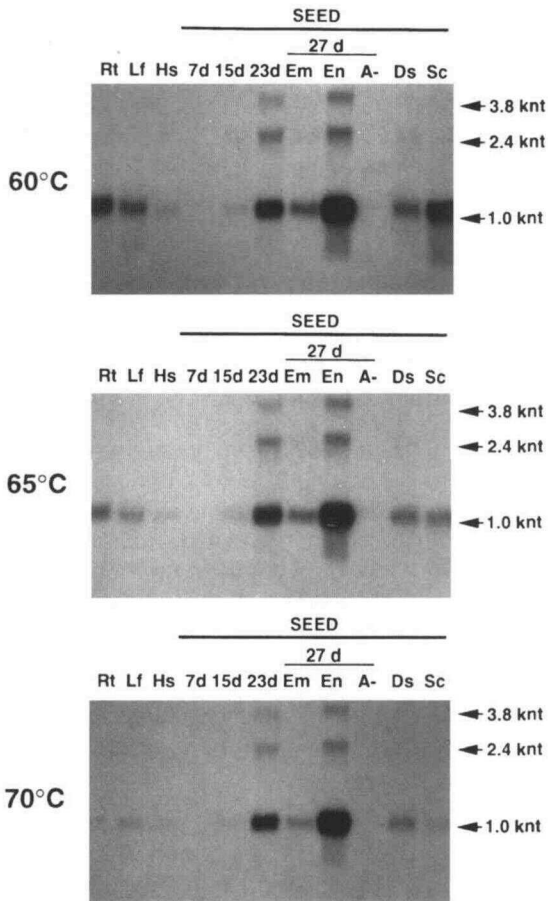


Figure 3. Northern blot of maize tissues for *Zlp* mRNA, with increasing stringency of rinse conditions. Rt, Root; Lf, leaf; Hs, husk. For seeds, 7d, whole seed 7 d postpollination (pp); 15d, whole seed 15 d pp; 23d, whole seed 23 d pp; 27d Em, embryo tissue only, 27 d pp; 27d En, "endosperm" (all nonembryo tissue) 27 d pp; 27d A-, whole seed nonpolyadenylated RNA 27 d pp; Ds, fully desiccated whole seed; Sc, scutellum of seed that underwent imbibition. The migration and three sizes of *Zlp*-related species is indicated with arrows, with sizes given in kilonucleotides (knt). Northern blots were hybridized and washed sequentially in 0.1× SSC at increasing temperature as described in "Materials and Methods."

species of approximately 14 kD from mercuric chloride-treated leaves may represent the product of the Chem4 gene (Frendo et al., 1992). Both the larger and smaller cross-reactive proteins co-purified as acidic proteins during Mono-Q anion-exchange chromatography (data not shown).

Expression of ZLP in Transgenic Plants

The coding region of *Zlp* gene (including the secretion signal peptide sequence; see above) was subcloned into plant binary expression vector pMON977. In the final construct, pMON8936, transcription of the coding sequence is under the control of the CaMV 35S promoter. Transformation of *A. thaliana* root explants resulted in recovery of five regenerant lines that yielded sufficient seed for propagation and testing.

Of these, two lines yielded ratios of kanamycin-resistant and -sensitive progeny in the R_2 generation that were consistent with single-gene integration events. Southern and western blots revealed that homozygous R_3 progeny of both lines harbored single *EcoRI* fragments containing the *Zlp* gene and that both lines expressed abundant material cross-reacting with anti-ZLP antiserum (data not shown). One line was arbitrarily chosen for purification of transgene-expressed ZLP.

ZLP constitutes a major protein species in total protein extracts of transgenic *A. thaliana* leaf tissue (Fig. 5A). The relative abundance of ZLP is even more pronounced in the IWF, where it constituted up to 20% of recoverable protein. The identity of the major protein species as ZLP is supported by its reaction in western blots (Fig. 5B) with antiserum against the maize seed 22-kD TLP (Huynh et al., 1992a). Smaller cross-reactive species in IWF may represent degradation products of ZLP. Cross-reaction of anti-ZLP antiserum with endogenous *A. thaliana* TLP homologs (Uknes et al., 1992; D.E. Malehorn and D.M. Shah, unpublished data) is demonstrated in ethephon-treated leaf tissue: the endogenous TLP(s) has a slightly greater mobility than ZLP. Express-

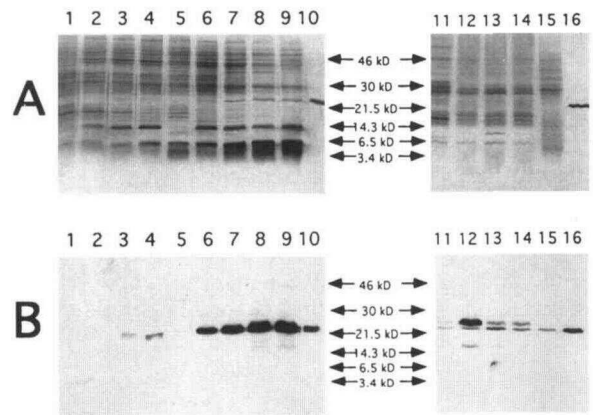


Figure 4. Western blot survey for ZLP expression. Tissue samples from various vegetative and seed tissues were homogenized with an overhead stirrer in 20 mM sodium acetate, pH 5.2, clarified by microcentrifugation, and supernatant proteins were boiled in sample buffer prior to SDS-PAGE. A, Silver-stained SDS-PAGE gels of total proteins, 5 mg/lane. Lane 1, Whole seed 7 d postpollination (pp); lane 2, whole seed 15 d pp; lane 3, whole seed 23 d pp; lane 4, whole seed 27 d pp; lane 5, embryo only, 27 d pp; lane 6, whole seed 34 d pp; lane 7, whole seed 44 d pp; lane 8, fully desiccated whole seed; lane 9, whole germinated seed; lane 10 and 16, 100 ng of purified ZLP (from transgenic *A. thaliana*); lane 11, water-sprayed leaf of 2-week-old seedlings; lane 12, mercuric chloride-sprayed leaf; lane 13, salicylate-sprayed leaf; lane 14, ethephon-sprayed leaf; lane 15, root of 2-week-old seedlings. B, Western blots of the same samples. Lanes 10 and 16, Ten nanograms of purified ZLP only. Western blots were reacted with rabbit antiserum against maize seed 22-kD protein (Huynh et al., 1992a), nearly identical with ZLP. Secondary antiserum was donkey anti-rabbit, labeled with horseradish peroxidase. Visualization of immunocomplexes was performed using enhanced chemiluminescence (ECL; Amersham). Migration and sizes of molecular mass markers are indicated by arrows.

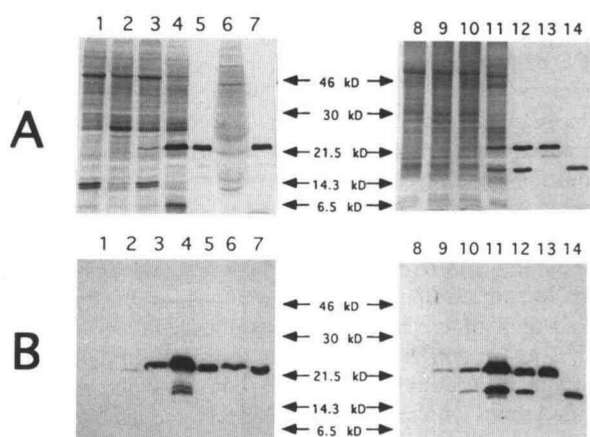


Figure 5. Purification of heterologously expressed ZLP. A, Silver-stained SDS-PAGE gels. Lane 1, Five milligrams of total protein from healthy wild-type *A. thaliana* leaf; lane 2, 5 mg of total protein from ethephon-treated wild-type *A. thaliana* leaf; lane 3, 5 mg of total protein from Zlp-transgenic *A. thaliana* leaf; lane 4, 5 mg of total protein from IWFs of Zlp-transgenic *A. thaliana* leaf; lane 5, 1 mg of purified ZLP from Zlp-transgenic *A. thaliana* leaf; lane 6, 5 mg of total protein from recombinant baculovirus-infected insect cells, culture supernatant. Lane is somewhat distorted by culture medium components. Lane 7, One milligram of purified ZLP from baculovirus expression system; lane 8, 5 mg of total protein from healthy wild-type *L. esculentum* (tomato, cv UC82B) leaf; lane 9, 5 mg of total protein from senescent and virus-diseased wild-type tomato leaf; lane 10, 5 mg of total protein from Zlp-transgenic tomato leaf; lane 11, 5 mg of total protein from IWFs of Zlp-transgenic tomato leaf; lane 12, co-purified 22- and "17-kD" ZLP proteins from Zlp-transgenic tomato leaf (the apparent 17-kD form is in fact a proteolytically nicked 22-kD form; the reduced 5-kD carboxy-terminal fragment has run off of the gel. See text for details). Lane 13, Resolved intact ZLP from Zlp-transgenic tomato leaf; lane 14, resolved nicked ZLP from Zlp-transgenic tomato leaf. The reduced 5-kD carboxy-terminal fragment has run off of the gel. B, Western blots of the same samples. Loadings are identical except for lanes 5, 7, 12, 13, and 14, in which only 100 ng of pure protein was loaded. Antiserum against maize seed 22-kD protein (Huynh et al., 1992a) was used, and visualization used horseradish peroxidase-linked donkey anti-rabbit antiserum, followed by enhanced chemiluminescence. Migration and sizes of molecular mass markers are indicated by arrows.

sion of the endogenous homolog(s) in healthy nontransgenic leaves, grown under conditions identical with the transgenic plants, was undetectable.

Transgene-expressed ZLP was purified from total leaf protein using cation-exchange chromatography. Amino-terminal peptide sequence (AVFTVVNQXPFTVWA; X presumed to be Cys) confirmed the identity of the purified protein as ZLP; the endogenous *A. thaliana* TLP homologs have distinctly different amino-terminal sequences (Uknes et al., 1992; D.E. Malehorn and D.M. Shah, unpublished data). Amino-terminal sequence also confirmed the proper cleavage of the maize signal peptide in this heterologous system.

The same plant transformation vector was used to transform tomato. Of more than 20 primary regenerant lines, five lines yielded sufficient seed for propagation and testing. All five lines expressed significant amounts of ZLP as judged by

western blots of total leaf proteins (data not shown). The line arbitrarily chosen for further purification of ZLP was ultimately shown to harbor two *EcoRI* fragments bearing the Zlp gene, indicating integration of more than one copy of the construct (data not shown).

Expression of ZLP in transgenic tomato leaf does not constitute a readily identifiable band among total leaf proteins analyzed by SDS-PAGE (Fig. 5A). However, ZLP is greatly enriched among the IWF proteins: western blots indicate the presence of the full-length ZLP and a strongly cross-reactive 17-kD protein (Fig. 5B). This smaller protein was likely not an endogenous TLP homolog, since total leaf proteins from healthy nontransgenic leaves had no detectable cross-reaction and even diseased nontransgenic leaves produced only a single cross-reactive species of 22 kD.

Both the 22-kD and the apparent 17-kD cross-reactive proteins co-eluted during ion-exchange chromatography at pH 7.6. However, when the mixed sample was adjusted to 20 mM sodium acetate, pH 5.6, and rechromatographed in this buffer, two peaks were resolved. The first peak consisted of a 22-kD protein with amino-terminal peptide sequence AVFTVVNQXPFTVWA (X presumed to be Cys), which confirmed the identity of this protein as ZLP as well as the correct cleavage of the maize signal peptide. The second peak yielded two discernible amino-terminal peptide sequences in equimolar amounts: AVFTVVNQXPFTVWA (as above) and ANDXHPTNYSRYFKG (X presumed to be Cys). The second sequence corresponds to ZLP residues 182 to 196. This result indicates that the 17-kD species is in fact a proteolytically cleaved derivative of ZLP: cleavage occurs after Ala¹⁸¹, but the carboxyl-terminal 5-kD fragment remains associated throughout purification, presumably by virtue of four original intramolecular (now intermolecular) disulfide bonds. This "nicked" ZLP was formerly identified as a 17-kD species because the reduced 5-kD fragment was not detected during routine SDS-PAGE. In higher percentage acrylamide gels, both 17- and 5-kD species were detected from the preparation of nicked ZLP; in addition, the smaller fragment was also cross-reactive in western blots (data not shown), indicating at least one recognizable epitope involving the last 46 residues of ZLP. The yield of "intact" to nicked ZLP was approximately 2:1, indicating that about one-third of all ZLP molecules recovered from tomato are cleaved between Ala¹⁸¹ and Ala¹⁸².

Antifungal Activity of ZLP

Heterologously expressed ZLP was tested for inhibitory activity against several fungi previously reported to be inhibited by corn seed TLPs (Roberts and Selitrennikoff, 1990; Huynh et al., 1992a). Paper discs were impregnated with 10 μ g of purified ZLP in <50 mL of 20 mM sodium acetate, pH 5.6. For inhibition testing of filamentous fungi (*T. reesei*, *A. solani*, and *N. crassa*), these discs were placed onto agar plates with hyphae-containing agar plugs at their center. Inhibition is indicated by occlusion of hyphal growth from the region beyond the disc. Discs impregnated with buffer only had no effect on outward growth of fungal hyphae.

Purified ZLP from all sources (insect cells, *A. thaliana*, and tomato, both nicked and intact) showed comparable inhibition at the 10- μ g level (Fig. 6) Inhibition by smaller amounts

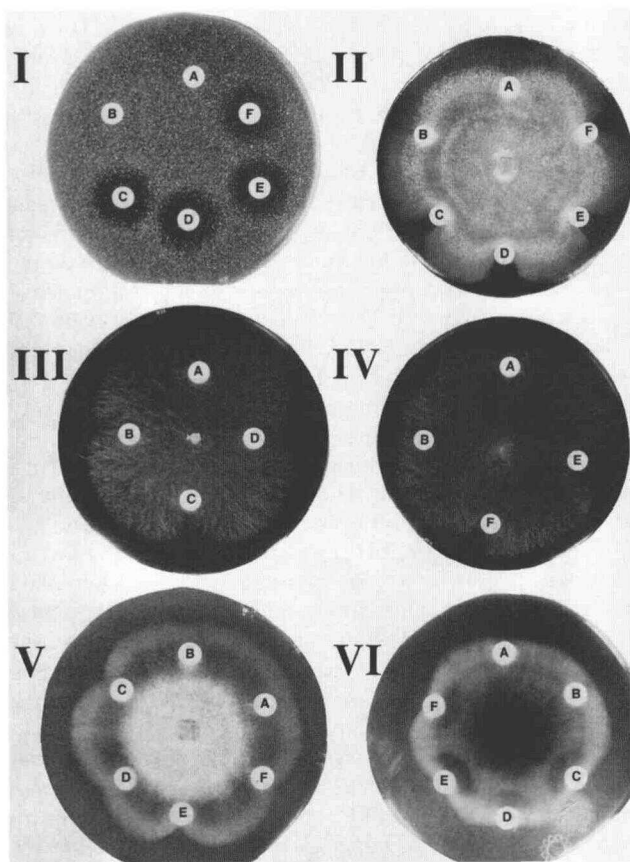


Figure 6. Fungal inhibition by purified ZLP. For all panels, A, 50 mL of 20 mM sodium acetate, pH 5.2 (protein samples on all other discs are in $<50 \mu\text{L}$ of this same buffer); B, 10 μg of purified thaumatin; C, 10 μg of purified ZLP from transgenic *A. thaliana*; D, 10 μg of purified ZLP from baculovirus expression system; E, 10 μg of purified intact ZLP from transgenic tomato; disc F, 10 μg of purified nicked ZLP (proteolytic cleavage after residue ala181). I, Inhibition of *C. albicans* in an agar pour plate containing 0.5 mg of nikkomycin/mL. Inhibition is indicated by clear halos where outward growth of embedded cells was prevented. II, Inhibition of hyphal growth of *T. reesei*. Inhibition is indicated by zones beyond discs where outward growth of hyphae is retarded. III and IV, Inhibition of hyphal growth of *N. crassa*. Transitory zones of inhibition relative to the controls were observed as in II, but hyphae penetrated these areas within several hours. V, Slight inhibition of *A. solani* hyphal growth, upper view. Outward growth of hyphae was only slightly retarded by ZLP, relative to controls. VI, Lower view of the same plate. Zones around ZLP discs promote premature darker pigmentation at the bottom of the fungal mat. For all panels, boiling of ZLP for 10 min destroyed all traces of fungal inhibition.

of ZLP was difficult to demonstrate unambiguously. Inhibition of *N. crassa* was transitory, and hyphal growth invaded the inhibition zone within several hours. In addition to slight *A. solani* hyphal inhibition, ZLP caused a darkening of fungal pigmentation in the inward portion of the disc zone; the cause of this pigmentation, and whether it is an indication of stress on the microorganism, is unknown.

In all cases, inhibitory activity of ZLP was completely destroyed by boiling. Commercially available thaumatin, re-

purified using conditions identical with those used to isolate ZLP, demonstrated no inhibitory activity against any organism.

Impregnated discs were also placed on agar pour plates containing viable *C. albicans* cells and subinhibitory concentrations of nikkomycin, a chitin synthase inhibitor. Inhibition resulted in the formation of a circular zone around the discs in which growth of the embedded cells was prevented. Comparable inhibition was demonstrated by ZLP purified from all sources (insect cells, *A. thaliana*, and tomato, both nicked and intact). Inhibition could be demonstrated using only 2 μg of purified ZLP, but a lower threshold was not determined. Inhibition was destroyed by boiling. Again, re-purified thaumatin showed no inhibitory activity, even when tested at the 100- μg level.

DISCUSSION

A heterologous TLP gene from *A. thaliana* was successfully used to identify a TLP cDNA clone (*Zlp*) from a maize mid-development seed cDNA library. The clone characterized is nearly identical with, but distinct from, the previously reported peptide sequences of MAI protein (Richardson et al., 1987), zeamatin (Roberts and Selitrennikoff, 1990), and antifungal 22-kD maize seed protein (Huynh et al., 1992a). Despite the differences in peptide sequence, the heterologously expressed and repurified ZLP did retain the previously described antibiotic activities reported for zeamatin and the maize seed 22-kD antifungal protein. Antifungal activity of heterologously expressed ZLP was not due to inadvertent copurification with hydrolases: colorimetric assays for chitinase and glucanase, sensitive to nanogram amounts of control hydrolases, were negative for ZLP preparations (data not shown); silver staining of ZLP preparations on SDS-PAGE revealed no discernible contaminants; and amino-terminal peptide sequencing of purified ZLP revealed no detectable secondary sequence.

Despite the near identity with MAI, purified ZLP did not prove to be an effective inhibitor of bovine trypsin, relative to a Bowman-Birk inhibitor; the effect of ZLP on trypsin hydrolysis was not substantially different from that of an inert control protein (data not shown). Details of MAI purification or trypsin inhibition have yet to be published; therefore, no direct comparison with ZLP could be made. It should be noted, however, that three of the six differences between ZLP and MAI reduce the homology noted between MAI and barley proteinase inhibitor and ragi amylase inhibitor (Richardson et al., 1987).

Expression of ZLP was observed primarily in seed but also to some extent in stressed leaf tissue. This would entail some overlapping aspects of seed developmental expression and leaf PR expression. This is reminiscent of the expression pattern of other basic TLP homologs, such as tobacco osmotin, which has been shown to be induced (at the mRNA level) not only in response to pathogenesis but also in response to developmental signals in roots, floral organs, and seeds (Singh et al., 1987; Memelink et al., 1990; Stintzi et al., 1991; LaRosa et al., 1992); in the case of osmotin, however, two gene copies are present that may differ in their regulation (Singh et al., 1989). Although the *Zlp* gene was shown to

reside on a relatively small *Xba*I fragment, we have not tested the possibility of multiple reiterations of this fragment in the maize genome, bearing differently expressed/regulated isoforms. Differential regulation of nearly identical isoenzymes of PR2-related barley glucanase has been demonstrated (Slakeski and Fincher, 1992).

The *Zlp* cDNA sequence predicts a basic, secreted TLP. This conflicts with the prevailing notion from dicots that acidic PR proteins, induced by stress, are secreted and that basic PR protein homologs, expressed in response to developmental signals, are retained intracellularly (Memelink, 1990). The distinction between acidic and basic TLPs, with regard to induction and localization, is becoming blurred as more homologs are described. Acidic TLPs have been identified in leaves in response to abiotic and biotic stresses (Bol and Linthorst, 1990; Dixon and Harrison, 1990; Linthorst, 1991), but they have also been purified from healthy seeds (Bryngelsson and Green, 1989). Basic TLPs are expressed in healthy roots, floral organs, and seeds (Singh et al., 1987; Huynh et al., 1992a; Nelson et al., 1992), but they have also been shown to be a component of pathogenesis response in leaves (King et al., 1988; Singh et al., 1989; Rodrigo et al., 1991; Stintzi et al., 1991).

The presence of a signal peptide and the absence of a carboxy-terminal extension suggest that ZLP is targeted to the extracellular space rather than the vacuole (Bednarek et al., 1990; Neuhaus et al., 1991), although retention via a peptide sequence internal to the mature protein is possible (Chrispeels and Tague, 1991). Although the compartmentalization of native ZLP in maize seed or leaves has not yet been examined, expression experiments in heterologous systems have indicated that ZLP can be exported from plant cells and insect cells. Amino-terminal peptide sequencing proved that in all cases the signal peptide was correctly processed. Although the general recognition and function of the ZLP secretion signal is permitted in these heterologous systems, recognition of an internal retention signal (if present) may not be possible if the recognition system is specific to *Z. mays*.

The high level of constitutive secretion of the basic ZLP protein in vivo (up to 20% of total extractable protein in leaf IWF) did not produce any apparent phenotypic change in growth, habit, or reproduction; in highly expressing plant lines, the level of ZLP expression was stable through three generations tested in tomato and four generations tested in *A. thaliana*. High levels of expression of ZLP were obtained despite a dramatic codon bias: in nearly 100% of degenerate codons, G or C is preferred in the wobble position. Such bias, also observed for monocot seed β -1,3-glucanases (Fincher, 1989; Simmons et al., 1992), has prompted speculation that codon bias might be a mechanism by which accumulated seed mRNAs could be rapidly translated upon germination, if isoaccepting tRNAs are correspondingly abundant. However, ZLP expression is highest in seeds prior to desiccation and is not appreciably induced upon germination.

The high steady-state level of ZLP expression that was observed in transgenic plants may be in part due to extreme stability of this protein; slow turnover would allow accumulation despite (potentially) slow kinetics of translation. The stability of heterologously expressed ZLP was remarkable:

bioactive intact ZLP could be recovered from insect cell culture supernatants that had been stored at 8°C more than 6 months. Bioactive intact ZLP could also be purified from senescent, even necrotic detached leaf tissue from transgenic plants. The apparent modest increase of ZLP levels in germinated seeds (from 1 to 2% of total extractable protein) may be due simply to the resistance of ZLP to degradation, relative to other catabolized seed proteins.

A proportion of ZLP expressed in transgenic tomato was shown to experience a secondary proteolytic cleavage. The mechanism of this cleavage is unknown, but it is tempting to invoke signal peptidase: the secondary cleavage site is between Ala residues, which is the consensus signal peptide cleavage site for TLPs. If the tertiary structure of ZLP resembles that of thaumatin, this site may be situated on an exposed loop of domain II, which could promote its recognition as a cryptic signal peptide cleavage site. Regardless of the means by which ZLP was cleaved in transgenic tomato, both the intact and nicked ZLP retained undiminished antifungal activity.

The description and expression of a *Z. mays* basic TLP gene has provided many useful tools for the examination of molecular, cellular, and plant biological questions. Heterologous expression of ZLP has confirmed reports of fungal inhibition, but it has cast doubt on the previous reports of protease inhibition. The success of transgenic plant engineering with the *Zlp* gene has provided useful information about heterologous expression of monocot genes despite a marked codon bias. The availability of stable transgenic tomato lines expressing high levels of ZLP will enable in vivo testing for resistance to *A. solani* and possibly other pathogens. The description of the *Zlp* cDNA will lead to the recovery of the *Zlp* genomic gene and promoter, which may provide a useful tool for comparing PR and developmentally regulated expression of TLPs. The availability of structural information about thaumatin makes ZLP a potential target for biorational engineering to study the mode of TLP antifungal action; ZLP might likewise provide a secreted, stable proteinaceous backbone for the expression of entirely unrelated bioactive domains.

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