A Biochemical and Molecular Characterization of LEP1, an Extensin Peroxidase from Lupin*

Nicholas J. Price‡§, Carla Pinheiro‡, Claudio M. Soares‡, David A. Ashford¶, Cândido Pinto Ricardo‡ and Phil A. Jackson‡**

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An analysis of apoplastic extensin cross-linking activity in vegetative organs of Lupinus albus indicated that leaves contain the highest specific activity. Assays of peroxidases fractionated from this material demonstrated that this activity could be largely attributed to a soluble and apoplastic 51-kDa peroxidase, denoted LEP1. Relative to other purified peroxidases, LEP1 demonstrates high extensin cross-linking activity and can be classified as an extensin peroxidase (EP). Optimal conditions for the in vitro oxidation of other phenolic substrates included 1.5–3.0 mM peroxide at pH 5.0. EP activity of LEP1 was low under these conditions but optimal and substantially higher with 100 µM peroxide and neutral pH, suggesting that physiological changes in pH and peroxide in muro could heavily influence the extensin cross-linking activity of LEP1 in vivo. Analysis of LEP1 glycans indicated 11–12 N-linked glycans, predominantly the heptasaccharide Man₃XylFucGlcNAc₂, but also larger structures showing substantial heterogeneity. Comparative assays with horseradish peroxidase isomorph C and peanut peroxidases suggested the high level of glycosylation in LEP1 may be responsible for the high solubility of this EP in the apoplastic space. A full-length cDNA corresponding to LEP1 was cloned. Quantitative reverse transcriptase-PCR demonstrated LEP1 induction in apical portions of etiolated hypocotyls 30–60 min after exposure to white light, prior to the onset of growth inhibition. Comparative modeling of the translated sequence indicated an unusually unstructured equatorial cleft across the substrate access channel, which might facilitate interaction with extensin and confer higher EP activity.

Extensin is a structural protein belonging to the family of hydroxyproline-rich glycoproteins (HRGPs)1 and a major component of dicot cell walls. This extracellular protein is typically 60–90 kDa, consisting largely of repeated sequences containing Ser(Hyp)₄–₆ motifs flanked by short stretches rich in Lys, Tyr, and His. Circular dichroism studies indicated that the extensin polypeptide is in a polyproline (II) conformation (1), and scanning electron microscopy of extensins has demonstrated an extended rod-like shape up to 100 nm in length (1, 2). This suggests that individual extensin molecules could span across cellulose microfibrils, which have been measured to be spaced 20–40 nm apart in primary cell walls (3).

Secreted extensin is covalently cross-linked to form an extensin network that intercalates with the cellulose-pectin networks. It is further associated with the cell wall structure through ionic links with acidic pectins. A covalent link between extensin and pectin has also been reported (4). The deposition of the extensin network is considered to be important during development for correct primary wall assembly (5, 6), the definition of cell morphology (7), the regulation of cell elongation (8, 9), embryogenesis (10), the formation of tissue load-bearing characteristics (11), cell wall adaptation to mechanical stress (12, 13), and for maintenance of cell wall integrity during fruit ripening (14). In addition, several studies (15–17) have indicated an important role for extensin in cell wall defensive responses.

Although cross-linking of extracellular HRGPs in Volvox carteri appears to be autocatalytic (18), the formation of the extensin network in higher plants requires peroxide and peroxidase (1). Class III peroxidases can oxidize a variety of physiological substrates and, in general, demonstrate little substrate specificity, making it difficult to attribute a specific function to individual peroxidases in vivo. The low substrate specificity of peroxidases with small aromatic substrates can be related to the wide open substrate access channel (SAC) and the absence of substrate-specific docking sites. Crystallographic studies of an HRP-C-ferulic acid complex demonstrated that this natural aromatic substrate binds proximal to the catalytic Arg-38 (19). Although natural substrates, bound benzhydroxamic acid (20) and Tris (21) can also be located at this site. The coordination of small aromatic substrates to this site within the open SAC appears to be mediated by hydrophobic interactions of the substrate with hydrophobic residues of the channel. Site-directed mutagenesis of HRP-C has demonstrated that Phe-179 is critical for efficient complex

horseradish peroxidase isomorph C; Px, peroxidase; RT, reverse transcriptase; RACE, rapid amplification of cDNA ends; PDB, protein data bank; PNP, pea isoform peroxidase; SBP, soybean seedcoat peroxidase; Hyp, hydroxyproline; LEP1, lupin extensin peroxidase 1; CTPP, C-terminal propeptide.

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‡ Present address: Dept. of Botany and Plant Sciences and the Center for Plant Cell Biology, 2190 Batchelor Hall, University of California, Riverside, CA 92521.

§ Present address: Dept. of Botany and Plant Sciences and the Center for Technologica Quimica e Biologica, Apartado 127, 2781-901 Oeiras, Portugal, § Research and Analytical Glycobiology, Department of Biology, University of York, P. O. Box 373, York YO10 5YW, United Kingdom, and ‡ Instituto de Agronomia, Tapada da Ajuda, 1349-017 Lisbon, Portugal.

1 The abbreviations used are: HRGPs, hydroxyproline-rich glycoproteins; LEP1, lupin extensin peroxidase 1; CTPP, C-terminal propeptide.

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formation with small, aromatic substrates (22). However, the coordination of large macromolecules such as extensin to within the required distance from the substrate-binding site might impose additional structural restraints in areas bordering or further removed from the SAC.

Several studies have demonstrated that the capacity to efficiently cross-link extensin is not a property of class III peroxidases in general but is limited to particular peroxidases, referred to as extensin peroxidases (EPs) (8, 23–27). Although these peroxidases may be capable of oxidizing other phenolic substrates in vitro (24), it seems reasonable to assume that EPs, rather than other class III peroxidases, are responsible for the catalysis of essential extensin deposition in vivo.

Only a few EPs have been purified to date (8, 24, 27), and they remain to be characterized in depth. In previous work we described LEP1, an EP in lupin which is associated with white light inhibition of etiolated hypocotyl growth (8). This same peroxidase also appears to play a part in defensive responses to fungal infection and UV irradiation (28). In this report we present the purification and characterization of this peroxidase as well as the first primary structure of an EP. Comparative modeling was utilized to predict the three-dimensional structure of LEP1, and we discuss structural aspects that could be related with its high capacity for extensin cross-linking.

**EXPERIMENTAL PROCEDURES**

**Preparation of Lupin Tissue and Organ Infiltrates—**Fifteen-day-old lupin plantlets were grown in the light or dark as described previously (29). Roots and etiolated and light-grown hypocotyl segments were obtained from the apical 3 cm of organs after removal of the apical meristem. Leaves of 2–3 cm were utilized. Shoot apical meristems were isolated by defoliation of the meristem under a bifocal microscope and isolation of the apical 1–2 mm. The apical 1–2 mm of roots provided the source of root apical meristems. Where necessary, plant material was sectioned to smaller segments to facilitate subsequent vacuum infiltration. All material was washed in 20 mM sodium phosphate (pH 6.8) and, unless stated otherwise, vacuum-infiltrated with the same buffer containing 1 mM KCl. Infiltrates were collected by centrifugation at 1,500 × g for 10 min at 4 °C through a 0.45-μm ultrafilter (Nalgene, Hereford, UK). Infiltrates were concentrated and equilibrated in 20 mM sodium phosphate (pH 6.8) by centrifugation through a 10-kDa membrane (Vivapen 600, Gloucestershire, UK). The total protein content of infiltrates was measured by the Bio-Rad protein assay kit as per the manufacturer’s instructions.

**Purification of Soluble and Ionically Bound Apoplastic Peroxidases—**All stages of peroxidase fractionations were performed at 4 °C. For the purification of soluble, apoplastic peroxidases, 750 g of leaves were homogenized in 2× volume/mg (fresh weight) of 50 mM Tris-HCl (pH 7.2) and centrifuged at 20,000 × g for 20 min. The supernatant was concentrated to 4 ml with Diaflow® (Amicon), using PY10 membranes (Amicon).

The soluble extract was then subjected to anion exchange chromatography (DE-52, Whatman) in a 10 × 1.6-cm column (XK16, Amersham Biosciences) equilibrated with 20 mM Tris-HCl (pH 8.0) and the peroxidase-containing fraction (non-binding) eluted in the same buffer. The fraction was then subjected to concanavalin-A affinity chromatography in a 2 × 0.5-cm column equilibrated with 0.5 M NaCl, 20 mM Tris-HCl (pH 7.4) with 1 mM CaCl2, and 1 mM MgCl2. Non-glycosylated proteins were eluted with 40 ml of equilibration buffer and the peroxidase-containing fraction in 20 ml of 35 mM α-D-mannopyranose in equilibration buffer. This fraction was then subjected to cation exchange chromatography (Mono-S, Amersham Biosciences) in a 0.5 × 1.5-cm column equilibrated with 20 mM sodium acetate (pH 4.5). After washing with 10 ml of acetate buffer, two minor peroxidase fractions (Pxo 60 kDa and Pxo 55 kDa) were eluted with 15 mM NaCl. The major peroxidase fraction (Pxo 51 kDa, also referred to as LEP1 in the text) was eluted within a 15–60 mM NaCl gradient over 20 ml.

For the isolation of Pxo 7, leaf cell walls were purified from 100 g (fresh weight) of leaves as described previously (30) and extracted with 20 mM sodium acetate (pH 4.5) containing 1 mM KCl. The extract was clarified by vacuum-assisted ultrafiltration through 0.45-μm filters (Sartorius) and equilibrated in 20 mM sodium acetate (pH 4.5) and concentrated to 4 ml with Diaflow®, using PY10 membranes (Amicon).

The concentrate was loaded onto a 5-ml SP-Sepharose column (Hi-Trap, Amersham Biosciences) equilibrated with the same buffer. After washing the column with 15 ml of equilibration buffer, a 0–0.5 M NaCl gradient was applied at 2 ml min⁻¹ over 25 min. Pxo 9.7 was eluted at 60–80 mM NaCl. Pxo 5.2 was isolated as described previously (28).

In all cases, column eluates were monitored for protein (650 nm) and peroxidase activity using horseradish peroxidase in the standard guaiacol assay. The purities of peroxidase-containing preparations was determined in 50 mM Tris-HCl (pH 7.2) by UV-visible spectroscopy using the R2 ratio, A450/A275, and quantified from the heme concentration using ε403 = 100 μmol 1 cm⁻¹ cm⁻¹ (31).

Assay of EP Activity and Kinetics of Studies of LEP1—The extensin cross-linking assay was essentially performed as described previously (8). The specific activity of EPs was determined in 0.5 M NaCl, using equilibration buffer containing 100 μg of extracted protein, 60 μg of grapevine extensin, and 4 h of incubation at 30 °C. Assays of fractionated or pure peroxidases utilized 5 pmol of each peroxidase, incubated for 30 min at 25 °C.

Kinetic studies with pure LEP1 (0.01 μg after Mono-S) utilized a UV-visible DU-70 (Beckman Instruments) spectrophotometer. The apparent Kₘ and Vₘₐₓ values for guaiacol, coniferyl alcohol, ferulic acid, and ascorbate were determined in 0.1 mM sodium acetate (pH 5) with 5 mM H₂O₂. For indole-3-acetic acid, assays were conducted according to Ref. 32. For the calculation of Kₘ and Vₘₐₓ values of LEP1 for H₂O₂, 20 mM guaiacol was used.

Extinction coefficient values (ε) for the different substrates are as follows: guaiacol ε₄₅₀ = 26.8 × 10³ μmol cm⁻¹ (30); coniferyl alcohol ε₃₅₄ = 7.5 × 10³ μmol cm⁻¹; ascorbate ε₃₃₄ = 5.5 × 10³ μmol cm⁻¹; indole-3-acetic acid ε₃₈₈ = 18.7 μmol cm⁻¹ (32). The molar extinction coefficient for ferulic acid was estimated in our laboratory as ε₃₈₀ = 11.3 μmol cm⁻¹ cm⁻¹. Apparent Kₘ and Vₘₐₓ values were calculated by a hyperbolic regression analysis program (Hyper.exe Version 1.1s, BioSoft, Cambridge, UK).

**Glycosylation of LEP1—**The glycans were released from 8.5 nmol of LEP1 by hydrazinolysis, labeled with 2-aminobenzamide, and separated by gel filtration as described previously (34). LEP1 glycan was quantified relative to 2 nmol of N,N'-diacetylchitobiose added as an internal standard immediately after the hydrazinolysis reaction. The number of glycans per mol of LEP1 was calculated after correcting for yield by reference to NapH-C (8 glycans/mol) (35) which was analyzed in an identical parallel experiment.

**RNA Isolation and PCR Amplification of LEP1 cDNA—**Total RNA was isolated from Lupinus albus leaves using the RNeasy system from Qiagen according to the supplier’s instructions. First strand cDNA synthesis utilized 2 μg of total RNA incubated at 42 °C for 1 h with Superscript™II RNase H⁻ reverse transcriptase (Invitrogen) and the oligonucleotide PoxS3 (5' - CACAGTACGAAACACTCGACCTGCAGCC - 3') containing 10 pmol each of the primers PoxS4 (5' - GTCGACTTTGTTTTGTCAGAAGA - 3') and PoxS2 (5' - CACAGTACGAAACACTCGACCTGCAGC - 3'). 0.1 mM dNTPs, and 2 units of Taq polymerase (Amersham Biosciences). PCR conditions employed an initial denaturation step at 94 °C for 3 min, followed by 31 cycles with a 1-min denaturing step at 94 °C and a 2-min elongation step at 72 °C. The annealing temperatures were 52 °C for 3 cycles, 50 °C for 3 cycles, and 56 °C for the remaining 25. A final extension step at 72 °C for 5 min was included. Selected PCR products were subcloned into pGEM-T vector (Promega) and sequenced.

The remaining 5' region LEP1 cDNA was obtained when the RACE kit from Invitrogen according to the manufacturer’s recommendations. 5'-RACE was performed by means of nested PCR using the LEP1-specific PoxS6 (5' - CTTTGAGATTITTTGTTGAACGAAAAGCATTATTT - GTGAGG - 3') and the anchored anchor and abridged universal anchor primers supplied with the kit. Following an initial denaturation step at 94 °C for 3 min, 35 cycles of 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C were run. The final extension step at 72 °C was carried out for 10 min. The 5'-RACE product was subcloned into µGEM-T vector (Promega) and sequenced.

**Quantitative RT-PCR—**To quantify lep1 gene transcripts, an RT-PCR protocol was performed on total RNA extracts of 10-day-old etiolated plantlets exposed to white light for 0, 0.5, 1, 3, 12, or 24 h. Segments of 1 cm length were obtained from hypocotyls just below the apical meristem and RNA isolated using the RNeasy kit (Qiagen). As a control, RNA was also extracted from lupins grown in darkness (Hi-F) and plants grown in light (Hi-L) (5'-CCCTGTACCATAGAGCCTTTAG - AAAAGC - 3'). Following the RT reaction as described above, PCR mixtures (50 μl) were prepared containing 2 μl of cDNA, 2.5 units of Taq DNA polymerase and buffer (Amersham Biosciences), 200 μM dNTPs, and 10 μM of the LEP1-specific primers PoxS6 (5' - CTTGAGATTITTTGTTGAACGAAAAGCATTATTT - GTGAGG - 3').
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TAGTGTAACGAACTAATACATATCTACCC-3′) and PosS7 (5′-ACAC-TTCGATACATGTTCAATGGTGGAGCAGG-3′). The PCR temperature program was 94 °C for 30 s, 56 °C for 2 min, and 72 °C for 2 min for 30 cycles with a final 5-min extension at 72 °C. Aliquots (5 μl) were taken every five cycles for each reaction. The reaction conditions for Hb were the same with the exception that the annealing temperature was 40 °C. Following amplification, aliquots of each PCR product were analyzed by electrophoresis in a 1.2% agarose gel and stained with ethidium bromide. The gels were visualized with UV light and photographed.

Molecular Modeling of LEP1—The structures of the class III peroxidases PNP (PDB code 1sch) (36), HRP-C (PDB code 1atj) (37), Arabidopsis thaliana peroxidase A2 (PDB code 1iap) (38), A. thaliana neutral peroxidase (PDB code 1lqg) (39), and SBP (PDB code 1bf1) (21) were used to derive the structure of LEP1 by comparative modeling techniques using the program Modeler version 4 (40). The x-ray structures were superimposed (using a 4.0-Å equivalence radius) to generate a sequence alignment that reflected the equivalence of residues in the structure. The LEP1 sequence was additionally aligned with several plant peroxidases with high homology to LEP1, and the resulting profile was aligned with the structural alignment of the x-ray structures. This additional procedure helped produce a less ambiguous alignment because it made the final result less dependent on particular point mutations in LEP1. The alignment was manually corrected in selected places and used to derive structural models. The models contained the home group, the catalytic water molecule, two calcium ions, and the complete polypeptide chain, except the C-terminal 23 residues (from Asp-304 to Tyr-327) that are not homologous with any of the available structures. The four conserved disulfide bridges were modeled explicitly.

An initial alignment was used to generate models that were checked using two criteria: identification of zones displaying restraint violations using Modeler, and several stereochemical and conformational criteria using PROCHECK (41). The alignment was modified several times in order to correct specific problems, with the generation of new structures each time. In the final cycle, 40 structures were generated and that with the lowest objective function value was chosen as the model of LEP1. The chosen model was further optimized by modeling the loop comprising Gly-112 to Asn-113 to Gly-114 because Asn-113 occupied a generously allowed zone of the Ramachandran plot. The final model had 93.3% of the residues in the most favored regions, 6.7% in additionally allowed zone of the Ramachandran plot. The four conserved disulfide bridges were modeled explicitly.

RESULTS

The Distribution of EP Activity in Vegetative Organs of Lupin—The in vitro oligomerization of extensin monomers by EP can be detected by Superose-12 gel filtration chromatography of reaction products (24, 25, 27). Fig. 1A depicts how pure grapevine EP (27) cross-links grapevine extensin completely to higher order multimers that elute in the void volume, whereas monomeric extensin is retained. Substitution of grapevine EP with saline infillates from various organs and tissues of L. albus allowed us to test their relative content of apoplastic EP activity. Extracts prepared in this way contain both ionically bound and soluble apoplastic proteins and are free from contamination from intracellular compartments (29). Fig. 1, B–G, indicates that EP activity is low or absent in apical meristems, shows minor levels in etiolated hypocotyls, but is considerably higher in roots, hypocotyls, and leaves obtained from photomorphogenic plants. The highest abundance of EP activity and the highest specific activity was observed in leaves. Similar levels of EP activity could also be recovered in aqueous infiltrates (Fig. 1H), indicating the peroxidases responsible are freely soluble constituents of the apoplastic space.

Purification of LEP1 from Leaves—Aqueous extracts of leaf homogenates were chosen as an abundant and convenient source for the purification of apoplastic and soluble leaf EP (see “Experimental Procedures”). Following concanavalin A-affinity chromatography, the EP-containing fraction consisted largely of two proteins of 51 and 61 kDa (Fig. 2, lane CA). Mono-S chromatography was able to resolve the two major proteins and minor quantities of a diffuse band of 55 kDa (lanes A–C). The UV-visible spectra of all three fractions displayed spectra typical of class III peroxidases, with a Soret peak at 403 nm and additional maxima at 502 and 640 nm. Their RZ values (Table I) also confirmed that these peroxidase fractions had been isolated without significant levels of contaminants. Isoelectric focusing of these peroxidases (data not shown) confirmed that they were basic (pI 8.8) and that all migrated together with the major soluble peroxidase of the apoplast previously referred to as B2 (29, 42). We have assayed the extensin cross-linking activity of these soluble peroxidases and of two major ionically bound peroxidases of leaves, Px5.2 and Px9.7 (28) (Table I). These assays revealed that 5 pmol of the major 51-kDa peroxidase resulted in near-complete cross-linking of 60 μg of grapevine extensin. This compares well with that observed for a tomato basic EP (24) and grapevine EP (27). Under these same conditions, the 55-kDa peroxidase cross-linked ~20% of extensin. The 61-kDa peroxidase, Px 9.7 and Px 5.2, demonstrated 5% cross-linking or less. The purified peroxidases HRP-C and PNP showed similarly lower levels of cross-linking activity (10–12%; see Table II). These data clearly indicate that extensin cross-linking activity in the leaf apoplast could be largely attributed to the 51-kDa peroxidase. They also demonstrate that relative to several other peroxidases, the 51-kDa peroxidase had much higher extensin cross-linking activity and justify the classification of this peroxidase as an EP, namely lupin EP1 (LEP1).

Substrate Specificity and Enzyme Kinetics—In order to determine the specificity of protein cross-linking activity of LEP1, several extensins and non-HRGPs were assayed as substrates, including Cot c, a structural protein from Bacillus subtilis which is insolubilized during spore coat formation and contains several contiguous tyrosine and lysine residues reminiscent of the proposed cross-linking site of extensins (43). As reported for a tomato EP, LEP1 was incapable of cross-linking the non-HRGPs bovine serum albumin and aldolase (24). Similar results were obtained with Cot c. However, LEP1 readily cross-linked grapevine, tomato, and potato extensins.

Attempts to measure more precise kinetic parameters for LEP1 activity with extensin proved impractical due to the high quantities of substrate needed to approach V_{max} under in vitro conditions. This is largely due to the high molecular mass of GvP1 (90 kDa) (27), and consequently, the high extension quantities required to reach mM levels are in excess of 90 mg/ml. Such high concentrations lead to filter clogging in Superose-12 chromatography and unreliable results. Similar difficulties have been reported for an acidic tomato EP with tomato extensin (25).

Assays with alternative substrates demonstrated that LEP1 showed no or relatively low reactivity with ascorbate and in-dole-3-acetic acid, respectively. LEP1 did show higher reactivity with guaiacol, coniferyl alcohol, and ferulic acid (Table II). Of interest, relative to values reported in the literature for other class III peroxidases, LEP1 showed a surprisingly low apparent affinity for guaiacol. Similar observations have been made with a basic tomato EP.2 The apparent affinities and V_{max} values for coniferyl alcohol and ferulic acid were considerably higher. It is therefore apparent that LEP1 is capable of efficiently cross-linking other physiologically relevant substrates in nuce. Conditions for maximal peroxidase activity with guaiacol, coniferol alcohol, and ferulic acid were proximal to pH 5.0 and peroxide concentrations above 1.5 mM. In contrast, maximal EP activity of LEP1 required neutral pH and peroxide concentrations near 100 μM. This compares well with that observed for a tomato basic EP (24) and grapevine EP (27). Under these same conditions, the 55-kDa peroxidase cross-linked ~20% of extensin. The 61-kDa peroxidase, Px 9.7 and Px 5.2, demonstrated 5% cross-linking or less. The purified peroxidases HRP-C and PNP showed similarly lower levels of cross-linking activity (10–12%; see Table II). These data clearly indicate that extensin cross-linking activity in the leaf apoplast could be largely attributed to the 51-kDa peroxidase. They also demonstrate that relative to several other peroxidases, the 51-kDa peroxidase had much higher extensin cross-linking activity and justify the classification of this peroxidase as an EP, namely lupin EP1 (LEP1).

2 P. Dey, personal communication.
might be the principal factors in determining LEP1 substrate utilization.

Glycosylation and Solubility of LEP1—Although LEP1 is basic (pI 8.8) and therefore positively charged at physiological pH, the secreted peroxidase remains highly soluble and resides principally in the extracellular spaces of all vegetative organs (29).

The number and size of LEP1 glycans was determined after hydrazinolysis and separation of the liberated and fluorescently labeled glycans. The chromatogram (Fig. 4) demonstrates that the most abundant glycan of LEP1 eluted at a volume equivalent to 6.5 glucose units, the same position as that observed for the major glycan moiety in HRP-C (44, 45) and soybean peroxidase (46), a complex heptasaccharide, Manα3(Manα6)(Xylβ2)Manβ4GlcNAcβ4(Fucα3)GlcNAc. Larger glycans were also present up to and including the biantennary complex tridecasaccharide Galβ3(Fucα4)GlcNAcβ4Manβ3(Galβ3(Fucα4)GlcNAcβ4Manα6)(Xylβ2)Manβ4GlcNAcβ4-

Fig. 1. Apoplastic extensin cross-linking activity of vegetative organs of L. albus. A depicts the cross-linking of grapevine extensin (GvP1; gray trace) by GvEP to higher order multimers (black trace) that elute proximal to the void volume (V0). The following panels represent the EP activities (quantified as % extensin cross-linked in top right corner): B, shoot meristem; C, root meristem; D, etiolated hypocotyl; E, hypocotyl from photomorphogenic plants; F, roots; G, leaf. H shows that EP activity in leaves could be extracted by infiltration with aqueous buffers. For B–H, 25 μg of each extract was added to 60 μg of extensin and incubated for 4 h at 30 °C in the dark after the addition of H2O2 to 100 μM.
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Instead, it is clear that both peroxidases saturated cell walls with considerably less quantities than PNP.

Cloning and Analysis of the Deduced Sequence of LEP1—

Purified LEP1 was trypsin-digested and fragments separated by reverse phase C\textsubscript{18} chromatography. Two peptides were chosen for sequencing and are indicated in Fig. 6. Alignment of the resulting sequences with known class III peroxidases structures demonstrated that peptide 1 showed best homology with poorly conserved sequences from the N-terminal portion of helix C, whereas peptide 2 corresponded to a hypervariable sequence from an inter-helical region and the N-terminal portion of helix F. In order to obtain a more complete primary structure for LEP1, truncated peroxidase cDNAs (~1 kb) were cloned from lupin leaves by 5'-RACE with a degenerate primer based on the conserved catalytic site of peroxidases (see "Experimental Procedures"). Three distinct peroxidase sequences were obtained in this manner (LalbPrx1, GenBank\textsuperscript{TM} accession numbers AF405326;LalbPrx2, accession number AF405327; LEP1, accession number AF403735). Clones of truncated (1,146 bp) LEP1 were identified by their deduced amino acid sequence homology (100%) to LEP1 peptides 1 and 2. The truncated sequences of LalbPrx1 and LalbPrx2 were <75% homologous with truncated LEP1 and did not contain sequences with 100% homology to LEP1 peptides 1 or 2.

To obtain the full-length cDNA of LEP1, 5'-RACE was performed using LEP1-specific primers designed from the truncated LEP1 sequence. Full-length LEP1 cDNA is 1351 bp long, containing a 1068-bp coding sequence. The entire sequence and the deduced primary structure of LEP1 is shown in Fig. 6.

The open reading frame corresponds to a deduced protein of 327 amino acids with a primary structure typical of plant peroxidases. There are eight conserved cysteines at positions 11, 44, 49, 91, 97, 176, 208, and 299 (HRP-C numbering), allowing for the formation of four conserved disulfide bridges (50). The amino acids sequence of the mature LEP1 also exhibited the conserved distal catalytic residues Arg-38 and His-42 and the proximal His-169 and Asp-246, a conserved signature of class III peroxidases (51).

A signal peptide cleavage site was predicted between Ala-28 and Gln-29 from a hydropathy profile (data not shown) and confirmed by alignment with sequences of peroxidases for which the first N-terminal residues in the mature protein are known (Fig. 6). Deduction of the signal peptide mass resulted in a polypeptide mass of 35,264 kDa which, with the additional contribution of heme (616 Da), compares favorably to that observed for the deglycosylated form of the mature LEP1 (~37 kDa). A theoretical pI of 6.0 for LEP1 was predicted as compared with pI 8.8 observed in native isoelectric focusing gels (30). However, similar discrepancies were seen with a zucchini peroxidase (52) and the pI 8.3 FBPI peroxidase from Phaseolus vulgaris (53), which has a predicted pI of 6.5.

The deduced LEP1 sequence also contains a putative 22-amino acid C-terminal propeptide (CTPP) that is generally considered to target the protein from the trans-Golgi to vacuoles in some proteins and is cleaved off in the mature protein (54–56). However, LEP1 is secreted into the apoplastic space (29) (this study). A zucchini peroxidase (52) and the French bean FBPI (53, 57) also contain putative CTPPs, although both peroxidases are at least partly directed to the cell wall.

The deduced amino acid sequence of LEP1 indicates this EP to have 12 putative N-glycosylation sites (Fig. 6). This is in agreement with the number of glycans in mature LEP1 determined by quantification of glycans reported above.

LEP1 is the first identified EP to be cloned. A BLAST search (58) identified several plant peroxidases with 75–81% similarity to LEP1. The strongest similarity was found with peroxi-
dases in the leguminosae family including *P. vulgaris* (FBP1, 81%; GenBank™ accession number AAD37427), *Medicago sativa* (PXDC, 75%; GenBank™ accession number AAB41811), and *Glycine max* (GMIPER1, 73%; GenBank™ accession number AAC98519). Interestingly, FBP1 is known to be capable of cross-linking HRGPs (57). As depicted by the cladogram (Fig. 7), other peroxidases from these species and lupin showed substantially less homology to LEP1. This indicated that LEP1 is closely related to a conserved subset of legume peroxidase (indicated at a root in Fig. 7).

Structurally, the subset of peroxidases showed further similarity in that they all contained 9–12 N-linked glycosylation sites and a putative CTPP. Fig. 7 also revealed that the closely related peroxidases included the soybean seed coat peroxidase (SBP) for which a crystal structure has been determined (21).

Expression of LEP1 in Etiolated Hypocotyls in Response to White Light—Previous analysis has shown that LEP1 is induced to a higher abundance in apical segments of hypocotyls undergoing growth retardation by white light (Jackson et al. (8)). To determine whether the increase in LEP1 abundance is due to changes in expression of *lep1*, Quantitative RT-PCR was performed on RNA extracted from 10-day-old etiolated hypocotyls exposed to white light, and the *lep1* transcript level was compared between samples isolated over a period of 24 h (Fig. 8). Products became clearly visible after 25 cycles of PCR. As can be seen in Fig. 6, *lep1* transcript was undetectable in apical portions of etiolated hypocotyls. Exposure to white light, however, induced an increase in expression of *lep1* between 30 min and 1 h after which *lep1* expression was maintained.

Structural Model of LEP1—The structural alignment of LEP1 with that of known crystal structures allowed us to model the tertiary structure of this EP and examine structural features that might be related to its high capacity for extensin cross-linking (Fig. 9).

Similar to that observed for SBP (21), the properties of LEP1 residues defining the heme cavity were similar to that in other known peroxidase structures, suggesting that the oxidative capacity of this peroxidase would not differ substantially. As also observed for SBP (21), the major differences appeared to be due to side chain substitutions introducing topographical differences in regions surrounding the SAC, which might influence substrate specificity. All known peroxidase structures display, to some degree, an equatorial cleft that traverses the SAC (Fig. 9B). In *Arabidopsis* peroxidases N and A2, the extent of the cleft is limited by bulky residues either side or to the left of the SAC, respectively. The structures of HRP-C, PNP, SBP, and LEP1 have less bulky residues in these regions and display a relatively unobstructed cleft extending across the enzyme. However, in these peroxidases, the equatorial cleft is partly occluded by the presence of two bulky Phe residues (Phe-142 and Phe-143, HRP-C numbering). These residues are substituted by serines in LEP1, resulting in a wider equatorial cleft which is deeper in the area immediate to the site of substrate interaction. This topographical feature is likely to be important for the orientation and closer approximation of macromolecular substrates to the active site.

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**Fig. 3.** Optimal pH (A) and peroxide concentrations (B) for LEP1 with extensin (closed diamonds), guaiacol (open squares), coniferyl alcohol (open triangles), and ferulate (open circles).
An examination of the electrostatic properties of LEP1 revealed that the regions proximal to the SAC in LEP1 appear to be particularly devoid of charged residues, whereas those of SBP, HRP-C, and PNP are predominantly acidic in nature (data not shown). However, LEP1 appears to have an unusual distribution of hydrophobic residues around the SAC (Fig. 9C). The hydrophobicity of the inter-helical loop above the SAC is contributed by Phe-68 alone, whereas in other peroxidase structures this patch is considerably more extensive. LEP1 also appears to be unique in that it contains an uninterrupted hydrophobic patch extending from residues immediately surrounding the SAC to the left extreme of the equatorial cleft.

**DISCUSSION**

A study of the distribution of extensin cross-linking activity in lupin led to the purification of LEP1 from leaves, a 51-kDa peroxidase with high extensin cross-linking activity. In a standard assay for extensin cross-linking activity, LEP1 demonstrated substantially higher apparent rates than observed with HRP-C, PNP, and four other lupin peroxidases. Similarly large differences in apparent rates between EPs and other class III peroxidases have been observed in tomato (24, 25), mustard (26), and grapevine (27). LEP1 was shown to have extensin cross-linking activity comparable with EPs identified in tomato (24) and grapevine (27). The partial biochemical and molecular characterization of LEP1 presented here represents the first such characterization of an EP.

It seems reasonable to assume that the high extensin cross-linking activity of EPs is of functional importance and that these peroxidases are required for the formation of the extensin network in vivo. However, LEP1 also displays high affinities for the physiologically important cell wall substrates, ferulate and coniferyl alcohol. This indicates that LEP1 shares the generally low substrate specificity of class III peroxidases and that it is potentially multifunctional. This initially suggests that the presence of alternative substrates at the site of LEP1 expression may heavily influence the functional outcome of its activity. However, whereas extensin cross-linking activity by LEP1 was optimal at neutral pH and 100 μM H$_2$O$_2$, optimal activity with other cell wall substrates required acidic pH (5.0) and H$_2$O$_2$ in the mM range. Similar optimal conditions for
extensin cross-linking were observed with an anionic tomato EP (23). Ovoperoxidase, an animal peroxidase that cross-links the fertilization membrane of sea urchin eggs by dityrosine links, also demonstrates a lower optimal hydrogen peroxide concentration for tyrosine oxidation than for other substrates (59). Although the reasons for these differences remain to be explained, they suggest that developmental and stress-related changes in in muro conditions could help regulate the EP activity of LEP1. Hydrogen peroxide is tightly regulated in developing hypocotyls and is maintained at low concentrations in actively growing tissues (60, 61). The apoplastic pH is also more acidic in actively elongating cells. Extensin cross-linking by LEP1 could therefore more readily occur in the apoplast of juvenile, post-elongation phases of lower acidity and H2O2 concentration, whereas the higher peroxide concentrations in more mature tissues could favor the choice of alternative substrates. Similarly, the differing optima for extensin cross-linking could favor extensin deposition during the earliest phases of the oxidative burst during defense responses, which is accompanied by alkalization of the apoplastic medium in several species (62, 63). Earlier work has indicated that this peroxidase is associated with the early phases of both growth restriction in hypocotyls (8) and defensive responses in leaf (28), where such conditions might prevail.

By RT-PCR with LEP1-specific primers it was demonstrated that in addition to leaves, LEP1 was expressed in hypocotyls and roots from photomorphogenic plants, confirming the widespread occurrence of this peroxidase in vegetative tissues (29). Recently, we reported that LEP1 was induced to higher levels in etiolated hypocotyls exposed to 3–6 h of white light, in a manner temporally and spatially coincident with irreversible inhibition of etiolated apical growth. Of all the peroxidases in hypocotyls, LEP1 appeared to be unique in this respect (8). Here we have utilized quantitative RT-PCR to demonstrate that the white light-induced increase in LEP1 is preceded by an increased transcription of LEP1 30–60 min after irradiation, indicating a close association of this gene with regulatory elements of light signal transduction pathways.

White light irradiation of etiolated seedlings leads to cessation of growth concomitant with accelerated formation of cell wall cross-links (64). Our studies with lupin suggest that one important developmental role of LEP1 is to contribute to this process through its catalysis of extensin deposition in the early stages of growth inhibition.
In leaves and hypocotyls, LEP1 is secreted into the apoplastic space where it resides largely as a freely soluble component (this study (29)). However, the sequence of LEP1 showed the presence of a putative CTPP that is generally considered to target the protein to the vacuole. Although such CTPPs lack homology, cDNA sequences of HRP-C (65) and the barley peroxidases BP1 (66) and BP2 (67) also show C-terminal extended sequences that are removed in the mature protein. However, other proteins containing a C-terminal extension, such as α-mannosidase, class I chitinase, and class I β-1,3-glucanase, were reported to be secreted to the apoplast via the vacuolar compartment (68). The key motif in a functional CTPP has been proposed to be three to four hydrophobic residues followed by one or two acidic amino acids (69). Such a motif is present in the CTPPs of HRP-C (70) and BP1 (66) but is not obvious in LEP1. The cDNAs of FBP1 and an anionic peroxidase from zucchini (GenBank™ accession number CAAT67680 (52)) encode a putative CTPP, but both are secreted to the cell wall. This therefore appears to strongly indicate that the presence of a CTPP is not a reliable marker for vacuolar targeting.

The primary structure of LEP1 indicated 12 putative N-glycosylation sites. The three-dimensional model of LEP1 demonstrates that all map to the peroxidase surface, where they are reasonably uniformly distributed. An analysis of glycans revealed that mature LEP1 has 11–12 glycans/mol, thus indicating that the majority of the putative sites are glycosylated. The sites most likely remaining unglycosylated correspond to Asn-286 and proximal to Asn-214 of HRP-C, both of which are free from carbohydrate in mature HRP-C and PNP.

The high level of glycosylation in LEP1 is likely to help maintain this alkaline peroxidase soluble in the apoplastic space. In comparative cell wall binding assays, saturating quantities of LEP1 and HRP-C (8 glycans) were 6× lower than that of lightly glycosylated PNP (3 glycans).

The ionic binding of peroxidase to cell walls is thought to involve the electrostatic interaction of basic and topographically contiguous surface residues with calcium pectate (71). A high number of uniformly distributed glycans at the peroxidase surface might be expected to sterically inhibit the approach of such basic patches to potential cell wall binding sites and thus ensure high solubility. However, the salt elution profiles of all three peroxidases from cell walls were qualitatively similar, requiring 25 mM for 50% recovery and 50 mM NaCl for maximal recovery. This clearly indicates that the electrostatic interaction of all three peroxidases is of similar strength but that the more heavily glycosylated HRP-C and LEP1 nevertheless saturate cell walls with substantially less quantities. One possible explanation is that the highly mobile and large glycans extending from bound peroxidase could conceal neighboring potential binding sites in the cell wall. As a result, more heavily glycosylated peroxidases would tend to effectively saturate the cell wall binding sites with relatively lower amounts. This, together with competition with other electrostatically interacting cell wall proteins for potential binding sites, would ensure that the majority of LEP1 remains freely soluble in the apoplastic space.

The high solubility of LEP1 is likely to be of physiological importance to its role as an EP in planta. Extensin network formation by ionically bound EPs such as those characterized in tomato (24, 25) would require co-localization of the EP and monomeric extensin in the same cell wall. The high solubility of LEP1, however, would allow it to diffuse away from its site of secretion, so that it can contact cell walls of neighboring cells bordering any contiguous apoplastic space. This may be particularly important during the early phases of cell corner formation, where extensin deposition can be heavy (72). Soluble apoplastic peroxidases in xylem have been considered important for the plugging of damaged vascular tissues (73). As a soluble component of the apoplastic space in epidermal and vascular tissues (29), LEP1 appears to be particularly suited to contributing extensin network formation to wound plugs in vascular or surface tissues.
LEP1 showed highest homology (up to 81%) with some legume peroxidases from the Papillinoideae subfamily of Fabales. Other peroxidases from these same species showed considerably less homology, indicating that LEP1 belongs to a conserved subset of peroxidases within these closely related species. In addition to sequence homology, peroxidases of this subset also contained CTPPs and a high level (9–12) of putative N-linked glycosylation sites. Many of this subset also contained an unusual putative glycosylation site immediately adjacent to a conserved Asn-70 (HRP-C numbering) and proximal to the SAC.

Interestingly, LEP1 shows highest homology with FBP1, a peroxidase known to have extensin cross-linking activity (57). It is therefore tempting to suggest that the LEP1 homologues in *P. vulgaris, M. sativa*, and *G. max* may also encode EPs.

It is notable that LEP1 showed considerably less homology with peroxidases from less genetically related dicot species, even though all dicot species are thought to employ extensin deposition for essential cell wall modification (7), which implies an equally widespread distribution of EPs. The highest homology with *A. thaliana* peroxidases was only 53% identity (GenBankTM accession number AF452388.1). This suggests that a high sequence homology with LEP1 is not essential for high extensin cross-linking activity. Instead, the capacity of peroxidases for EP activity may be related to relatively small and localized variations in peroxidase structure.

Comparison of the model tertiary structure of LEP1 with available peroxidase structures revealed that moderately few amino acid substitutions in this peroxidase results in moderately few available peroxidase structures revealed that moderately few amino acid substitutions in this peroxidase results in an unusually distinct equatorial cleft, which is also relatively deeper than the clear equatorial cleft in LEP1 has the capacity to orientate extensin across the deposition for essential cell wall modification (7), which implies an equally widespread distribution of EPs. The highest homology with *A. thaliana* peroxidases was only 53% identity (GenBankTM accession number AF452388.1). This suggests that a high sequence homology with LEP1 is not essential for high extensin cross-linking activity. Instead, the capacity of peroxidases for EP activity may be related to relatively small and localized variations in peroxidase structure.

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60. Schopfer, P. (1994) *Plant Physiol.* **104**, 1269–1275
61. Cano, A., Artés, F., Arnau, M., Sanchez-Bravo, J., and Acosta, M. (1996) *J. Plant Physiol.* **147**, 721–728
62. Bolwell, G., Davies, D., Gerrish, G., Auh, C.-K., and Murphy, T. (1998) *Plant Physiol.* **116**, 1379–1385
63. Zhang, S., Du, H., and Klessig, D. (1998) *Plant Cell* **10**, 435–449
64. Kutschera, U. (1996) *J. Exp. Bot.* **47**, 1387–1934
65. Fujiyama, K., Takeda, H., Shibayama, S., Kobayashi, K., Choi, J.-K., Shinmyo, A., Takano, M., Yamada, Y., and Okada, H. (1988) *Eur. J. Biochem.* **173**, 681–687
66. Rasmussen, S., Welinder, K., and Hejgaard, J. (1991) *Plant Mol. Biol.* **16**, 317–327
67. Theilade, B., and Rasmussen, S. (1992) *Gene (Amst.)* **118**, 261–266
68. Kunze, I., Kunze, G., Broeker, M., Manteuffel, R., Meins, F., and Muntz, K. (1998) *Planta* **205**, 82–99
69. Nakamura, K., and Matsuzaka, K. (1993) *Plant Physiol.* **101**, 1–5
70. Theilade, B., Rasmussen, S., Rosenkrands, I., Frokier, H., Heggaard, J., Theilade, J., Pihakaski-Maunsbach, K., and Maunsbach, A. (1993) in *Plant Peroxidases: Biochemistry and Physiology* (Welinder, K., Rasmussen, S., Penel, C., and Greppin, H., eds) pp. 321–324, University of Geneva, Geneva, Switzerland
71. Carpin, S., Crévecœur, M., de Meyer, M., Simon, S., Greppin, H., and Penel, C. (2001) *Plant Cell* **13**, 511–520
72. Swords, K. M. M., and Staehelin, L. A. (1993) *Plant Physiol.* **102**, 891–901
73. Biles, C., and Abeles, F. (1991) *Plant Physiol.* **96**, 597–601
74. Cooper, J. B., and Varner, J. E. (1984) *Plant Physiol.* **76**, 414–417
75. Nicholls, A. (1992) *GRASP: Graphical representation and analysis of surface properties*, Columbia University, New York
76. Kraulis P. J. (1991) *J. Appl. Cryst.* **24**, 946–950
77. Merritt E. A., Bacon D. J. (1997) *Methods Enzymol.* **277**, 505–524