

Expression of genes encoding PR10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules[☆]

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Abstract

Pathogenesis-related proteins of the PR10 class have been found in many plant species, are induced under various stress conditions and act as common allergens. Here we demonstrate the presence of two PR10 proteins in yellow lupine (*Lupinus luteus* L. cv. Ventus). Both 17 kDa proteins, referred to as LIPR10.1A and LIPR10.1B, are composed of 156 amino acids, and have 76% parities identity (91% similarity). Identity to homologues from other plants ranges from 25 to 67% (46–82% similarity). Patterns of their expression in lupine organs and tissues were investigated using Western blotting and immunocytochemistry. Both proteins are constitutively expressed in roots, but expression is significantly decreased in young and mature root nodules (9–26 days post infection (dpi)), but not in senescent nodules (36 dpi). Immunocytochemical staining localised the proteins in the parenchymatous tissues of the root and senescent nodule, primarily in the cortex. The PR10 proteins were not detected in nodule bacteroid tissue. Expression in aerial parts of the plant is generally lower and only one of the proteins, LIPR10.1B, is expressed constitutively in the stem, leaf and petiole, while the other, LIPR10.1A, is only present in the stem and is induced in senescent leaves. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

One of the important features of plant response to stress is the increased expression of distinct proteins, including chitinases, glucanases, enzymes of phenylpropanoid pathway, thionins, osmotins, peroxidases, protease inhibitors, proteases, proline-rich glycoproteins and proteins of unknown function. The stress-induced proteins, described as ‘pathogenesis-related’ (PR) have been classified into 12 groups according to their properties or sequence homology [1]. Proteins of the PR10 class

seem to be ubiquitous in the plant kingdom, their homologues found in various species belonging to both dicotyledonous and monocotyledonous plants [2]. Also, based on sequence homology, common allergens present in birch pollen grains [3], celery [4] and apple [5] are included in this group. The PR10 proteins are small (around 17 kDa), slightly acidic and resistant to proteases. Although a precise cellular localisation of PR10 proteins has not been determined, the absence of an apparent signal peptide and their structural properties indicate that they are cytosolic [6,7]. Tertiary structure of the birch pollen allergen Betv1 was recently determined by X-ray crystallography [8]. The protein contains a long C-terminal α -helix, surrounded by a seven-stranded β -sheet. Additionally, two shorter α -helices are located near the N-terminus. The amino acid se-

[☆] The nucleotide sequences of the full-length cDNA clones LIPR10.1a and LIPR10.1b were registered in the EMBL GenBank under accession numbers X79974 and X79975.

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quence analysis of almost 90 related PR10 proteins revealed the presence of conserved glycine residues (at positions 46, 48, 49 and 51 in Betv1) that form a so-called glycine reach loop (P-loop). This motif has been found in many nucleotide binding proteins [9]. An additional lysine residue at position 54 of Betv1 may be responsible for binding of the phosphoryl group of the nucleotide. The position of the P-loop on the surface of the PR10 protein molecule suggests that it is important for its biological function.

Several laboratories have reported that PR10 proteins accumulate around sites of pathogen invasion [10–15], wounding [16,17] and are induced by other environmental stress [6,18], suggesting their involvement in a general defence mechanism. The physiological function and any contribution of PR10 proteins to a defence mechanism remain unknown. However, high amino acid sequence homology and the similarity of the expression pattern with that of ginseng ribonuclease suggest that an RNase activity associated with these proteins may be involved in the defence reaction [19,20]. It has recently been shown that birch pollen allergen Betv1 has RNase activity *in vitro* [21,22]. Because of amino acid sequence similarity, PR10 proteins have been classified as ribonuclease-like PR proteins [1]. There are also suggestions that PR10 proteins play an important role in plant development since they have been identified in dry seeds [7,23], constitutive expression was observed in roots [18,24–26], and many plants have the constitutive expression of PR10 proteins in their stems [7] or various parts of the flowers [3,7,17,27–29]. Senescent leaves also often have elevated levels of PR10 protein expression [18]. Some PR10 homologues appeared to be induced by phytohormones like cytokinin [30], abscisic acid [31] or ethephon, an ethylene releasing compound [32].

Major latex proteins (MLPs), induced by wounding in bell pepper [33], also present in opium poppy, melon, strawberry, *Arabidopsis* and tobacco, have low (much less than 25%) homology to PR10 proteins. However, secondary structure predictions for PR10 and MLP groups are similar, suggesting that weak homology between them may be significant [34]. Interestingly, these two groups of proteins have never been found to coexist in one plant species.

The interaction between legume plants and rhizobia results in the formation of a new plant organ, the nitrogen-fixing root nodule, where bacteria are present intracellularly, in so-called infected cells. These cells are completely packed with bacteria and obvious host defence response does not occur. The surface determinants of rhizobia (lipopolysaccharides, LPS and exopolysaccharides, EPS) as well as the host membrane, that always separates the bacteria and host cytoplasm, most likely control the avoidance of the defence response in symbiotic interactions [35–37].

Although, rhizobia appear to be successful in avoiding defence reactions in nodules, it is striking that even in the wild type interaction defence responses are induced [38]. Elicitation of defence mechanisms in roots by symbiotic bacteria may be a part of the mechanism by which the plant controls infection and, therefore, regulates nodulation (feedback control of nodulation). It has been shown by immunolocalisation assay that PR proteins associated with the defence mechanism in plants accumulated in the necrotic cells induced by rhizobia. However, a number of defence genes, enzymes of phenylpropanoid pathway, peroxidases, chitinases and PR proteins, were constitutively expressed in uninfected roots of both non legume and legume plants. Therefore, it can be proposed that the constitutive defence mechanism is a part of the root developmental program. The expression of pea RH2 protein in root epidermal cells [26] represents a good example of the gene encoding pathogenesis-related protein of PR10 class which is developmentally regulated and shows organ-specific expression.

In contrast to pathogenic invasion which leads to cell necrosis and cell death, symbiotic bacteria are beneficial to their host plant. There may be similarities between pathogenic and symbiotic modes of infection and at least some elements of the molecular recognition mechanisms may be common to both types of interactions. It is postulated that rhizobial symbionts can be considered as ‘prokaryotic plant parasites’ [39], ‘refined parasites of legumes’ [40] or ‘sympathogenesis’ [41]. During the early stages of plant-rhizobia interaction the symbiotic bacteria are recognised as a pathogen and the plant defence mechanism is normally operating. In the latter stages of symbiosis development, when the process of nodulation becomes beneficial to host plant, the suppression of

the plant defence mechanism is observed. The ability of plants to distinguish between beneficial and parasitic interactions is an example of their genome adaptation to diverse environmental conditions. Since there is only a single publication available on the expression of gene encoding PR10 protein *MtN13* during symbiosis development in *Medicago truncatula* root nodules [42], we decided to describe in this paper the characterisation of two yellow lupine proteins of class PR10 and their corresponding cDNA clones. We also present their expression pattern and localisation studies in roots and developing nodules after inoculation with the symbiotic bacterium *Bradyrhizobium* sp. (*Lupinus*).

2. Materials and methods

2.1. Plant growth conditions

Lupinus luteus L. cv. Ventus plants were grown in sterile perlite at 23°C with a 16 h day and 8 h night photoperiod. One day old imbibed seeds were inoculated with *Bradyrhizobium* sp. (*Lupinus*), strain USDA 3045.

2.2. Protein extraction and purification

The extraction and purification of soluble proteins from plant tissues was carried out according to the procedure described by Sikorski et al. [43] with some modifications of chromatographic steps: 3-day-old lupine seedlings were frozen in liquid nitrogen and ground to a powder using a mortar and pestle and 1 g tissue was suspended in 3 ml buffer E (50 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5 M sucrose). The homogenate was left on ice for 1 h and extraction was carried out by gentle stirring. The protein extract was then centrifuged twice at 15 000 × *g* and 30 000 × *g* for 15 min at 4°C. The supernatant was collected and fractionated by ammonium sulphate precipitation. The fraction of 40–80% saturation containing PR10 proteins was dialysed against buffer D (20 mM Tris–HCl (pH 8.0), 5% glycerol, 10 mM β-mercaptoethanol). For further purification of the PR10 proteins the 40–80% ammonium sulphate fraction was applied to a 30 ml DE52 cellulose column and the proteins were fractionated by stepwise elution with one column volume each of

buffer D containing 0.05, 0.1, 0.3 and 0.5 M NaCl. Fractions eluted with 0.3 M NaCl were pooled, dialysed against buffer D and submitted to a MonoQ HR 10/10 column in the linear gradient of 0–1 M NaCl in buffer D. Fractions eluted with 0.1–0.2 M NaCl, containing LIPR10.1A and LIPR10.1B proteins, were concentrated and 5.0 mg protein samples were separated by size exclusion chromatography on a Superose 6 HR 10/30 column (column buffer D containing 0.05 M NaCl). The PR10-containing fractions were purified to homogeneity by reversed phase chromatography on a Pro RP HR 5/10 column in a linear gradient of 0–80% acetonitrile in the presence of 0.1% trifluoroacetic acid (TFA) and 0.01% β-mercaptoethanol (sample containing 1.0 mg of protein was treated with 10% HCOOH, centrifuged and applied to the column). The LIPR10.1A protein was eluted at 48.8% CH₃CN and LIPR10.1B at 43.5% CH₃CN.

2.3. Screening of yellow lupine cDNA library

The yellow lupine cDNA library was constructed in UNI ZAP XR expression vector (Stratagene). The poly(A)⁺ RNA used for cDNA synthesis was isolated from lupine roots of non infected plants, harvested 8-, 15- and 21-days after germination. The amplified library contained 3.5 × 10¹⁰ pfu per 1 ml (represents 3.0 × 10⁶ recombinants). Two synthetic deoxyoligonucleotide probes: Oligo A (TTYGCNTTYGARAAY-GARCA, 20-mer, 128 × degenerated) and Oligo B (TTYGCNTTYGARGAYGA, 17-mer, 64 × degenerated) were chemically synthesised based on the determined N-terminal amino acid sequences of LIPR10.1A and LIPR10.1B proteins. The probes were labelled with γ-[³²P]ATP (S.A., 5000 Ci/mmol) using T4 polynucleotide kinase as described by Sambrook et al. [44] and used for the screening of yellow lupine root cDNA.

2.4. Northern hybridisation

Total RNA from the roots and nodules at different stages of development was prepared by the acid guanidine isothiocyanate–phenol–chloroform extraction procedure described by Chomczynski and Sacchi [45]. The RNA blots were hybridised to ³²P-labeled 3' end cDNA probes generated by *Bam*HI/*Xho*I digestion of the entire

cDNA clones encoding either LIPR10.1A or LIPR10.1B (an internal *Bam*HI cleavage site was used to split the cDNA to ensure the specificity of probes).

2.5. Western blot analysis of soluble protein extracts

The LIPR10.1A protein has been overexpressed in *Escherichia coli* expression system using vector pET-3a and purified to homogeneity [46]. The homogenous recombinant protein was used as an antigen to inject a female New Zealand rabbit. The injection was carried out with 100 µg of protein with Freund's complete adjuvant (1:1, v/v) and repeated 2 and 4 weeks later with the same amount of protein. Antiserum was collected 2 weeks after the last boost.

Soluble proteins for Western blot were extracted from the plant tissue with buffer containing 0.1 M sodium phosphate (pH 7.5), 10% glycerol and 10 mM β-mercaptoethanol (3 ml per 1 g of tissue) using a mortar and pestle. The homogenate was centrifuged twice at $15\,000 \times g$ for 15 min to remove cell debris. The protein content was measured according to Bradford [47] and electrophoresis was performed in a 15% polyacrylamide gel in the presence of sodium dodecylsulphate (SDS) according to Laemmli [48]. The samples containing 10 ng of recombinant protein or 10 µg of soluble plant protein extract were applied on the gel for Western blot analysis. The separated proteins were transferred onto an Immobilon P membrane, using the MilliBlot-SDE transfer system according to the Millipore protocol, and incubated for 2 h with anti-LIPR10.1A polyclonal antibodies. Immunodetection was carried out with the biotin/streptavidin-AP system of Amersham.

2.6. Immunocytochemical localisation of PR10 proteins

Plant material was sampled 9, 26 and 40 days after bacterial inoculation. Fragments of the roots and roots with nodules were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). The 12 µm sections were cut from the paraffin embedded tissue. After deparaffinization and PBS soaking the sections were incubated in the solution containing rabbit anti-LIPR10A serum in PBS in the presence of 3% bovine serum

albumine for 1.5 h at 37°C. The primary antibody solution was washed out three times for 30 min at room temperature. Further steps of detection were performed using the biotin/streptavidin-AP system from Amersham, according to the standard procedure. Pre-immune serum or PBS was used as negative controls.

2.7. Sequence Analysis

All PR10 protein sequences retrieved from the GeneBank database were aligned using the CLUSTALW program [49]. Alignment of amino acid sequences has been used to construct a neighbour-joining tree, using programs from the PHYLIP package [50]. Percent support for each branch was estimated by analysing 1000 bootstrap resamplings. The tree was displayed with TREEVIEW [51]. Alignment of the amino acid sequences was displayed using GENEDOC [52].

3. Results

3.1. Identification and purification of yellow lupine PR10 proteins

Electrophoretic analysis of soluble protein extracts from yellow lupine 3-day-old seedlings led to the identification of two abundant ca. 17 kDa proteins, LIPR10.1A and LIPR10.1B. Both proteins were purified from 3-day-old seedlings according to the flow diagram presented in Fig. 1. Sequence analysis of N-terminal 30 amino acid residues of LIPR10.1A and LIPR10.1B revealed a high level of similarity to intracellular pathogenesis-related PR10 class proteins.

3.2. Screening of lupine cDNA library and identification of cDNA clones encoding PR10 proteins

Full-length cDNA clones Llpr10.1a (775 nt) and Llpr10.1b (727 nt) were isolated from the cDNA library using degenerate deoxyoligonucleotide probes OligoA and OligoB (as described in Section 2) and sequenced. Both clones contained a single open reading frame encoding 156 amino acid polypeptides with molecular weight of 16.9 and 16.7 kDa. The N-terminal regions of the predicted amino acid sequences were identical to the corre-

sponding sequences derived from protein sequencing. The deduced amino acid sequences have significant homology to the PR10 class proteins from other plants and have the conserved amino acids characteristic for this group (Fig. 2A). The putative glycosylation site NYS is located at positions 82–84 of each polypeptide. The conserved glycine-rich motif GXGGXGXXK between residues 46 and 54, described as the P-loop, is considered as a possible phosphate binding site [9]. The hydropathy profiles of the deduced polypeptides (data not shown) show no dominating hydrophobic or hydrophilic regions, suggesting that the PR10 proteins do not contain a signal peptide. The putative polyadenylation signal AATAAA was found in the 3' untranslated region of both cDNA clones.

3.3. Northern analysis of PR10 transcripts

Total RNA was isolated from lupine root tissue at different stages of plant development (dry seeds, imbibed seeds and developing root, up to 30 days) and analysed by northern hybridisation with 3' end fragments of cDNA clones Llpr10.1a and Llpr10.1b. The lack of cross-hybridisation of

probes was confirmed by dot blot analysis (data not shown). As shown in Fig. 3A and C, transcription of both Llpr10.1a and Llpr10.1b starts between 6 and 24 h after imbibition and a high level of both transcripts is detected during the root development of non inoculated plants. Fig. 3B and D present Northern analysis of the transcripts in the infected zone of the root and in the developing nodule. Results for both LIPR10.1A and LIPR10.1B proteins are similar: the bands corresponding to both the *pr10.1a* and *pr10.1b* transcripts appear in the lanes containing the preparations from 3- to 15-day-old infected roots and 36-day-old nodules. The *pr10.1b* transcript is present additionally in 19-day-old nodules. There were no detectable *pr10.1a* and *pr10.1b* transcripts in 22–30- or 19–30-day-old nodules.

3.4. Immunochemical analysis of PR10 proteins in yellow lupine: Western blotting and in situ localisation

Organ specificity was determined by a series of immunoblotting of the protein extracts from the leaves, petiole, stem, root and root nodules. These analyses were performed using rabbit anti-LIPR10.1A antibody, which has approximately the same immunoreactivity with both proteins. Equal amounts of the purified LIPR10.1A and B proteins were placed in two lanes of each blot as a size reference. The general expression pattern is shown in Fig. 4A. LIPR10.1B protein is present in all the tissues tested with approximately equal intensity (slightly lower in the leaves). However the LIPR10.1A protein is present only in the roots and stems but not in the mature leaf or petiole. Details of PR10 protein expression in leaves taken from the upper, middle and lower part of the same plant, are presented in Fig. 4B. The LIPR10.1B intensity varies depending on the stage of leaf development; it is low in the preparations from young and mature leaves, but in senescent leaves it increases. Additionally, the LIPR10.1A protein is present in senescent leaves.

To detect the PR10 proteins at different stages of nodule development, equal amounts of nodule protein extracts (10 µg of total protein) were applied to an electrophoresis gel and treated as above (Fig. 4C). Both LIPR10.1A and LIPR10.1B proteins were present in all stages of development, but PR10.1A is present in larger amounts in

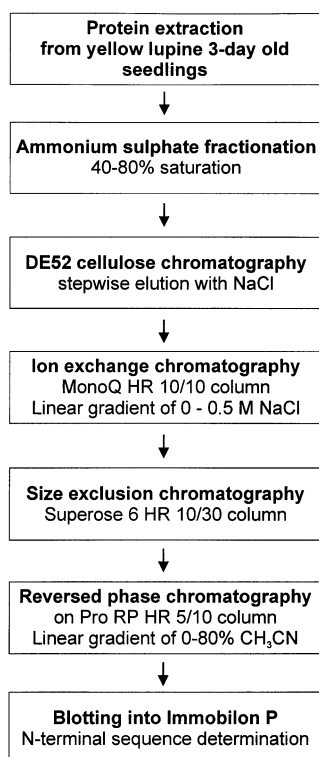


Fig. 1. Flow diagram of yellow lupine PR10 proteins purification.

A.

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*      20      *      40      *      60      *      80      *
LlPR10.1A : MGIFAFENEQSSIVAPAKLYKALTKSDSDEIVPHVI-EPIQNVSEIVEGNGGPGTIKKLIIAI-HDGHTSFVLHKKDAIDEANLTYNYSIIGS : 88
LlPR10.1B : MGVFAFEDHPSAVAQAKLHKALTKSDDIIPKVI-EQIQSVSEIVEGNGGPGTVKKIIAS-HGGHTSYVLHKKDAIDEASFEYNYSIIVGS : 88
PsABR17 : MGVFVFDDSYVSTVAPPKLYKALAKADEIVPHVI-KEAQGVSEIVEGNGGPGTIKKLSIL-EDGKTNVYLHKKDAIDEANFGYNYSLVGS : 88
LaPR10 : MGIFTFEDSSTSTVAPAKLYKALVADANILIPKAV-EAIQSVSEIVEGNGGPGTIKKLTFTI-EDGETKYVLHKKDEIDEANLGYNYSIIVGS : 88
PvPR10A : MGVTTFEDSTTSFVAPATLYKAVAKADTIFPKALPDSFKSVSEIVEGNGGPGTIKKISFV-EDGETKFVLHKKDESIDEANLGYSYSIIVGS : 89
MsPR10 : MGVFNFEDSTTSIVAPARLYKALVTSDNLIIPKVI-DAIQSSEIVEGNGGPGTIKKPTFV-EGGETKYDLHKKVDLDDVNFAYNYSIIVGS : 88
MtN13 : MGVTSESEYVSSLSAEKLYRGIVEGNIIYPKALPRFIEKAETLEGGGPGTIKKLTFTV---GDFGSKQHIDMVDRENCAITYSVYEG : 87
BvBetv1 : MGVFNYETSTTSVIPAARLFKAFILSGDNLFPKVPAPQAISSVENIEGNGGPGTIKKISFP-EGFPFKYVKDRVDEVDHTNFKYNSVIEG : 89
AgApigl : MGVTQTHVLELTSSVSAEKIHQGFVLDVDTVLKPAAPGAYKSVEIK-GDGGPGTLKIIITLP-DGGPITMTLRLDGVNKEALTFDYSVIDG : 88
MdMald1 : MGVTTFENEFTSEIPPSRLKAFVLDADNLIPKIAQAIKQAEILEGNGGPGTIKKITFG-EGSQYGYVKHRIDSDIDEASYSYSYTLIEG : 89
PgRNS1 : -GVQKTEVSEATSTVPAQKLYAGLLLDIDDLKPAFPQAIKSSEIIEGDSGVGTVKLVTLG-EASQENTMKQRIDALDKDALTYYTSIIGS : 88
PgRNS2 : -GVQKTETQAISEFVPAEKLFHKSFLDMDTVPKPAFPEGIKSVQVLEGGVGTIKNVTLG-DATPFNTMKTRIDALDEHAFTYTYTIIGS : 88
StSTH2 : MGVTSTYTHETTTPIAPTRLFKALVVDSDNLIPKLMF---QVKNIEAESD-GSIKMNFEV-EGSPIKYLKHKTHVVDKDLVTKYSMIEG : 84
AoPR1 : MSSGSWSHSEVAVNVAAGRMHKAAMLDWHNLGPKIVPDFIAGGSVSVSGDSVGTIREIKINNPAPFESYVKERLDFVDHDKFEVKQTLIVEG : 90

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100      *      120      *      140      *      160
LlPR10.1A : EGLDES-LEKISYESKILPGPDGSGIGKINVKFHTKGD-V-LSETVRDQAKFKGLGLFKALEGGYVLAHPDY----- : 156
LlPR10.1B : TGLDES-LEKITFESKLLSGPDGSGIGKIKVKFHTKGD-V-LSDAVREAAKARGTGLFKALEGGYVLANPNY----- : 156
PsABR17 : PGLHES-LEKVAFETIILAGSDGGSIVKISVKYHTKGDAA-LSDAVRDETKAGTGILKALEGGYVLANPGY----- : 157
LaPR10 : VGLPDT-VKKITFETKLVEGVNGSGSGIKVTIKIETKGDAA-PNEQEGKAAKARGDAFFKALETYLSAHPDYN----- : 158
PvPR10A : VALPET-AEKITFDSKLSGDPNGGSLIKLSITYHSKGDAP-PNEDELKAGKAKSDSLFKALEGAYLLANP----- : 156
MsPR10 : GGLPDT-VKKITFESKLSAGPDGSGTAKLTVKYFTKGDAA-PSEEEIKGGKARGDGLFKALEGGYVLANPDY----- : 157
MtN13 : IALSDQPLEKIVFEFKLVPTPEEGCIVKSTTKYYTKGDDIELSKDYLEAGIERFEGFTKALEGSEFLANPDYNKDSN : 163
BvBetv1 : GPLGDT-LEKISNEIKIVATPDGGSILKISNKYHTKGDHE-VKAEQVKASKEMGETLLRAVESYLLAHS DAYN--- : 160
AgApigl : DILLGF-IESIENHVVLPPTADGSGICKTTAIFHTKGDVAV-VEENIKYANEQNTALFKALEAYLLAN----- : 154
MdMald1 : DALTDT-LEKISYETKLIVAC-GSGATIKSISHYHPKGNIE-IKEEHVKVGKEKGHGLFKLIESYLLKGHPDAYN--- : 159
PgRNS1 : DILLDI-IESIVNHFTIVPTPDGGSIVKNTTIYNTIGDAV-IPEENIKDATEKAGLIFKALEGAYLLAN----- : 154
PgRNS2 : DILLDI-IESIENHFKIVP-TDGETITQTIIYNTIGDAV-IPEENIKDATDKSIQLFKALEGAYLLAN----- : 153
StSTH2 : DVLGDK-LESISYDLKFEAHNGGCVCKSITEYHTKGDYV-LKDEEHNEGQKGMELFKLIVAYLLANPSVYA--- : 155
AoPR1 : GGLGKM-FECATTHFKFEPSSNGGCLVKVTASYKILPG---VADESAAKEGITHNMKATEAYLLANPTAYV--- : 158

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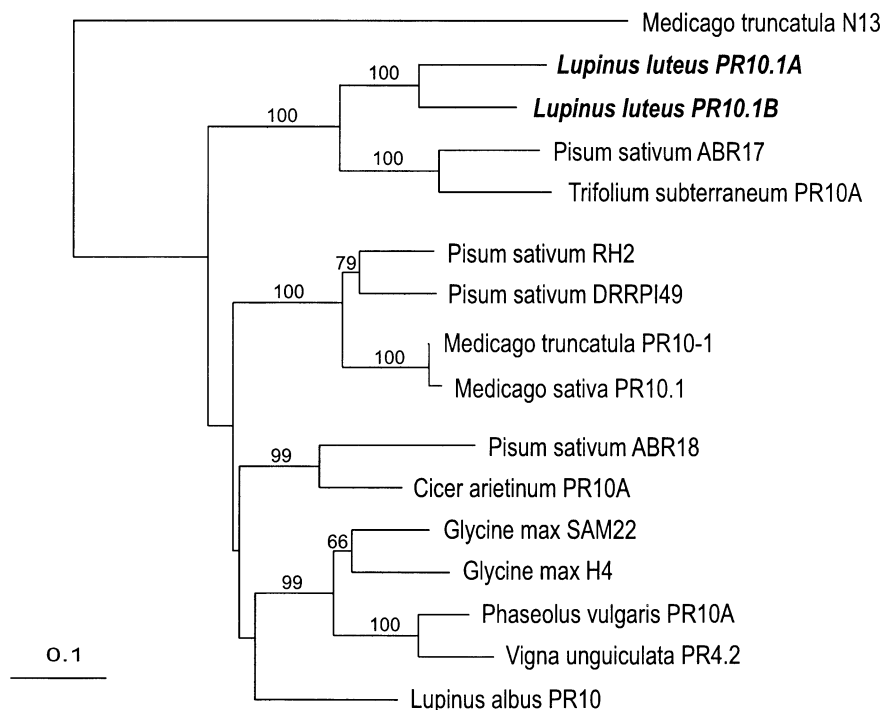
B.

Fig. 2.

young nodules (9 and 15 days post infection (dpi)). Both proteins were reduced to barely detectable levels in the mature nodule with PR10.1B present in slightly larger amounts, and both proteins present in approximately equal amounts in senescent nodule tissue (Fig. 4C). Fig. 4D shows the detection of leghaemoglobin level, a marker protein of effective symbiosis.

To determine the location of the PR10 proteins, immunocytochemical staining of the PR10 proteins was performed on root and nodule sections that represent distinct developmental stages corresponding to changes in PR10 protein expression during nodule development. Uninoculated, 9-day-old roots (Fig. 5B) exhibited uniform distribution within the primary root cortex and epiblem, but the PR10 proteins were not detected in root stele. In roots with 9-day-old nodules (Fig. 5D) the majority of the PR10 proteins was localised in the root cortex, as in the uninoculated 9-day-old root. However, the root stele of the nodulated root contained PR10 proteins. No PR10 protein was observed in the young nodule cortex or bacteroid tissue. In mature 26-day-old nodules (Fig. 5F) low protein levels were detectable in the nodule protective tissue and inner cortex, excluding the layer of cortical cells immediately adjoining the bacteroid tissue. The label was absent from the bacteroid tissue and nodule vascular bundles. The PR10 proteins also accumulated in the root stele with the exception of cambium and within the remains of the root cortex. In 40-day-old nodules (Fig. 5H) the PR10 protein was observed within all the nodule cortical tissues with the exception of the innermost layer adjoining the infected cells, resulting in an increase of the overall level of expression. The nodule vascular bundles and bacteroid tissue were free of label, except for single cells within the senescent zone. In the root stele an identical pattern of labelling was observed as in the 26-day-old material.

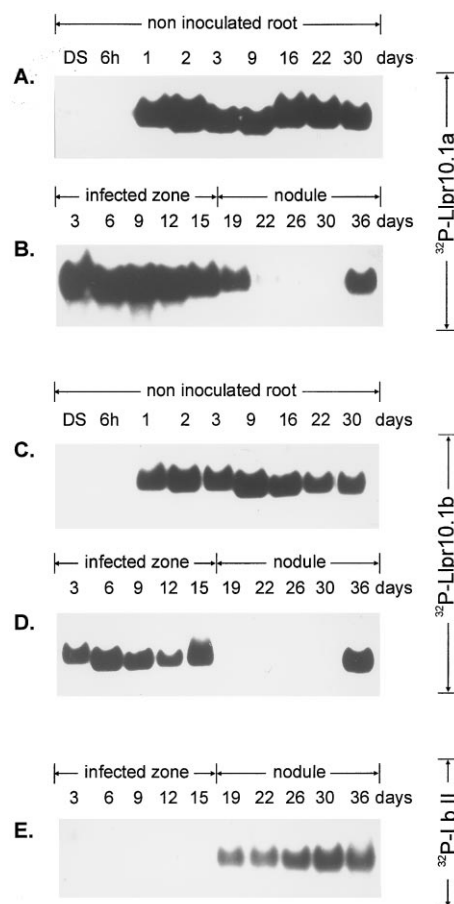


Fig. 3. Northern analysis of *L. luteus* total RNA of dry seeds (DS) and developing root (from 6 h after imbibition up to 30 days). The ^{32}P -labeled *Bam*HI/*Xho*I DNA fragments of Llpr10.1a cDNA and Llpr10.1b cDNA were used as a probes. Total RNA (20 μg) was applied per lane. Total RNA was isolated from dry seeds, developing root, inoculated root and developing nodule. The RNA blots were hybridised to ^{32}P -labeled 3'-end fragments of cDNA encoding LlPR10.1A and LlPR10.1B proteins: (A, B) hybridisation to ^{32}P -Llpr10.1a cDNA; (C, D) hybridisation to ^{32}P -Llpr10.1b cDNA; (E) control hybridisation to ^{32}P -LlbII; (A, C) non inoculated root; (B, D) *Bradyrhizobium* sp. (*Lupinus*) inoculated root and nodule. The plant material was harvested at different time points of plant development. DS-dry seeds; LlbII-*L. luteus* leghaemoglobin II cDNA.

Fig. 2. Analysis of amino acid sequences of PR10 protein class. (A) Alignment of the selected amino acid sequences of the PR10 proteins. Black boxes indicate 100% similarity. The residues with similarity over 75% are indicated in gray. The following sequences were taken from the EMBL GeneBank: *Medicago sativa* MsPR10 (X98867), *Pisum sativum* PsABR17 (Z15128), *Phaseolus vulgaris* PvPR10A (X61365), *Lupinus albus* LaPR10 (AJ000108), *Medicago truncatula* MtN13 (Y10455), *Mahus domestica* MdMald1 (AF074721), *Betula verrucosa* BvBetv1 (Z72436), *Apium graveolens* AgApig1 (Z48967), *Solanum tuberosum* StSTH2 (M29041), *Asparagus officinalis* AoPR1 (X64452) and SWISS-PROT protein sequence database: *Panax ginseng* PgRNS1 (P80889) and PgRNS2 (P80890). (B) Phylogenetic tree of the PR10 proteins from legume plants. Branch lengths reflect sequence diversity counted as the number of substitutions per site. The scale is corrected for multiply substitutions. The numbers indicate percent support for each branch, computed from 1000 bootstrap resamplings (bar corresponds to 0.1 substitution per site). The tree was constructed by neighbour-joining method using programs from PHYLIP package [50] and visualised by TreeView [51].

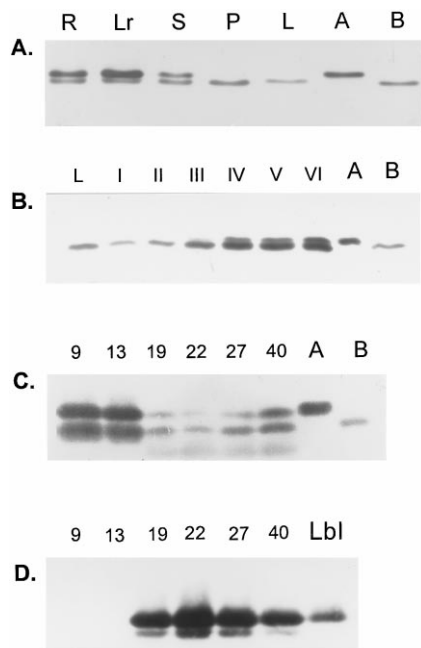


Fig. 4. Western blotting analysis of PR10 proteins in different lupine organs (A–C, detection with anti-LIPR10.1A). (A) Soluble protein extracts from root (R), lateral root (Lr), stem (S), petiole (P) and leaf (L) of 4-week-old non inoculated plant; LIPR10.1A and LIPR10.1B, purified recombinant proteins. (B) Soluble protein extracts from leaves of 8-week-old lupine harvested from different parts of the plant: I, young leaf (upper); II, III, mature leaf (middle); IV, V, VI, senescent leaf (lower); L, leaf of 4-weeks old plant; LIPR10.1A and LIPR10.1B, purified proteins. (C) Soluble protein extracts from nodules. (D) Soluble protein extracts from nodules incubated with anti-LbI. LIPR10.1A, LIPR10.1B and LbI, purified recombinant proteins. Protein extract (10 μ g) from each tissue and 10 ng of purified LIPR10 and LbI was analysed by Western blotting and detected with anti-LIPR10.1A and anti-LbI antibodies (diluted 1:10 000). The biotin/streptavidin-AP system was used to develop the protein blot.

The results presented above support the following model for the PR10 protein expression in roots and nodules. Both proteins were found at a high level in the root parenchymatous tissues, primarily in the cortex. In the early stages of root nodule development the PR10 proteins are inhibited, and 9 days after infection PR10 proteins are nearly absent from all nodule tissues. During the steps of nodule maturation PR10 expression remains low and a small amount of the protein can only be detected in the parenchymatous tissues, mostly in the nodule cortex. Further development and nodule senescence correlate with the increase of PR10 proteins in nodule cortex, so that in the 40-day-old nodule PR10 levels are comparable to the root

cortex. The changes in both protein levels appear to be similar, based on the results of the Northern and Western hybridisations. Since the proteins can not be distinguished in the immunocytochemical analysis due to their immuno-crossreactivity, it is not known whether the distribution of each homologue within the plant tissue is similar or different.

4. Discussion

The sequences of two yellow lupine PR10 proteins are 76% identical and reveal high homology with the other legume PR10 proteins (53–67% identity), and a 45% identity to birch pollen allergen BvBetv1. The most distantly related sequence, AoPR1 from a monocotyledonous plant, has only 25% identity to LIPR10. The identity values higher than 25% indicate not only a common origin of the PR10 class proteins, but also suggest a similar secondary structure for the proteins and the possibility that they have a common function. The blocks of highest homology correspond mainly to the regions of the well defined secondary structure (α -helices or β -strands), except for the glycine-rich loop between amino acid positions 46 and 54, which is one of the most conserved fragments and may be responsible for the protein activity (Fig. 2A).

Selected amino acid sequences were used to construct a phylogenetic tree representing genetic relations between the PR10 proteins of the subfamily *Fabaceae* (Fig. 2B). Topology of the tree is consistent with the taxonomy of plants at the level of subfamilies. The bootstrap values for the appropriate branches are too low, indicating rather the lack of useful genetic information in the sequences. The presence of the homologous PR10 proteins within one plant species indicates the duplications of the PR10 genes. Thus, the PR10 proteins are generally encoded by gene families. The diversity of PR10 genes in legumes is more variable among the species, suggesting a lack of concerted evolution.

The available data allow us to distinguish at least five paralogous groups of the PR10 genes in legumes. The most divergent group is represented by a single sequence, *M. truncatula* N13, the only gene expressed specifically in root nodules. Both lupine sequences fall into one group, but the presence of other homologues in lupine is possible. In fact, two lupine sequences seem to be paralogous to each other due to their relatively high divergence.

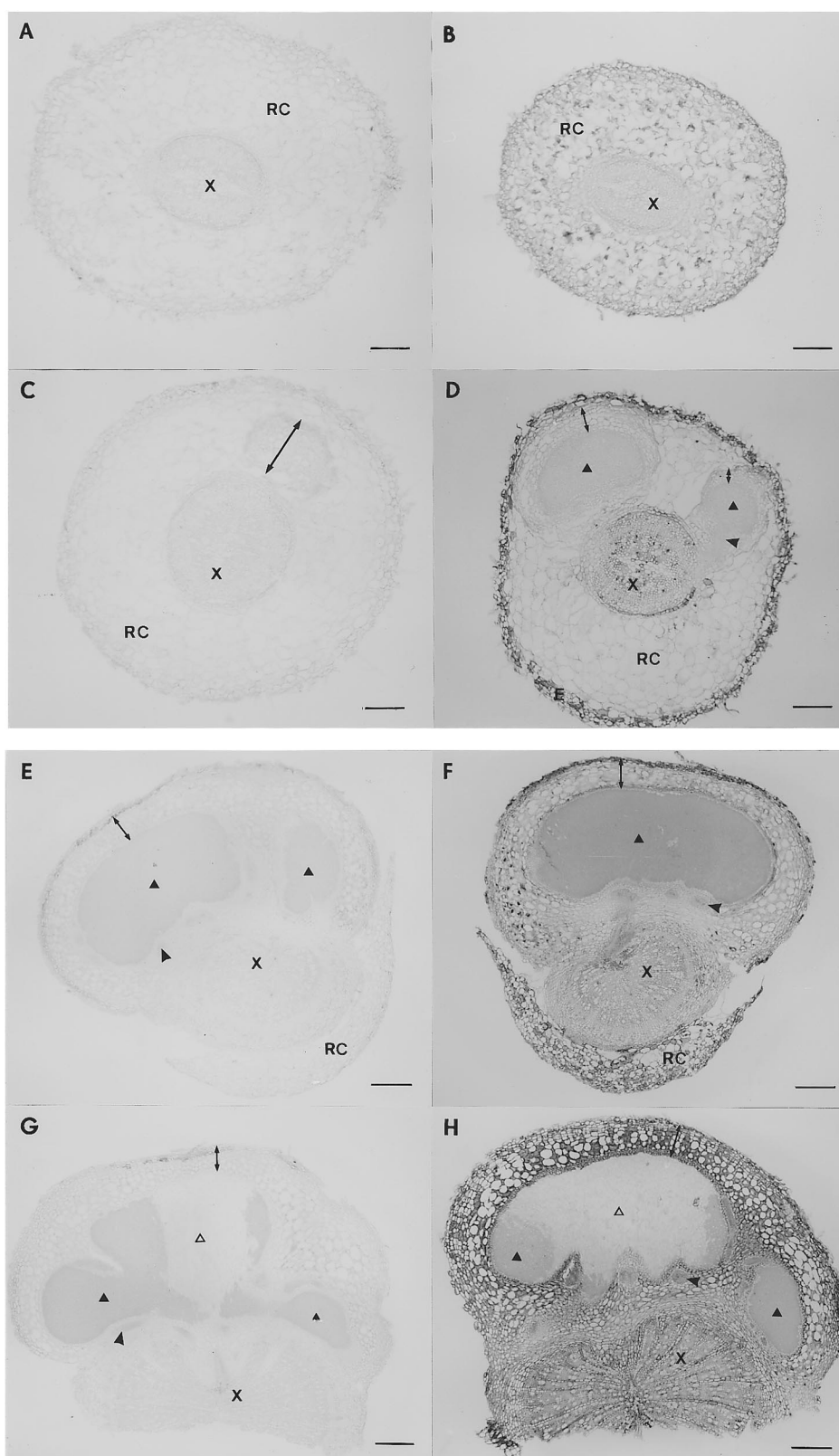


Fig. 5. Immunocytochemical localisation of PR10 proteins in lupine root and developing nodule: 9 days old root (A, B); nodule, 9 dpi (C, D); nodule, 26 dpi (E, F); nodule, 40 dpi (G, H). The sections A, C, E, G were incubated with pre-immune serum as the negative control. The sections B, D, F, H were incubated with anti-PR10.1A antibody. E, exodermis; RC, primary root cortex; X, xylem in the root stele; double-sided arrow, nodule cortical tissues; filled triangle, active bacteroid tissue; open triangle, degraded bacteroid tissue; arrow head, nodule vascular bundle. Bars: A and D, 227 μ m; B and C, 200 μ m; E and F, 417 μ m; G, 476 μ m; and H, 400 μ m. The antigen–antibody reaction was visualised as described in the legend to Fig. 4.

PR10 proteins were identified in plants in response to either biotic (pathogen infection) or abiotic stress (wounding or environmental stress like chemical pollutants, UV radiation). However, constitutive expression was also reported in different plant organs and tissues, primarily in roots, but in some cases in the leaves or various parts of the flowers. Large amounts of PR10 proteins present in fruits and vegetables or in the pollen grains of certain trees act as the common allergens. The biological function of PR10 proteins has not yet been determined, but their abundance, wide range of the plants, and different patterns of expression suggest that they play a significant and universal role in plant physiology, including defence mechanisms as well as development.

Two yellow lupine PR10 homologues are abundant in the roots, where their expression is constitutive. The level of PR10 proteins in the aerial parts of the plant is lower. Both proteins are present in the stem, but only the PR10.1B is constitutively expressed in the leaf and petiole. The increase of the PR10.1B protein, accompanied by the appearance of small amount of PR10.1A, was observed during the senescence of the leaves. The similar effect of the PR10 content increase in the lupine leaf was observed in response to infiltration with the pathogenic bacteria *Pseudomonas syringae* (data not shown).

The major difference between the levels of PR10 proteins in the roots and aerial parts of the plant, and observation of the expression pattern in developing and senescent lupine leaves suggests that the phytohormones may be involved directly or indirectly in the regulation of the PR10-encoding genes. This is supported by the recent findings describing the activation of PR10 gene expression by cytokinins in periwinkle callus [30], by abscisic acid in pea [31] and by ethylene in subterranean clover [32].

The main purpose of the presented work was to study the expression of pathogenesis-related proteins during the development of symbiosis with nitrogen fixing bacteria. A decrease of PR10 transcripts and protein accumulation in nodules between 9 and 30 days after inoculation with *Bradyrhizobium* sp. (*Lupinus*) and the return to a high level in senescent nodules 36 days after infection was observed. A decrease of PR10 protein content is correlated with the increase of the level of leghaemoglobin, a marker of effective symbio-

sis. The PR10 proteins are located outside of the bacteroid tissue, primarily in the parenchymatous tissue of the nodule cortex, suggesting that PR10 inhibition is not connected directly with the nitrogen fixation process. Since it is known that PR10 proteins are induced in leaves in response to the invasion of pathogenic bacteria, it seems apparent that the suppression of a defence mechanism is part of the recognition of the bacteria as a microsymbiont. However, the involvement of these proteins in some more general, developmental process, connected with the formation of nodules or other tissues might provide the most likely explanation, additionally supported by the reported presence of a nodule-specific PR proteins MtN1 and MtN13 in *M. truncatula* [42].

The *MtN1* gene, homologous to two cysteine-rich, pathogen inducible proteins from the pea (pI39 and pI230) is induced at the early stages of symbiosis development (24 dpi), whereas *MtN13*, closely related to genes encoding pathogenesis-related proteins of PR10 family, represents the first specific marker detected in the outer nodule cortex (induced 3 dpi). These genes were not found to be pathogen-inducible. The other member of the PR10 family, MtPR10-1 (constitutively expressed in roots), was induced in leaves during pathogen invasion. The divergence between MtN13 and MtPR10-1 is a result of a gene duplication event, which allows independent evolution and regulation of the two genes (one specifically expressed in nodule and other in root). The *MtN13* gene was not expressed in root tissue (including the root cortex), but required the formation of an active nodule meristem. A high expression of MtN13 was also observed in the cortex of mature nodules. These two facts indicate, that the nodule cortex developed from the nodule meristem and displays properties different from epidermal and cortical root tissue. The pattern of *MtN1* and *MtN13* genes expression suggests that they represent a class of nodulation-specific defence genes. The expression of these genes seems to be specifically triggered by rhizobia like nodule specific leghemoglobin genes, which are activated during symbiosis development. It was proposed by Gamas et al. [42], that MtN13 is a symbiotic homologue of MtPR10-1 and that both proteins possess a similar function. The *MtN13* gene is induced in symbiotic and MtPR10-1 in pathogenic interactions. Following this idea, the possible

function of these defence proteins could be to prevent pathogenic infections of the root nodule, which is a potential carbon source for soil micro-organisms. A high homology of MtN13 to pea RH2 protein, specifically expressed in root epidermal cells and proposed to contribute to a constitutive defence mechanism [26], supports the hypothesis of the protective role of MtN13 in nodules. Further similarity of MtN13 protein to *Panax ginseng* PR10 proteins exhibiting the ribonuclease activity [19,20] lead to the conclusion that MtN13 could possess such activity involved in mediating of cytotoxic effects.

The alternative hypothesis of a protective role against pathogens concerns the possible role of MtN1 as a defence protein expressed in response to inoculation of plant by *Rhizobium*. However, this speculation is contrary to the hypothesis that effective symbiosis development involves the suppression of plant defence mechanisms [40,53].

The other possibility is that MtN1 and MtN13 are involved in a developmental program not directly related to the plant defence mechanism. It should be pointed out that the exact biological role of PR10 proteins during pathogenic invasion still remains unknown. The expression of some PR proteins was also observed in the absence of pathogens, e.g. during developmental processes and organogenesis [54–59]. Furthermore some defence proteins such as peroxidases and chitinases are considered to play different roles during nodule development and defence responses to pathogens [60–62]. The PR10 proteins belong to the wide group of proteins described as ‘pathogenesis-related’. It is important to consider the relationship of these proteins with the plant response to pathogens. It is possible that the protein is produced to act directly against the pathogen, being either cytotoxic to the infected cells or simply harmful to the pathogen, but it can be involved in a physiological mechanism that is enhanced during pathogenesis but that also occurs under other conditions. If the first possibility were true, it would allow us to describe these proteins as defensive; otherwise, they are only pathogenesis-induced. Although other authors have discussed both of these possible functions, the data available in the literature as well as the results of our own work suggest that the PR10 proteins play a more general role in plant physiology rather than that of purely defensive proteins.

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