



Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots

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Abstract

An abundant 17 kDa protein which was isolated and characterized from 10-day old healthy root tissue of white lupin (*Lupinus albus*) proved to have a high sequence similarity to pathogenesis-related proteins found in other species. Subsequently, a corresponding clone (*LaPR-10*) was identified in a cDNA library prepared from the same tissue that exhibited a high amino acid sequence similarity to a number of the PR-10 family proteins. The clone contains an open reading frame encoding a polypeptide of 158 amino acids, with a predicted molecular mass of 16 905 Da and an isoelectric point of 4.66. Southern blot analysis indicates that *LaPR-10* is likely a single-copy gene, or a member of a small gene family. The clone was expressed in *Escherichia coli*, and its protein product was purified to near homogeneity. Both the native and the recombinant proteins were immunorecognized by antibodies raised against pea PR-10 proteins, and exhibited a ribonucleolytic activity against several RNA preparations, including lupin root total RNA. Characterization of its enzymatic properties indicates that the *LaPR-10* protein belongs to the class II ribonucleases. We present evidence that the white lupin 17 kDa protein is constitutively expressed during all stages of root development and, to a lesser extent, in other plant parts. In addition, we demonstrate the presence, in the *LaPR-10* amino acid sequence, of a number of motifs that are common to most PR-10 proteins, as well as a RGD motif that is shared only with the alfalfa SRG1 sequence.

Introduction

The term ‘pathogenesis-related’ (PR) proteins was proposed (Antinov *et al.*, 1980) to indicate a class of proteins that are synthesized in plants in response to pathogenic infection. A more recent definition (Carr and Klessig, 1989) qualifies PR proteins as host-encoded polypeptides that are not present in healthy plants, but are synthesized only in response to pathological or stress-related situations, the most important criterion being the induction of their synthesis. The

production of PR proteins represents a component of the active defense repertoire. This repertoire includes the cell-wall hydroxyproline-rich glycoproteins (Sommer-Knudsen *et al.*, 1998), the hydrolytic enzymes chitinases and β -1,3-glucanases and the extracellular matrix proteins (Boller, 1987), as well as the enzymes of phenylpropanoid metabolism (Hahlbrock and Scheel, 1989); all of which are involved in the defense reactions to biotic and/or abiotic elicitors. In contrast, PR-like proteins (van Loon *et al.*, 1994) are defined to be homologous to the PR family with respect to their amino acid sequence, structural homology and corresponding enzymatic activity, but are expressed principally in a developmentally controlled,

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AJ000108 (*LaPR-10*).

tissue-specific manner, as opposed to a pathogen-related induction or expression.

Whereas the classification of PR proteins into 5 families was based initially on molecular mass, isoelectric point, localization and biological activity (Stinz *et al.*, 1993), the latest proposition divides PR proteins into 11 families based on their sequence or predicted sequence of amino acids, serological relationships and biological activity (van Loon *et al.*, 1994). The PR-10 family comprises proteins that are intracellular, acidic, with a molecular mass of 16–19 kDa, lacking a signal peptide, resistant to proteases, and whose constitutive expression is observed in developing roots of several species (Walter *et al.*, 1990). Parsley PR-1 has been suggested to represent the 'type member' of an ubiquitous class of intracellular defense-related proteins, the PR-10 family (van Loon *et al.*, 1994).

PR-10 proteins have been reported in a number of species throughout the plant kingdom, including pea (Fristensky *et al.*, 1988), bean (Walter *et al.*, 1990, 1996), soybean (Crowell *et al.*, 1992), alfalfa (Breda *et al.*, 1996) and potato (Matton and Brisson, 1989), as well as several monocots, including asparagus (Warner *et al.*, 1992), lily (Huang *et al.*, 1997) and rice (Midoh and Iwata, 1996). Studies on healthy roots of yellow lupin (*Lupinus luteus*) led to the identification of two homologous PR-10 proteins whose gene structure has recently been elucidated (Sirkorski *et al.*, 1995). These authors also reported that the genes induced under pathological conditions show an identity with those that are constitutively expressed (Sirkorski *et al.*, 1998). This implies that yellow lupin PR-10 proteins may be involved in development, as well as in the general defense mechanism. However, the biological function(s) of this class of proteins remains unknown, although it has been proposed that they possess a ribonuclease activity (van Loon *et al.*, 1994). While most PR-10 proteins reveal some structural similarity to ginseng ribonuclease (Moiseyev *et al.*, 1997), they also show high amino acid sequence similarity to the tree pollen allergens (Breiteneder *et al.*, 1989), as well as the major food allergens of celery (Breiteneder *et al.*, 1993) and apple (Vanek-Krebitz *et al.*, 1995).

Lupinus albus (white lupin) is an important legume seed crop (Hill, 1997), and its healthy roots accumulate 17 kDa protein in a constitutive manner. The gene encoding this protein has recently been cloned, and its sequence deposited under the GenBank accession number AJ000108 (Bantignies *et al.*, 1998). We here provide direct evidence that both the lupin PR-10-like

protein and the gene product of its cDNA clone exhibit ribonuclease activity, and this may represent a component of the constitutive pre-infectious defense mechanism of the plant.

Materials and methods

Plant material and tissue extraction

Seeds of white lupin (*Lupinus albus* L. cv. Primovski; Leguminosae) were obtained from Labon Inc. (Boucherville, Québec) and germinated in vermiculite under greenhouse conditions. Tissues were collected on a daily basis from the time of sowing up to 7 days and then weekly up to 6 weeks. They were frozen in liquid nitrogen and stored at -80°C . Fresh or frozen tissue was extracted with 0.9% NaCl pH 6.4 (1:4, w/v), and the homogenate was filtered through cheese-cloth before centrifugation at $10\,000 \times g$ for 15 min. The crude extracts were rendered lipid-free (Vessel and Flügge, 1984) prior to protein analysis.

SDS-PAGE and protein measurement

SDS-PAGE was performed as described by Laemmli (1970) using 4% and 15% polyacrylamide in the stacking and resolving gels, respectively, and the molecular weight markers were obtained from BioRad. Proteins were detected with Coomassie Blue stain, and their amounts were measured by the BioRad protein assay kit, using BSA as standard.

Western blot analysis

After electrophoresis, proteins were transferred onto a nitrocellulose membrane according to the BioRad semi-dry Trans-Blot apparatus protocol, using Tris-borate-EDTA (TBE) buffer for transfer. The blots were blocked with non-fat dry milk before being incubated with polyclonal antibodies raised against the pea root-specific PR-10 protein ABR 18 (Itturriaga *et al.*, 1994), diluted to 1:1000 to 1:2500, or preimmune serum diluted to 1:1000. Immunodetection was carried out with alkaline phosphatase-conjugated anti-rabbit IgG as secondary antibody, using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

White lupin LaPR10	MGIFTTFEDES	TSTVAPAKLY	KALVADANII	IPKAVEAIQS	VENVEGNGGP	50
White lupin motifs	<u>C</u>				<u>M</u>	
Soybean SAM22	<u>C</u>		C	P	M	P
Pea RH2			C	P		
Bean PR10a	C				P	M
Pea ABR18		G				M
Alfalfa SGR1						M
Yellow lupin			C			M
Pea ABR17				P		M
Birch Betv1					C	M
Ginseng RNase1	M	C				M

White lupin LaPR10	GTIKKLTIE	DGETKYVLHK	IEEIDEANLG	YNYSIVGGVG	LPDTVEKITF	100
White lupin motifs	<u>P</u> <u>A</u> <u>C</u>			<u>G</u>	<u>M</u>	
Soybean SAM22	<u>P</u> <u>A</u> <u>C</u>		C		B	
Pea RH2	A	C		G	B	M
Bean PR10a	P	A	C	C		B
Pea ABR18	PA	C			G	M/B
Alfalfa SGR1	PA	C	C		G	B
Yellow lupin	P			G	G	B
Pea ABR17	PA	C			G	M
Birch Betv1	PA	C			GC	M/B
Ginseng RNase1	P	C	P			M

White lupin PR10	ETKLVGVNG	GSIGKVTIKI	ETKGDAPNE	QEGKAAKARG	DAFFKAIETY	150
White lupin motifs	<u>M</u> <u>M</u>	<u>P</u> <u>C</u>	<u>C</u>		<u>RG</u> <u>D</u>	(100)
Soybean SAM22	<u>M</u> <u>M</u>	<u>P</u> <u>C</u>	<u>C</u>			(80.3)
Pea RH2	M	P	C	C	M	(77.8)
Bean PR10a			C			(74.6)
Pea ABR18	M		C			(74.6)
Alfalfa SGR1	M	P	P	C	RG	DM
Yellow lupin	M		C	C/P	M	(69.4)
Pea ABR17	M	P	C		M	(66.8)
Birch Betv1		P	C			(64.1)
Ginseng RNase1		G	C		P	(41.8)

Figure 1. Common motifs observed among the PR-10 amino acid sequences with highest homology: soybean SAM22 (Crowell *et al.*, 1992), pea RH2 (Mylona *et al.*, 1994), bean PR-10a (Walter *et al.*, 1990), pea ABR18 (Itturriaga *et al.*, 1994), alfalfa SGR1 (Truesdell and Dickman, 1997), yellow lupin LIR18A (Sirkorski *et al.*, 1995), pea ABR17 (Itturriaga *et al.*, 1994), birch Betv1 (Breiteneder *et al.*, 1989) and ginseng RNase1 (Moiseyev *et al.*, 1997). Motifs are identified at their amino acid positions as derived from the Motifs Program of the Genetics Computer Group, and designated as: A, cAMP- and cGMP-dependent protein kinase phosphorylation site; B, pathogenesis-related Betv1 family signature; C, casein kinase II phosphorylation site; G, N-glycosylation site; M, N-myristoylation site; P, protein kinase II phosphorylation site; RDG, cell attachment site. Motifs found in the white lupin PR-10 sequence are underlined. Values in parenthesis indicate % amino acid sequence similarities among the 11 cDNA clones.

Isolation of the native protein and partial amino acid sequencing

Root protein extracts were subjected to SDS-PAGE, then transferred to a polyvinylidene difluoride membrane (Matsudaira, 1987). The Coomassie-stained 17 kDa protein was excised and subjected to automated gas phase sequencing in an Applied Biosystems ABI Model 470A sequenator equipped on-line with an ABI Model 130A phenylthiohydantoin analyser (Hewick *et al.*, 1981). All experiments on the ribonucleolytic activity of the native PR-10-like protein were conducted using the SDS-PAGE-purified 17 kDa protein.

cDNA cloning

One degenerate inosine-containing oligonucleotide primer, 5'-AT(T/C/A)TT(T/C)ACITT(T/C)GA(A/G)GA(T/C)GA(A/G)GTIAC-3', was synthesized based

on the N-terminal amino acid sequence of lupin PR-10 protein. And a second oligonucleotide primer was based on an internal region with a conserved amino acid sequence among other plant PR-10 proteins, 5'-CCIGGCCICCC(G/A)TTICCC(C/T)TC-3'. These were used for PCR amplification at an annealing temperature of 40 °C for 35 cycles of polymerization using *Taq* polymerase (Biocan Scientific). The PCR products were analyzed by electrophoresis, purified from an agarose gel, and then cloned into *pCRII* vector (Invitrogen). A clone containing a 144 bp fragment, which had a high sequence similarity to PR-10 genes, was identified by DNA sequencing and used for screening a cDNA library prepared from 10-day old *L. albus* roots (Attucci *et al.*, 1995). The screening of 2×10^5 lambda phage plaques, at a moderate stringency, yielded positive clones which were purified by further rounds of plating and hybridization. Plasmids were isolated by *in vitro* excision, using Exassist helper

phage (Stratagene) and a full-length cDNA clone, as determined by its DNA sequence and comparison with other PR-10 genes, was selected for further characterization. The nucleotide sequence was determined by the dideoxynucleotide method for double-stranded DNA using the Sequenase kit (US Biochemicals).

Northern blot analysis

Total RNA samples, 15 μ g each, were electrophoresed using a formaldehyde denaturation agarose gel and transferred onto Hybond-N nylon membranes (Amersham). The blots were prepared for hybridization with *LaPR-10* probe (Multiprime kit, USB) and washed according to Sambrook *et al.* (1989). The blots were then exposed to Fuji RX film with intensifying screens at -80°C for 1 day.

Southern blot analysis

DNA was isolated from 2-week old roots (Della-porta *et al.*, 1983), and 10 μ g samples were digested individually with 100 units each of the restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III, *Hin*fI, *Stu*I and *Xho*I (MBI Fermentas). The products were electrophoresed in 0.8% agarose gel and transferred by capillary blotting onto nylon membranes (Amersham). Radiolabelling of the PR-10 probe was carried out by the random priming method using the Amersham Multiprime Labelling system, and the membrane was hybridized as described by Sambrook *et al.* (1989). After hybridization, the blot was washed at room temperature for 5 min in $2\times$ SSC containing 0.5% SDS, for 15 min in $2\times$ SSC containing 0.1% SDS, for 30 min at 37°C in $0.1\times$ SSC containing 0.5% SDS, and for 1 h at 68°C in $0.1\times$ SSC containing 0.5% SDS, then rinsed in $0.1\times$ SSC before exposure on a Fuji RX film at -80°C for 1 day.

Subcloning the coding region of the cDNA insert into the *pTrcHisC* expression vector

An oligonucleotide primer (5'-TTTCGAAGGATCCGTATTTTT-3') was designed to incorporate a *Bam*HI site at the beginning of the ORF, starting at nucleotide 71 in the cDNA insert of *LaPR-10*; it changes only the first two codons of the coding sequence. The cDNA clone possesses a natural *Xho*I restriction site at nucleotides 574–579 in the 3' UTR. *Taq* polymerase (Biocan) was used for PCR amplification at an annealing temperature of 55°C , using the N-terminal primer in conjunction with the M13 universal primer. After

digestion with *Bam*HI and *Xho*I, the PCR product was ligated to the *pTrcHis-C* vector (Invitrogen), and used to transform the *Escherichia coli* TOP10 strain.

Expression and partial purification of *LaPR-10* protein

A single bacterial colony of *LaPR-10* transformant was grown in Luria-Bertani (LB) medium containing ampicillin (100 μ g/ml) at 37°C , and allowed to grow until reaching an OD_{600} of 0.7. Expression of the fusion protein was induced by the addition of 1 mM isopropyl-1-thio- β -D-galactopyranoside, followed by a 2 h incubation. After centrifugation (10 min, $2000\times g$), the bacterial pellet was sonicated at 0°C and recentrifuged. The supernatant was chromatographed on a Ni-NTA affinity resin according to the manufacturer's instructions (Qiagen), and the affinity-purified protein was passed through an Amicon cell (YM10 membrane) in order to concentrate the sample and remove imidazole.

Nucleic acid degradation

The SDS-PAGE-purified 17 kDa protein was assayed for RNase and DNase activity for 1 to 6 h at room temperature. The reaction mixtures contained 6 μ g of wheat mRNA, or 15 μ g each of total RNA or DNA prepared from white lupin roots, or *Serratula tinctoria* (Asteraceae) leaves which served as a control. The proteins were removed from the reaction mixtures by extraction with phenol-chloroform, and the nucleic acids were separated on 1.2% agarose gels for RNA or 0.8% gels for DNA, then stained with 0.003% ethidium bromide.

Ribonuclease activity in acrylamide gels

Acrylamide gels were prepared under RNase-free conditions according to the method of Yen and Green (1991), using Torula yeast tRNA as the substrate with some modifications. Electrophoresis of protein preparations, denatured with 8 M urea, was performed under SDS-denaturing, non-reducing conditions using 15% acrylamide separating gels containing 2.4 mg/ml yeast tRNA, and 4% stacking gel. The acrylamide gels were pre-run and the buffer changed before sample loading. After protein separation, the gels were washed twice with aqueous 25% isopropanol to remove SDS and residual urea, and twice in water to remove the isopropanol, followed by incubation in water for at least 1 h at room temperature. The gels were stained

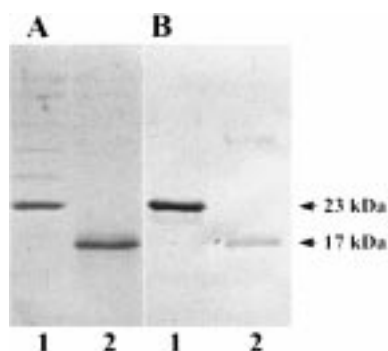


Figure 2. SDS-PAGE profiles (A) and immunoblots (B) of white lupin PR-10 proteins. Lane 1, Ni-affinity-purified recombinant protein (23 kDa); lane 2, SDS-PAGE-purified native protein (17 kDa). Both wells were loaded with 5 μ g protein and stained with Coomassie blue in A. Proteins of a duplicate gel were transferred onto a nitrocellulose membrane and immunodetected with anti-ABR18 antibody (1:2500 dilution) in B, as described in Materials and methods.

with 0.2% toluidine blue in the incubation buffer for 10 min (Yen and Green, 1991), destained in water (1 $\times$ 10 min and 2 $\times$ 20 min), equilibrated in 10% glycerol and finally mounted in cellophane. Proteins exhibiting RNase activity were seen as bright bands against a blue background. BioRad pre-stained protein markers were used to evaluate the apparent molecular weights.

Results

Isolation and characterization of lupin root LaPR-10

SDS-PAGE of 10-day old healthy lupin root tissue showed an abundant protein which migrates at a molecular mass of 17 kDa. Microsequence analysis yielded a 10 amino acid sequence [Gly-Ile-Phe-Thr-Phe-Glu-Asp-Glu-Ser-Thr] near its N-terminus, which exhibited 70% amino acid sequence identity with the stress-induced soybean H4 protein (Crowell *et al.*, 1992), a member of the PR-10 family. One degenerate oligonucleotide based on that sequence, and a second primer corresponding to the protein's P-loop structure, Gly-Xaa-Gly-Gly-Xaa-Gly (Gajhede *et al.*, 1996) that was deduced from a conserved region of other plant PR-10 proteins (Figure 1), were synthesized. A 144 bp fragment with high sequence similarity to the PR-10 family was obtained by PCR amplification, and was used to screen the lupin root cDNA library. A full-length cDNA clone was isolated and subsequently sequenced. The 772 bp clone contains a single open

reading frame (ORF) which encodes a 158 amino acid PR-10-like protein (Bantignies *et al.*, 1998). The predicted translation product of the clone has a calculated molecular mass of 16905 Da and an isoelectric point of 4.66. The absence of a hydrophobic N-terminal region in the protein sequence, characteristic of a signal peptide, as well as the absence of cysteine residues (Figure 1), indicate that this protein is likely intracellular (van Loon *et al.*, 1994).

Sequence analysis

In order to identify residue that may be important for the structure and biological function(s) of the lupin PR-10 protein, a protein database search using the PILEUP and MOTIFS programs from the Genetics Computer Group was conducted with the *LaPR-10* ORF. This search revealed significant (ca. 33–80%) similarity to 20 related amino acid sequences (data not shown); the highest similarity (ca. 67–80%) was observed among the genes that were previously identified from the same family, the Leguminosae (Figure 1). In addition, the alignment of these sequences revealed the presence in the lupin sequence of a number of well conserved motifs (Aitken, 1990), as was also the case for several PR-10 proteins with high amino acid sequence similarity (Figure 1). These motifs include the N-glycosylation site NYSI, several phosphorylation and N-myristoylation sites, and the cell-attachment determinant tripeptide RGD that is shared only with the alfalfa SRG1 sequence (Truesdell and Dickman, 1997) (Figure 1). Noted by their absence in the lupin PR-10 protein, pea ABR17, and ginseng RNase 1 are the Betv1 pathogenesis-related family signature, as well as the amino acids cysteine and tryptophan (Figure 1).

Expression of LaPR-10 in E. coli and properties of LaPR-10 proteins

Expression of the recombinant form of *LaPR-10* in *E. coli* strain Top 10 was carried using the *pTrcHisC* vector, which gave rise to a fusion product with a polyhistidine tag as part of a short leader sequence of the N-terminus of the protein. The affinity-purified recombinant protein migrated at 23 kDa in SDS-PAGE (Figure 2A). Both the native (17 kDa) protein, isolated from lupin roots, and the recombinant protein were immunorecognized by polyclonal antibodies raised against pea ABR18 (Itturriaga *et al.*, 1994) PR-10 proteins, although other minor immunoreactive bands were also observed for the recom-

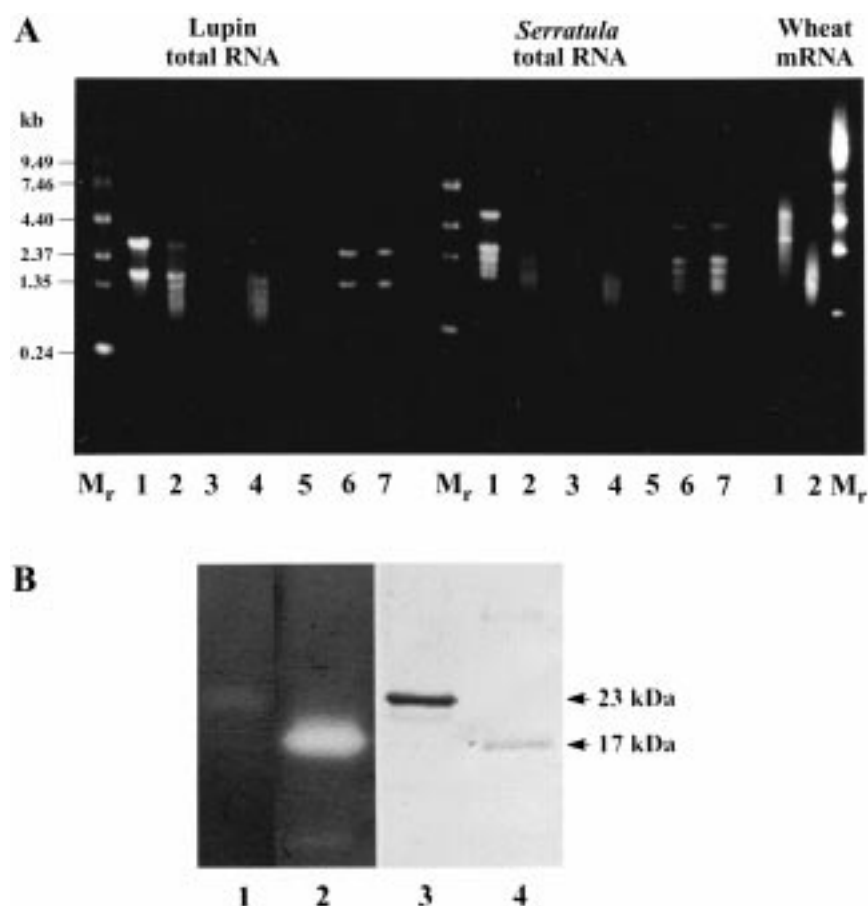


Figure 3. A. Ribonuclease activity of the recombinant LaPR-10 gene product (10 μ g) tested against lupin root total RNA (5 μ g), *Serratula* leaf total RNA (5 μ g) and wheat germ mRNA (6 μ g). After removal of protein, the RNA hydrolysis products were separated in 1.2% agarose gels as described in Materials and methods. Lanes: 1, RNA of the indicated tissue; 2, RNA+PR-10, 3 h incubation; 3, RNA+PR-10, 6 h incubation; 4, RNA+PR-10+RNasin, 3 h incubation; 5, RNA+RNase A, 10 min incubation; 6, RNA+protein of *E. coli* which does not contain the cDNA clone; 7, RNA+PR-10 in acetate buffer pH4.8. M_r, molecular weight markers (kb). B. RNase activity of the recombinant (lane 1) and native (lane 2) PR-10 proteins (10 μ g each) in acrylamide gels containing 2.4 mg/ml yeast tRNA. The RNase-positive bands (arrows) were eluted, and re-electrophoresed in SDS-PAGE for immunodetection with anti-ABR18, prepared as in Figure 2B: lane 3, recombinant protein (arrow); lane 4, native protein (arrow).

binant protein preparations (Figure 2B). Extracts of *E. coli* containing the vector without the insert gave negative results (data not shown). In addition, neither the native nor the recombinant proteins were stained with the glycoprotein Schiff's base reagent (Allen, 1994), indicating that they are not glycosylated (data not shown).

Ribonuclease activity of LaPR-10 protein

RNase activity of LaPR-10 was assayed using two different methods. In the RNA degradation assay, the recombinant protein exhibited ribonucleolytic activity against total RNA isolated from lupin roots and *Serratula tinctoria* leaves, as well as wheat mRNA.

Significant ribonucleolytic activity was shown by the migration of the degradation products in the agarose gel (Figure 3A, lanes 2 and 3). A control protein sample, which was purified by the Ni-NTA resin from a culture of *E. coli* without the *LaPR-10* cDNA clone, did not degrade any of the RNA samples tested (Figure 3A, lane 6). On the other hand, RNasin, a specific inhibitor of pancreatic-type RNases (Hofsteenge, 1997), partially inhibited the RNase activity of the recombinant protein (Figure 3A, lane 4). The degradation of total RNA of lupin root or *Serratula* leaf was inhibited in 10 mM acetate buffer pH 4.8 (Figure 3A, lane 7), but not in citrate buffer of the same molarity and pH (data not shown). There was no nucleic acid

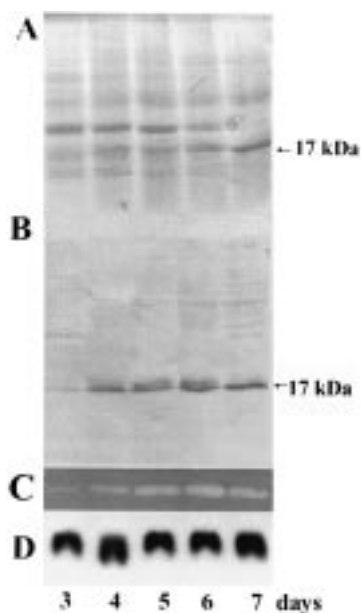


Figure 4. Characterization of white lupin root PR-10 protein during early development (3–7 days). A. Coomassie-stained SDS-PAGE profiles (10 μ g protein). B. Immunoblots of A, prepared as in Figure 2B. C. RNase activity in yeast-acrylamide gels (10 μ g protein), prepared as in B. D. Northern blot of *LaPR-10* transcripts prepared with equal amounts (15 μ g) of total RNA as described in Materials and methods.

degradation of either lupin root or *Serratula* leaf DNA (data not shown).

Both the native and recombinant proteins were found to degrade yeast RNA in polyacrylamide gels (Figure 3B, lanes 1 and 2). In order to verify the identity of the enzymatically active proteins, the latter were eluted from the acrylamide gels and re-electrophoresed. Both the native and recombinant proteins migrated at their expected molecular size, and were immunorecognized by the anti-ABR18 antibody (Figure 3B, lanes 3 and 4).

The RNase activity of the 17 kDa protein is insensitive to EDTA (ca. 1–10 mM), has no requirement for the divalent cations Ca^{2+} , Mg^{2+} or Mn^{2+} , and is completely inhibited by DTT (data not shown). Furthermore, the enzyme reaction can be catalyzed in water or Tris-HCl buffer, with an optimal activity at pH 6.0–6.5 and pH 7.5, respectively.

Accumulation of LaPR-10 protein and RNase activity during development

Coomassie-stained SDS-PAGE profiles of lupin root proteins show that the 17 kDa *LaPR-10* band gradually increased after germination, and reached a maximum

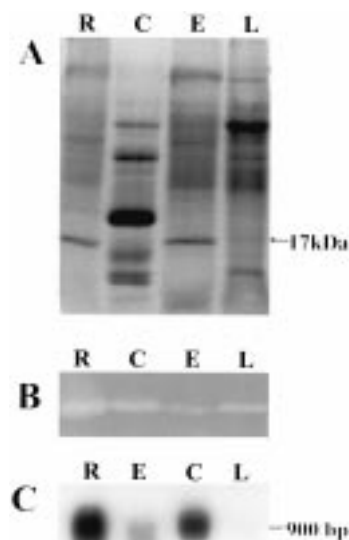


Figure 5. Expression of PR-10 protein in different organs of 8-day old lupin seedlings. A. Coomassie-stained SDS-PAGE (10 μ g protein). B. RNase activity against yeast tRNA in acrylamide gel (5 μ g protein), prepared as in Figure 2B. C. Northern blot of *LaPR-10* transcripts (15 μ g total RNA). Plant organs: R, root; C, cotyledon, E, epicotyl; L, leaf.

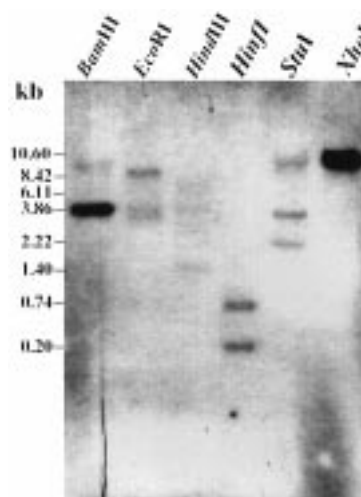


Figure 6. Southern blot analysis of *LaPR-10* gene. Genomic DNA was isolated from white lupin, digested with the indicated restriction enzymes, and the membrane was probed with a 721 bp *EcoRI-XhoI* fragment of *LaPR-10*. M_r markers are indicated on the left.

accumulation 6–7 days after the seeds were sown (Figure 4A), and this finding is also corroborated by western blot analysis (Figure 4B). However, the amount of *LaPR-10* protein gradually declines over the next several weeks during root development (data not shown), possibly as a result of a slow protein turnover process. Acrylamide gels containing yeast

RNA show that the PR-10 protein of 3–7-day old roots exhibits a ribonucleolytic activity (Figure 4C) as determined by the method of Yen and Green (1991). In addition, northern blot hybridizations performed with lupin root total RNA show a high abundance of the *LaPR-10* transcripts at all stages of early root development (Figure 4D), including the seeds after 24 h soaking (data not shown). However, the increasing protein accumulation over time (Figure 4A) as compared with the relatively constant levels of mRNA transcripts (Figure 4D) suggest that protein levels are regulated post-transcriptionally. The PR-10 protein can be detected, but in smaller amounts than the roots, in the epicotyls>cotyledons>leaves (Figure 5A); these tissues also exhibited RNase activity in the gel electrophoresis assay (Figure 5B). Northern blot analysis indicated that the mRNA transcript levels were highest in the roots, and were also detected in the cotyledons and hypocotyls (Figure 5C).

Southern blot analysis

For Southern hybridization analysis, genomic DNA isolated from white lupin was probed with a 721 bp *EcoRI-XhoI* fragment from the *LaPR-10* cDNA sequence. The latter is devoid of restriction sites for *BamHI* and *XhoI*, and these enzymes produced bands with 3.86 kb and 10.60 kb, respectively, which hybridized with the *PR-10* probe (Figure 6). Digestion with the endonuclease *HinfI* which is present once in the cDNA sequence, and with *HindIII* which has two sites within 10 bp of each other, produced 2–3 bands which hybridized with the probe, each with a molecular size ranging from 0.2 to 6.11 kb (Figure 6). Although there are no restriction sites for the enzymes *StuI* and *EcoRI* within the cDNA sequence, 2–3 of the bands hybridized to the *PR-10* probe suggesting the presence of introns in the genomic sequence. Taken together, these simple restriction patterns, especially the two small fragments from the *HinfI* digestion, suggest that *LaPR-10* exists as a single-copy gene per haploid genome, although the restriction fragment pattern does not exclude the possibility of the presence of a small gene family.

Discussion

In this paper, we report on the isolation and characterization of a 17 kDa protein and its corresponding cDNA clone, designated as *LaPR-10*, from healthy

white lupin roots. This cDNA clone encodes a polypeptide belonging to the PR-10 family (van Loon *et al.*, 1994) that shares high (67–80%) amino acid sequence similarity with those reported from the Leguminosae, and 45% similarity with the parsley PR1 (Somssich *et al.*, 1988), the type member of this ubiquitous class of defense-related proteins. It is no surprise, therefore, that the predicted protein sequence shares some 40 conserved amino acid residues with the members of this family, as compared with only five residues with those of the 12 other unrelated species (data not shown). The fact that *LaPR-10* protein is constitutively expressed primarily in the root, and that its amino acid sequence lacks the pathogenesis-related motif (Breiteneder *et al.*, 1989), designates it as a PR-10-like member of the PR-10 superfamily (van Loon *et al.*, 1994).

Both the recombinant protein expressed in *E. coli* and the 17 kDa native protein purified from the roots were shown to cleave several RNA preparations, including lupin root total RNA, as demonstrated by two different techniques (Figure 3A, B). This feature has only been reported with the birch pollen Betv1 proteins, both in the native and recombinant forms (Bufe *et al.*, 1996; Swoboda *et al.*, 1996). Most of the published reports on PR-10 proteins infer a putative RNase function based on some amino acid sequence similarity to the ginseng callus RNases 1 and 2 (Moiseyev *et al.*, 1994). However, neither of the ginseng PR-10-like cDNA clones (Moiseyev *et al.*, 1997) has been expressed in a heterologous system, nor has the catalytic activity of the gene products been demonstrated in order to justify the claim that these cDNA clones encode RNases. In addition, the ginseng RNase1 sequence shares only 42% similarity with that of lupin, and lacks a number of conserved motifs known to occur in legume PR-10 proteins (Figure 1).

The recently reported X-ray and NMR structures of birch pollen Betv1 (Gajhede *et al.*, 1996) indicate the presence of a P-loop structure with the sequence GXGGXG (positions 47–52), which is well conserved in the lupin *LaPR-10* (Figure 1), as well as in other PR-10 proteins. This P-loop structure resembles a motif found in many nucleotide-binding proteins (Saraste *et al.*, 1990) and, therefore, may indicate a phosphate-binding site, a likely place for the binding of an RNA phosphate group that may be correlated with a ribonucleolytic activity. The fact that this ribonucleolytic activity was only partially inactivated by the human placental ribonuclease inhibitor (RNasin) indicates that its specificity may be targeted to an RNase

type different from the pancreatic-type ribonuclease A. This is also corroborated by the inhibition of enzyme activity in acetate buffer (Bariola and Green, 1997). This ribonucleolytic activity is present during all stages of root development and, to a variable extent, in other organs of the plant. In addition, it has no signal peptide in its sequence, it cleaves all types of RNA tested with a pH optimum of 6.0 to 7.5, is insensitive to EDTA, and does not require divalent cations for activity. Therefore, this RNase activity may be considered a class II-type ribonuclease according to the traditional biochemical classification (Bariola and Green, 1997).

A common feature, which has not previously been highlighted in the PR-10 protein family, is the presence of a number of conserved motifs that may be related to specific functions. White lupin LaPR-10 shares with the other plant PR-10 proteins several phosphorylation sites that are characteristic of cAMP-dependent protein kinases, as well as casein kinase II and protein kinase C (Figure 1). These motifs are indicative of an intracellular regulatory role for this protein in a variety of metabolic processes (Aitken, 1990). Another motif of frequent occurrence in PR-10 protein sequences involves the covalent N-myristoylation of glycine, an abundant amino acid residue in these proteins (Figure 1). Although this post-translational modification has not been well characterized in plants, it is believed to play a role in the modification of cellular activities, especially in the association of the cytosolic G-proteins with the plasma membrane of the cell in response to extracellular signals (Borgese et al., 1996; Vaandrager et al., 1996). However, the conservation of these consensus motifs among most PR-10 proteins may serve as a diagnostic feature of this important gene family.

In spite of the fact that the LaPR-10 is likely an intracellular unglycosylated protein, which lacks a signal peptide for protein targeting, its sequence contains a single N-glycosylation consensus sequence, namely Asn-Xaa-Ser/Thr (positions 82–84, Figure 1). Whereas this motif is conserved in most of the PR-10 sequences examined, it does not necessarily mean that these proteins are glycosylated, since protein folding plays an important role in the regulation of N-glycosylation (Pless and Lennarz, 1977). In addition, it has been shown that about 50% of the sites that have a proline (replaced by isoleucine in most PR-10 proteins) residue C-terminal to a serine, are not glycosylated (Bause, 1983).

One rare motif present in the LaPR-10 protein sequence is the cell surface attachment tripeptide RGD

(Ruoslahti and Pierschbacher, 1987). This sequence, which is also conserved in the alfalfa SGR1, is crucial for its interaction, with a surface receptor, an integrin, and plays a role in cell-substratum adhesion which is mediated by various adhesive proteins including fibronectin, collagens and slime-mold discoidins. Furthermore, the RGD motif was found in a pea protein with receptor activity (Swart et al., 1994) for the Ca^{2+} -dependent, rhizobium-specific rhicadhesin (Smit et al., 1989).

The lupin root PR-10 protein was neither inducible by *Botrytis cinerea*, a fungus not known to cause a disease to white lupin, nor by the lupin's compatible symbiont, *Rhizobium lupini*; control experiments were also used (data not shown). This feature is corroborated by the absence of the Betv1 pathogenesis-related motif in its protein sequence (Breiteneder et al., 1989). However, its high level of constitutive expression in lupin roots implies a role in the plant defense mechanism. It is conceivable, therefore, that such a cytosolic protein with ribonucleolytic activity could operate on the invading pathogen after its successful penetration into the root tissues, which may account for the relative resistance of white lupin to pathogens (Hill, 1997).

To our knowledge, this is the first report of a PR-10-like protein with RNase activity from a leguminous species. Its ribonucleolytic activity has been demonstrated unequivocally in the recombinant protein, as well as in the purified native protein. The fact that it is strongly expressed during all stages of root development, as well as in other organs, suggests that it is a component of the constitutive, pre-infectional repertoire of the plant's defense mechanism.

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