Expression of *Lupinus albus* PR-10 proteins during root and leaf development

M.P. PINTO*, A. RIBEIRO**, A.P. REGALADO***, C. RODRIGUES-POUSADA***
and C.P.P. RICARDO***,****,[1]

*Escola Superior Agrária, Apt 310, 2001-904 Santarém, Portugal**
*Instituto de Investigação Científica Tropical/Centro de Investigação das Ferrugens do Cafeeiro, 2784-505 Oeiras, Portugal***
*Instituto de Tecnologia Química e Biológica, 2781-901 Oeiras, Portugal****
*Instituto Superior de Agronomia, 1349-017 Lisboa, Portugal*****

Abstract

Based on the NH_2-terminal sequence of three PR-10 isoforms previously identified in *Lupinus albus* leaves and a conserved amino-acid region in the PR-10 proteins from leguminosae, a pair of oligonucleotides was designed and used to amplify the corresponding cDNA fragment from a *L. albus* leaves cDNA library. A fragment of DNA of 200 bp was isolated from the polymerase chain reaction (PCR) mixture and subsequently used to screen the cDNA library. A cDNA coding for a PR-10 protein of 158 amino acid residues was cloned and sequenced. Subsequent studies involving Northern and Western blot analysis have shown that the PR-10 protein isoforms are differentially expressed during the development of the healthy lupin plant. High mRNA and protein contents were detected in roots and hypocotyls of both 7- and 20-d-old plants. In young leaves, the mRNA and protein contents were low and increasead in mature leaves. Tissue printing experiments with root sections suggest that the proteins are extracellular and are mainly associated with the vascular tissues in mature roots.

Additional key words: cDNA cloning, differential protein expression, intercellular spaces, vascular tissues, white lupin.

Introduction

Many pathogenesis-related (PR) proteins are differentially expressed in different organs of healthy plants, suggesting that their genes are not only regulated by stress factors, but are also under developmental regulation. For example, in healthy tobacco plants, mRNAs encoding basic isoforms of PRP-1, PRP-2 and PRP-3 are highly expressed in roots, whereas in leaves they can only be detected after infection with tobacco mosaic virus or after ethephon treatment (Memelink *et al.* 1990). The acidic PR-1 proteins seem to be expressed in healthy leaves, the highest content being found in the oldest leaves and decreasing along the axis of the plant (Grüner and Pfitzner 1994). It was also shown that in barley leaves PR proteins accumulate in the intercellular spaces during leaf senescence (Tamás *et al.*1998).

In *Lupinus albus* leaves we have previously identified three isoforms of defense-related proteins (PR-p16.5a, PR-p16.5b and PR-p16.5c) showing high similarity with members of the PR-10 class (Pinto and Ricardo 1995). Proteins of this class are found in several plant families and are characterized by sharing similarity with the Betv1 protein from the pollen allergen of birch (Chiang and Hadwiger 1990, Crowell *et al.* 1992, Walter *et al.* 1990, Löchk *et al.* 1990, Breiteneder *et al.* 1989, Matton *et al.* 1990, Warner *et al.* 1992, Dubos and Plomion 2001). These proteins are thought to be ribonucleases due to its structural similarity with two ribonucleases identified in ginseng calluses (Moiseyev *et al.* 1997). Recently, a PR-10 protein with ribonuclease activity was identified in *L. albus* roots (Bantignies *et al.* 2000).

Received 30 January 2004, *accepted* 25 October 2004.
Abbreviations: PAGE - polyacrylamide gel electrophoresis; PCR - polymerase chain reaction; PR - pathogenesis-related; PVDF - polyvinylidene difluoride; PVP - polyvinylpyrrolidone; SDS - sodium dodecyl sulfate; SSC - saline-sodium citrate buffer.
[1] Corresponding author; fax: (+351) 21 443 36 44; e-mail: ricardo@itqb.unl.pt

Although the PR-10 proteins have been first related to the defence mechanism of plants, in response to pathogen infection and abiotic stress, there is increasing evidence that they are developmentally regulated. In fact, in soybean and bean, genes coding for PR-10 proteins are expressed at high levels in roots and in senescent leaves, but transcript levels are low in young leaves (Crowell *et al.* 1992, Walter *et al.* 1996). In potato, strong expression of the PR-10a protein (formerly STH-2) has been observed in the papillae and uppermost cell layers of the stigma of healthy plants (Constabel and Brisson 1995). Also, the wound inducible AoPR1 gene from asparagus is highly expressed in stems of mature transgenic tobacco plants, as well as in the seed coats (Warner *et al.* 1994).

Recently, two gene families encoding two PR-10 protein subclasses have been identified in *Lupinus luteus*: subclass LlPR10.1, consisting of three isoforms of 156 residues, and subclass LlPR10.2, consisting of two isoforms of 158 residues (Sikorski *et al.* 1999, 2000). All isoforms of the two subclasses are constitutively expressed in roots and are down regulated during nodule development in roots infected with a symbiotic bacterium (Sikorski *et al.* 2000). However, there is evidence of differential expression between members of the same subclass in *L. luteus* tissues. While LlPR10.1A isoform is constitutively expressed in roots and stems but is absent in non-senescent leaves, the LlPR10.1B isoform is constitutively expressed in the whole plant (Sikorski *et al.* 1999).

In this paper, we report the cloning and characterization of a *L. albus* leaf cDNA encoding a protein of subclass PR10.2 and show evidence for the differential expression of PR-10 protein homologues in *L. albus* tissues and for their extracellular location.

Materials and methods

Plants and growth conditions: *Lupinus albus* L. cv. Rio Maior plants were grown in a growth chamber under a 12-h photoperiod (irradiance of 160 μmol m^{-2} s^{-1}), dark/light temperature of 17/22 °C, and relative humidity of 60 - 70 %, on washed coarse sand watered twice a week with a nutrient solution (Johnson *et al.* 1957, Shea *et al.* 1968). Plants 7- and 20-d-old were mostly used in the experiments.

Protein extraction and separation: Leaf samples were homogenized in 30 mM Tris-HCl pH 7.5, containing 20 mM 2-mercaptoethanol and 5 % (m/v) polyvinyl-pyrrolidone (PVP). The homogenate was centrifuged for 20 min at 10 000 *g* and the resultant supernatant was centrifuged for 30 min at 80 000 *g* to obtain the soluble fraction. Protein electrophoresis in native basic gels was performed in 10 % polyacrylamide gels, according to Laemmli (1970), except that sodium dodecyl sulfate (SDS) was omitted.

Immunoblotting: Proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The blots were incubated with the primary antibody (serum anti PR-p16.5; Pinto and Ricardo 1995) diluted 1:5000, which was detected with alkaline phosphatase-labelled anti-rat IgG and developed with bromochloroindolyl phosphate/nitroblue tetrazolium substrate.

Tissue printing: Tissue prints of root cross sections were obtained according to the method of Cassab and Varner (1987) and developed with the method described above for immunoblots.

Isolation of a cDNA probe: Using a pair of degenerated oligonucleotides, a 0.2 kb fragment was amplified from a *L. albus* leaves cDNA library. One of the nucleotides was designed based on the NH_2-terminal sequence of the PR-10 proteins identified in *L. albus* leaves (5'-GGRATHTTYACRTTYGARGAYGA-3', corresponding to the first eight amino acid residues of the protein: GIFTFEDE; Pinto and Ricardo 1995). The other oligonucleotide was designed based on a conserved region of legume PR-10 proteins (5'-TTRGTYTCRCCRTCYTCRACRAA-3', reverse primer).

A 2 mm^3 volume of the cDNA library in 8 mm^3 of water were heated at 70 °C for 10 min and 0.5 mm^3 of each primer (50 pmol mm^{-3}), with 2.5 mm^3 of enzyme buffer, 1.5mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 units of Taq polymerase (*Pharmacia*, Uppsala, Sweden) were added. The polymerase chain reaction was performed on a Trio-Thermoblock™ (*Biometra*, Göttingen, Germany) according to the following protocol: five cycles 30 s at 94 °C, 1 min at 38 °C, 30 s at 72 °C followed by 30 cycles: 30 s at 92 °C, 1 min at 40 °C and 30 s at 72 °C.

The DNA was precipitated from the reaction mixture by addition of 3M sodium acetate and 2.5 volumes of ethanol. After dissolving in 10 mm^3 of water, the DNA was separated on a 2 % low melting agarose gel. A major fragment of 200 bp was excised from the gel and the DNA was extracted. This DNA fragment was subsequently reamplified by PCR and used as a probe to screen the DNA library, after labeling with ^{32}P by random oligonucleotide priming (*Amersham*, Uppsala, Sweden, Megaprime DNA labeling system).

Screening of cDNA library: The construction of the cDNA library in the Uni-ZAPXR vector, with mRNAs isolated from mature *L. albus* leaves has been described (Regalado *et al.* 2000). A total of 10^5 phage plaques were plated at low density, lifted to a set of duplicate nitrocelulose filters and hybridized to the ^{32}P-labeled probe. The hybridizations were carried out overnight, at 42 °C in 40 % formamide containing 6 × saline-sodium citrate buffer (SSC), 1 × Denhardt's solution and 100 μg cm^{-3} of denatured salmon sperm DNA.

DNA sequencing and computer analysis: The nucleotide sequences were determined using the dideoxy chain termination method (Sanger *et al*. 1977) and an automatic sequencer (*Model 373A*, *Applied Biosystems*, Foster City, USA). Sequence data were analysed with the University of Wisconsin Genetic Computer Group Software Package (Program Manual for the Wisconsin Package, version 8, Genetics Computer Group, Madison, WI). Homology searches were done by the *BLASTn* and *BLASTX* Programs (National Center for Biotechnology Information). Sequence alignments were performed with the *Multalin* Program (Corpet 1988).

RNA extraction and Northern blot analysis: RNA extraction and blotting was carried out as described in Regalado and Ricardo (1996). The filters were hybridized to the ^{32}P-labelled probe (*Amersham* Megaprime DNA labeling system) overnight, at 60 °C in 6 × SSC, 5 × Denhardt's solution, 0.5 % SDS and 25 μg cm^{-3} of denatured salmon sperm DNA.

Results

Sequence analysis of the isolated cDNA clone: Based on the NH_2-terminal sequence of the three PR-p16.5 proteins from *L. albus* and a conserved amino-acid region in the PR-10 proteins from leguminosae, a pair of degenerated oligonucleotides was designed and used to amplify the corresponding cDNA fragment from a *L. albus* leaves cDNA library. A fragment of 0.2 kb was isolated from the PCR reaction mixture and subsequently used to screen the cDNA library. This screening resulted in the isolation of two cDNA clones of 750 bp. Sequencing results reveals that one of the isolated clones encodes a PR-10 protein of 158 aminoacid residues (Fig. 1), being therefore termed pLaPR-10.2A (EMBL GenBank accession number ABO706.18), in accordance to the PR-10 subclasses identified in *L. luteus* (Sikorski *et al.* 2000). Sequence comparison between the protein deduced from LaPR-10.2A and the other lupin PR-10 proteins has shown 93% identity to the PR-10 protein identified in *L. albus* roots (158 amino acid residues), and 92.4 % identity to the LlPR10.2A protein from *L. luteus*, which is one of the isoforms of subclass LlPR10.2 (158 amino acid residues; Fig. 1). High identity (88 %) was also observed with the other gene of subclass two, the LlPR10.2B. The other cDNA clone has not been entirely sequenced, but preliminary results show that it also codes for a PR-10 protein.

The sequence of the protein deduced from clone LaPR-10.2A has 95 % identity to the NH_2-terminal sequence of the PR-16.5 proteins previously identified in *L. albus* leaves (Pinto and Ricardo 1995) showing that this clone represents a new isoform of these proteins.

PR-10 expression in different organs of *L. albus* plants: Using LaPR-10.2A as a probe, we detected high levels of transcript in roots and hypocotyls of both seven and twenty day-old plants (Fig. 2*A*). No significant levels could be detected in leaves of 7-d-old plants or in young leaves of 20-d-old plants. However, high levels could be detected in fully expanded leaves of 20-d-old plants.

We used the antiserum previously raised against the PR-10 proteins identified in the leaves of *L. albus* to probe a western blot of native proteins from different organs. High levels of the two major previously identified isoforms were detected in roots and hypocotyls of either 7- or 20-d-old plants (Fig. 2*B*). As expected, lower contents of the proteins were found in leaves, where the proteins accumulate only after stress treatment (Pinto and Ricardo 1995). Although not so marked as in the Northern blots, the content of the proteins seems higher in fully expanded leaves than in young leaves. An additional isoform, not found in leaves, was detected in roots and hypocotyls of both 7- and 20-d-old plants.

Study of PR-10 protein isoforms during root development: Since lupin PR-10 proteins were highly expressed in roots, we decided to analyse the expression of these isoforms during root development. The contents of the two major proteins were almost undetectable in the embryonic axis of the dry seeds, but the increase of protein accumulation was observed up to 5 d after germination and remained constant thereafter (Fig. 3*A*). The observed increase was coincident with a decrease of high molecular mass proteins during root development (Fig. 3*B*). Two new isoforms were detected 4 d after germination (Fig. 3*A*). However, these isoforms were not present in the following days.

Immunolocalization of PR-10 proteins in tissue prints of roots: In roots of 20-d-old plants intense staining was observed around the xylem vessels and in the area of

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LaPR10.2A     MGIFTFEDES TSTVAPARLY KALVKDADTI IPKAVEAIQS VETVEGNGGP 50
LaPR10        MGIFTFEDES TSTVAPAKLY KALVADANII IPKAVEAIQS VENVEGNGGP
LlPR10.2A     MGVFTFQDES TSTIAPAKLY KALVTDADII IPKAVETIQS VEIVEGNGGP
LlPR10.2B     MGVFTFQDEY TSTIAPAKLY KALVTDADII IPKAVETIQS VEIVEGNGGP
LlPR10.2C     MGVFTFQDES TSTIAPAKLY KALVTDADII IPKAVETIQS VEIVEGNGGP
LlPR10.2D     MGVFTFEDES TSTIAPARLY KALVKDADAI IPKAVEAIQS IETVEGNGGP
LlPR10.1A     MGIFAFENEQ SSTVAPAKLY KALTKDSDEI VPKVIEPIQS VEIVEGNGGP
LlPR10.1B     MGVFAFEDEH PSAVAQAKLF KALTKDSDDI IPKVIEQIQS VEIVEGNGGP
LlPR10.1C     MGVFSFEEET ISIVAPSKLF KALTKDSDEI IPKVIEPIQS VEIVEGNGGP

LaPR10.2A     GTIKKLTLIE GGETKYVLHK IEEIDEANLG YNYSIVGGVG LPDTVEKITF 100
LaPR10        GTIKKLTFIE DGETKYVLHK IEEIDEANLG YNYSIVGGVG LPDTVEKITF
LlPR10.2A     GTIKKLTFIE GGESKYVLHK IEAIDEANLG YNYSIVGGVG LPDTIEKISF
LlPR10.2B     GTIKKLTFIE GGESKYVLHK IEAIDEANLG YNYSIVGGVG LPDTIEKISF
LlPR10.2C     GTIKKLTFIE GGESKYVLHK IEAIDEANLG YNYSIVGGVG LPDTIEKISF
LlPR10.2D     GTIKKLTLIE GGETKYVLHK IEAVDEANLR YNYSIVGGVG LPDTIEKISF
LlPR10.1A     GTIKKIIAIH DGHTSFVLHK LDAIDEANLT YNYSIIGGEG LDESLEKISY
LlPR10.1B     GTVKKITASH GGHTSYVLHK IDAIDEASFE YNYSIVGGTG LDESLEKITF
LlPR10.1C     GTIKKITAVH GGHTSYVLHK IDAIDEASLT YDYSIVGGTG LDESLEKITF

LaPR10.2A     ETKLVEGVNG GSIGKVTIKI ETKGDAKPNE EEGKAAKVRG DAFFKAIENY 150
LaPR10        ETKLVEGVNG GSIGKVTIKI ETKGDAKPNE QEGKAAKARG DAFFKAIETY
LlPR10.2A     ETKLVEGANG GSIGKVTIKI ETKGDAQPNE EEGKAAKARG DAFFKAIESY
LlPR10.2B     ETKLVEGANG GSIGKVTIKI ETKGDAQPNE EEGKAAKARG DAFFKAIESY
LlPR10.2C     ETKLVEGANG GSIGKVTIKI ETKGDAQPNE EEGKAAKARG DAFFKAIESY
LlPR10.2D     ETKLVEGANG GSIGKVTIKI ETKGDAQPNE EEGKAAKARG DAFFKAIENY
LlPR10.1A     ESKILPGPDG GSIGKINVKF HTKGDVL.SE TVRDQAKFKG LGLFKAIEGY
LlPR10.1B     ESKLLSGPDG GSIGKIKVKF HTKGDVL.SD AVREEAKARG TGLFKAVEGY
LlPR10.1C     ESKIFSGPDG GSIGKINVKF HTKGDVL.SD TVREEAKFKG IGLFKAVEGY

LaPR10.2A     LSAHPEYN 158
LaPR10        LSAHPDYN
LlPR10.2A     LSAHPDYN
LlPR10.2B     LSAHPDYN
LlPR10.2C     LSAHPDYN
LlPR10.2D     LSAHPEYN
LlPR10.1A     VLAHPDY.
LlPR10.1B     VLANPNY.
LlPR10.1C     VLANPNY.
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Fig. 1. Sequence comparison between different members of the PR-10 proteins identified in *L. albus*, LaPR-10 (accession number AJ000108) and LaPR-10.2A (accession number ABO706.18), and *L. luteus*, LlPR-10.1 (accession numbers P52778, P52779 and AF180941) and LlPR-10.2 (accession numbers AF170091, AF170092, AF322225, AF322226). *Dark shading* indicates conserved amino acid residues, on the basis of the LaPR-10.2A sequence.

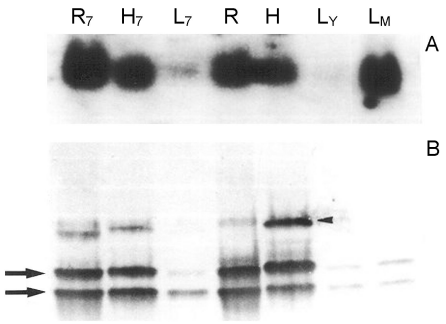

Fig. 2. Differential expression of PR-10 gene in vegetative organs of uninfected *L. albus* plants. *A* - Northern blot analysis using ^{32}P-labelled LaPR-10.2A as probe (10 µg RNA per lane). *B* - Western blot analysis of proteins separated by anodic PAGE, using the antiserum raised against the lupin PR-10 proteins (p16.5 a,b and c) as probe. R_7, H_7, L_7 - roots, hypocotyls and leaves of 7-d-old plants, respectively; R, H, L_Y, L_M - roots, hypocotyls, young leaves and mature leaves of 20-d-old plants, respectively; *large arrows* point to the major previously identified PR-10 proteins in *L. albus* and the *small arrow* points to new homologues of these proteins.

phloem, above the vascular cambium, when using the antiserum raised against the p16.5 proteins (Fig. 4). Some staining could also be detected in the pith but much less intense. No labeling was observed in tissue prints treated with preimmune serum (data not shown). The same intense staining around the vascular tissues could be observed in roots of 8-d-old plants. However, in this case, an abundant staining could also be detected throughout the cortex and pith (Fig. 5*A*). These results further suggest that the PR-10 proteins are located in the cell walls and the intercellular spaces.

Discussion

Previously, we described three lupin proteins, PR-p16.5a, b and c, which were almost undetectable in healthy leaves, but accumulated 24 h after inoculation with a pathogen or salicylic acid (Pinto and Ricardo 1995). The N-terminal amino acid sequence of these proteins shared high similarity with some PR-10 proteins. We here described the identification and nucleotide sequence of a cDNA isolated from a *L. albus* leaf cDNA library that encodes a PR-10 protein of 158 amino acid residues. The NH_2-terminal sequence of the deduced protein matched the NH_2-terminal sequence of the previously identified PR-p16.5 protein suggesting that the PR-p16.5 proteins from *L. albus* are members of the PR-10 class.

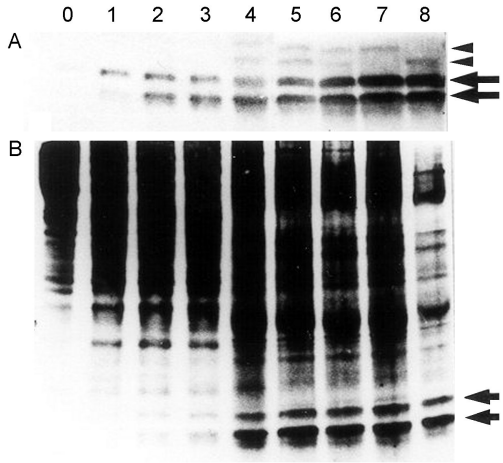

Fig. 3. Analysis of lupin PR-10 proteins (separated by anodic PAGE) during root development. *A* - Western blots probed with the antiserum raised against the p16.5 proteins. *B* - Total proteins stained with Coomassie Blue; 0 to 8 d after germination; *large arrows* point to the major previously identified PR-10 proteins and the *small arrow* points to new isoforms of these proteins.

The results obtained indicated that several PR-10 proteins are present in *L. albus* and that they are developmentally regulated. In fully expanded leaves, PR-10 protein expression was higher than in young leaves (Fig. 2*B*), where the mRNA levels were almost undetectable (Fig. 2*A*). This increase in expression of PRs during maturation of leaves has also been observed in the acidic PR-1 proteins of tobacco (Grüner and Pfitzner 1994).

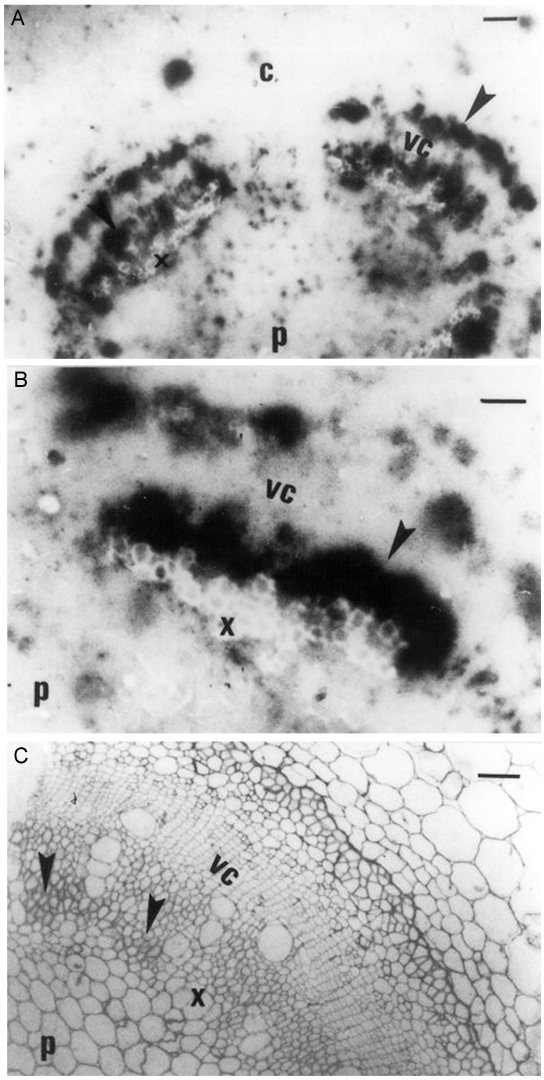

Fig. 4. *A*, *B* - Immunodetection of PR-10 proteins in tissue prints of the most differentiated part of roots of 20-d-old *L. albus* plants, using the antiserum raised against the p16.5 proteins. *A* - *bar* = 25 μm; *B* - *bar* = 50 μm. *C* - Anatomy of a root section stained with *O*-toluidine; *bar* = 25 μm; c - cortex; p - pith, vc - vascular cambium, x - secondary xylem. In the tissue prints, regions reacting with the antiserum appear as dark patches and the *arrows* point to staining in vascular tissues; in the anatomy section the *arrows* point to cells with lignified secondary walls.

The highest contents of *L. albus* PR-10 proteins were found in roots and hypocotyls of young and mature plants (Fig. 2*B*). Furthermore, expression of the proteins increased gradually in developing roots until 5 d after germination and thereafter they were present at least until day 20 after germination (Fig. 3*A*) and then they might decay, as judged from the lower mRNA levels in roots of 20-d-old plants.

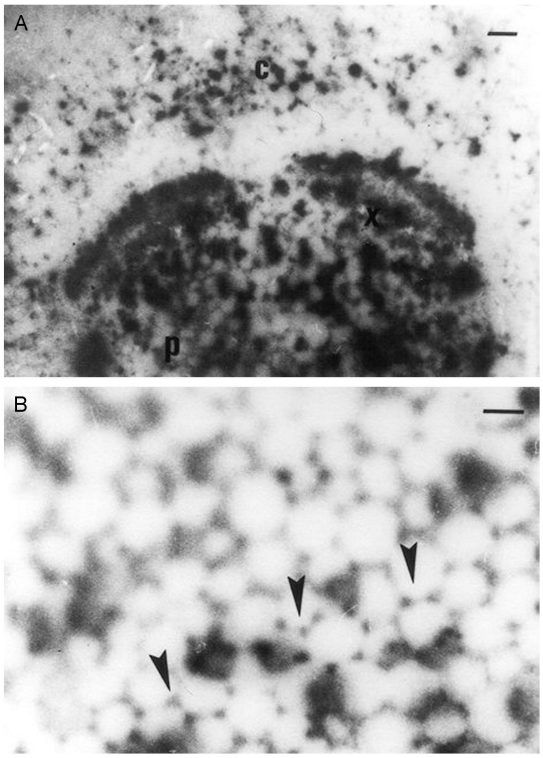

Fig. 5. *A*, *B* - Immunodetection of PR-10 proteins in tissue prints of the most differentiated part of the roots of 7-d-old *L. albus* plants, using the antiserum raised against the p16.5 proteins. *A* - *bar* = 50 μm; *B* - magnification of the cortex; *bar* = 25 μm; x - xylem; *arrows* point to staining in intercellular spaces.

As observed in leaves (Pinto and Ricardo 1995), we detected several PR-10 homologues in other *L. albus* organs. Although Bantignies *et al.* (2000) have detected only one PR-10 protein in roots of *L. albus* plants, we have detected five isoforms that are differentially expressed during root development. Our results are in accordance with those obtained for *L. luteus* roots by Sikorski *et al.* (2000), who identified two gene subfamilies encoding five homologues overall – two of subclass LlPR10.2 (158 amino acid residues) and three of subclass LlPR10.1 (156 amino acid residues). These authors also observed differential expression for the homologues of the same subclass (Sikorski *et al.* 1999). Comparison of *L. albus* PR-10 protein sequences (Fig. 1) suggests that in *L. albus* PR-10 proteins are encoded by a multigene family similarly to *L. luteus* and other plant species (Chiang and Hadwiger 1990, Crowell *et al.* 1992, Matton *et al.* 1990, Walter *et al.* 1996, Sikorski *et al.* 2000).

The tissue printing experiments show that in mature roots, the PR-10 proteins are mainly associated with the vascular tissue, as it was observed in infected *L. albus* leaves (Pinto and Ricardo 1995). However, the proteins were not detected in the vascular cambium, which is characterized by undifferentiated cells, suggesting that the proteins are mainly expressed in differentiated cells. Contrarily, in young root tissues, the expression of the proteins does not seem to be restricted to vascular tissues, since the proteins could be detected throughout the parenchyma cells as well as in the vascular cells. Similar results have been described for the PR-10c protein from bean (Walter *et al.* 1996). The authors have used the *Ypr10* gene fused to the glucuronidase reporter gene and have observed strong GUS activity in developing xylem of mature roots. However, in young roots, the expression of GUS was not restricted to vascular tissues.

Association of PR-10 proteins with vascular tissues of mature organs has also been observed in other plant families, suggesting that this is a common feature of these proteins. In potato, strong expression of the PR-10a protein was observed in the vascular cells of stems and petioles after plant infection with *Phytophthora* (Constabel and Brisson 1995), and in asparagus, specific expression of the *AoPR1* gene occurs in the stem when it becomes lignified (Warner *et al.* 1994). Possibly, in young tissues the PR-10 proteins are involved in the development of secondary cell walls and as the tissue matures they become involved in the subsequent differentiation of the vascular cells.

The tissue printing experiments have shown that the *L. albus* PR-10 proteins are located extracellularly in roots, in accordance with the ultrastructural results obtained in infected leaves (Pinto and Ricardo 1995). However, like other PR-10 genes reported so far, the sequenced cDNA clone did not have a recognizable signal peptide for extracellular targeting. It is possible that these proteins are secreted by a different system, as happens with some extracellular animal proteins that also do not have a recognizable targeting signal in the primary structure (Muesh *et al.* 1990).

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