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Lupinus albus L. Pathogenesis-Related Proteins That Show Similarity to PR-10 Proteins[1]

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We describe a group of three acidic proteins, pathogenesis-related (PR)-p16.5a, PR-p16.5b, and PR-p16.5c, that accumulate in the leaves of *Lupinus albus* L. cv Rio Maior plants when infected with the fungus *Colletotrichum gloeosporioides* Penz. These proteins co-migrate in sodium dodecyl sulfate-polyacrylamide gels as a single band of 16.5 kD, behaving as charge isomers, and are related to several members of the defense-related PR-10 protein family. Localization of the proteins was investigated by techniques of tissue printing and immunogold electron microscopy; they are predominantly associated with the vascular system and are localized extracellularly. The accumulation of PR-p16.5a, PR-p16.5b, and PR-p16.5c also seems to be induced by cucumber mosaic virus and by two forms of abiotic stress, salicylic acid and ultraviolet, suggesting a general defense role for these proteins.

When a pathogen attacks a host plant and overcomes the plant's defense mechanisms, disease results, leading to the eventual death of the plant. However, in most cases the plant is able to prevent spreading of the infection by rapidly inducing a number of defense responses: synthesis of antimicrobial substances called phytoalexins (Darvill and Albersheim, 1984); reinforcement of cell walls by deposition of polysaccharides (like callose), lignin, and structural proteins like hydroxyproline-rich glycoproteins (Hahn et al., 1989); and accumulation of defense-related proteins, which attack microbial cell walls (such as hydrolases), toxic proteins, proteinase inhibitors, and several low-molecular-mass proteins initially defined as PR proteins (Bowles, 1990). First characterized in tobacco plants reacting hypersensitively to tobacco mosaic virus, five groups or families of PR proteins (PR-1 to PR-5) have now been found in most plant species studied (van Loon, 1989). PR-2 and PR-3 groups have hydrolytic activity (β-1,3-glucanase and chitinase activities, respectively), and members of the other groups have recently been shown to have antifungal activities (Stintzi et al., 1993; Ponstein et al., 1994). Furthermore, in many plant species proteins were detected with molecular masses and acidic natures similar to those of the PR-1 class, but with no structural relation with the PR-1 proteins of tobacco. Instead, they share similarity with the pollen allergen Betv-1 from white birch, which is considered an ubiquitous new class of defense-related proteins (Somssich et al., 1988; Breiteneder et al., 1989; Chiang and Hadwiger, 1990; Matton et al., 1990; Walter et al., 1990; Crowell et al., 1992; Warner et al., 1992). According to the nomenclature for PR proteins recently suggested by van Loon et al. (1994), this class of proteins is now referred to as PR-10. Having been found in both dicotyledonous and monocotyledonous plant families, the PR-10 proteins are thought to have evolved from a common ancestor and to have similar functions. Recently, a significant similarity was found between a RNase from ginseng and a PR-10 from parsley, suggesting that these proteins are RNases (Moieyev et al., 1994). However, such enzymatic activity has not been demonstrated so far for any protein of the PR-10 family.

In this paper we describe a group of proteins induced in *Lupinus albus* in response to pathogenesis, salicylic acid, and UV stress. They have low molecular masses, acidic natures, and N-terminal amino acid sequences that show similarity with the PR-10 proteins. Polyclonal antibodies against these proteins were produced and used to study their cellular localization.

MATERIALS AND METHODS

Plant Growth and Inoculation

Lupinus albus L. cv Rio Maior plants were grown in a growth chamber under a light regime of 12 h, at 17/22°C (dark/light), on washed coarse sand watered twice a week with a nutrient solution (macroelements according to Shea et al., 1968; microelements according to Johnson et al., 1957).

The fungus *Colletotrichum gloeosporioides* Penz. and the CMV were obtained from the Phytopathology Department of Estação Agronómica Nacional (Oeiras, Portugal).

Two-week-old plants were inoculated in the first pair of leaves using Carborundum as an abrasive, with either a spore suspension of *C. gloeosporioides* (10^6 spores/mL), a 5 mM salicylic acid solution, or an extract of CMV-infected tomato leaves. The plants inoculated with the spores were incubated for 24 h in a moist chamber. Control plants were treated with sterile water.

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Abbreviations: CMV, cucumber mosaic virus; PR, pathogenesis-related.

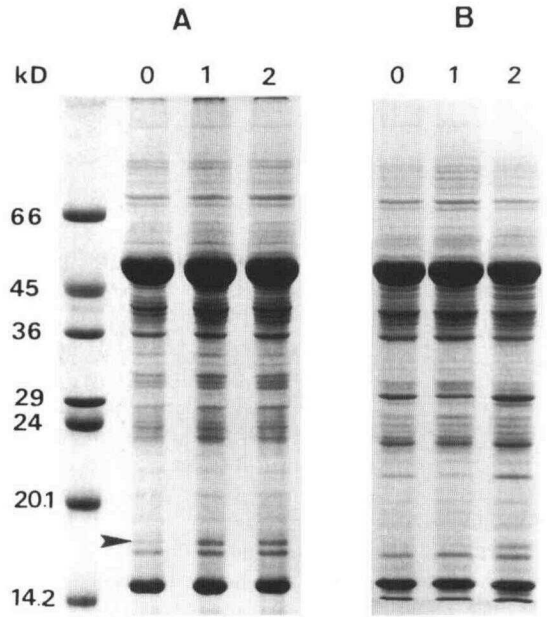

Figure 1. Analysis of leaf soluble polypeptides of infected *L. albus* plants on SDS-polyacrylamide gels. A, First pair of leaves; B, second pair of leaves. Lanes 0, Noninoculated plants; lanes 1, 24 h after inoculation; lanes 2, 8 d after inoculation. Gels were stained with Coomassie blue R. First lane on the left contains molecular mass markers; arrow indicates accumulation of PR-p16.5.

For UV stress studies, 2-week-old plants were submitted to 30 min of UV-C light (254 nm, 5 W m^{-2}) provided by a Philips (Eindhoven, The Netherlands) UV TUV 30W/G30T8 lamp.

Protein Extraction

Leaf samples were homogenized in 30 mM Tris-HCl buffer, pH 7.5, with 20 mM 2-mercaptoethanol and 5% (w/v) polyvinylpolypyrrolidone. 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. For partial purification, proteins precipitated with $(NH_4)_2SO_4$ (70–80% saturation) were solubilized in water, desalted in Sephadex G-25 (PD-10 column, Pharmacia), and loaded onto a DEAE cellulose column (Whatman DE-52) equilibrated with 20 mM His, pH 6.0. The proteins were eluted with 0.1 M NaCl dissolved in the equilibration buffer and concentrated by ultrafiltration in a Centricon-10 (Amicon, Beverly, MA).

Proteins from the intercellular fluid were obtained according to Parent and Asselin (1984), and proteins from the cell walls were extracted as described by Jackson and Ricardo (1994).

Protein Separation

Protein electrophoresis under denaturing conditions was performed in 12% SDS-polyacrylamide gels according to the Laemmli system (Laemmli, 1970). The same system was used for native basic gels, except that SDS was omitted and a 10% polyacrylamide gel was used as the resolving gel. IEF was performed on 5% polyacrylamide gels with 8 M urea and 2% Ampholines, pH 3.9 to 10 (Pharmacia).

Production of Polyclonal Antibodies

The gel band corresponding to the lupin PR-p16.5 was electroeluted after separation of proteins in SDS-PAGE; 25 μg of protein were injected with an adjuvant in 6-week-old Wistar rats. The animals were boosted twice at 3-week intervals. The antiserum was collected 2 weeks after the last boost. For affinity purification, bands corresponding to protein PR-p16.5c and to proteins PR-p16.5a plus PR-p16.5b were cut from western blots and incubated with anti-PR-p16.5 serum. Antibodies were eluted with 0.1 M Gly, pH 2.5, 1% (w/v) BSA, 0.2% (w/v) Tween 20. After neutralization, the purified antibodies were dialized against 10 mM phosphate buffer, pH 7.5, and concentrated in a Centricon-10 (Amicon).

Immunoblotting

Proteins were electroblotted onto polyvinylidene difluoride membranes. The blots were incubated with the primary antibody, diluted 1:5000, which was detected with alkaline phosphatase-labeled anti-rat IgG developed with bromochloroindolyl phosphate/nitroblue tetrazolium substrate.

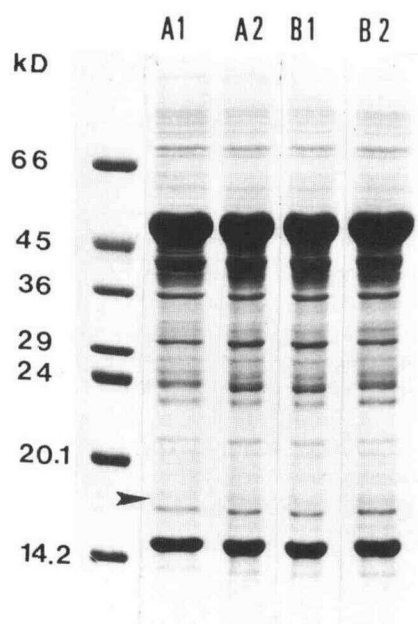

Figure 2. Effect of abrasion of *L. albus* leaves with Carborundum and inoculation with sterile water on the polypeptidic pattern. Lanes A, Leaves dusted with Carborundum and inoculated with water; lane A1, 24 h after treatment; lane A2, 8 d after treatment. Lanes B, Nontreated leaves, collected at the same times as in A. The first lane on the left contains molecular mass markers. Arrowhead indicates PR-p16.5.

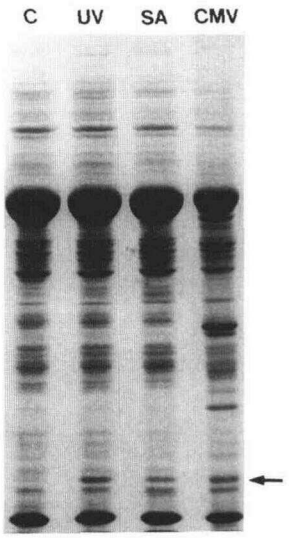

Figure 3. Analysis of leaf soluble polypeptides of *L. albus* plants subjected to different stresses. Lane C, Nonstressed plants; lane UV, plants subjected to UV light (254 nm, 5 W m^{-2}, 30 min); lane SA, plants sprayed with a 5 mM solution of salicylic acid; lane CMV, plants inoculated with CMV. Proteins were extracted 24 h after treatment; arrow indicates PR-p16.5.

N-Terminal Amino Acid Sequencing

The sequencing was performed on western blots using an Applied Biosystems protein sequencer (model 477A) on-line with an HPLC system (model 120A).

Tissue Printing

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

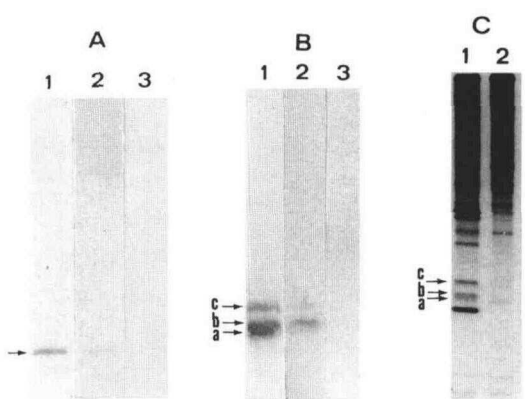

Figure 4. Specificity of anti-PR-p16.5 antiserum. A, Western blot of denatured proteins separated by SDS-PAGE; B, western blot of native proteins separated by PAGE under nondenaturing conditions. Lanes 1, Plants inoculated with *C. gloeosporioides*, blot probed with anti-PR-p16.5 antiserum; lanes 2, noninoculated plants, blot probed with anti-PR-p16.5 antiserum; lanes 3, plants inoculated with *C. gloeosporioides*, blot probed with preimmune serum. C, Total soluble proteins separated by PAGE under nondenaturing conditions. Lane 1, Plants infected with *C. gloeosporioides*; lane 2, noninfected plants. a, b, and c, PR-p16.5a, PR-p16.5b, and PR-p16.5c, respectively.

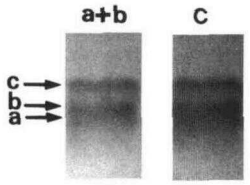

Figure 5. Immunological relationship among proteins PR-p16.5a, PR-p16.5b, and PR-p16.5c. Soluble proteins were separated by PAGE under nondenaturing conditions and electroblotted onto a polyvinylidene difluoride membrane. Lane a+b, Blots probed with antiserum purified against PR-p16.5a plus PR-p16.5b; lane c, blots probed with antiserum purified against PR-p16.5c. Arrows indicate PR-p16.5a (a), PR-p16.5b (b), and PR-p16.5c (c).

Immunogold Transmission EM

Fixation, embedding, and labeling of tissue were performed according to Pereira et al. (1992). The primary antibody was anti-PR-p16.5 serum diluted 1:500, and the secondary antibody was anti-rat IgG coupled to 10-nm gold particles, diluted 1:20. Control sections were incubated with preimmune serum instead of anti-PR-p16.5 serum.

RESULTS

Analysis of Soluble Polypeptides of Stressed Lupin Leaves on SDS-Polyacrylamide Gels

A soluble polypeptide band of M_r 16,500 was found to increase in leaves of *L. albus* plants infected with the fungus *C. gloeosporioides* before the development of any symptoms (24 h after inoculation) as well as in leaves showing necrotic lesions (8 d after inoculation) (Fig. 1A). Increased levels of the M_r 16,500 band were also detected in noninoculated leaves in the later stages of infection (Fig. 1B),

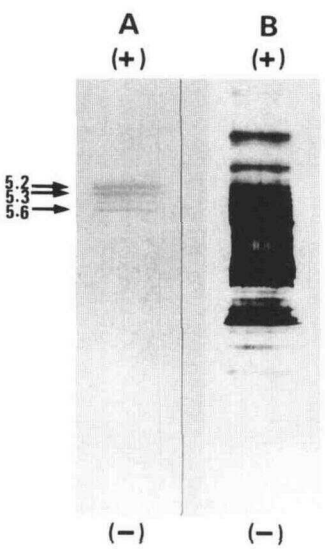

Figure 6. IEF of *L. albus* soluble leaf proteins in 5% polyacrylamide gels with 8 M urea and 2% Ampholine, pH 3.5 to 10. Lane A, Western blot of soluble proteins probed with anti-PR-p16.5 antiserum; lane B, gel of soluble proteins stained with silver. pI values of the three detected proteins were 5.2, 5.3, and 5.6. +, Anode; −, cathode.

Table I. *N-terminal sequence of PR-p16.5c and comparison with the predicted primary structure of soybean stress-induced H4 (Crowell et al, 1992), pea disease-response protein DRRG49C (Chiang and Hadwiger, 1990), bean PR protein PvPR1 (Walter et al, 1990), and potato PR protein pSTH21 (Matton et al, 1990)*

* and •, Identical and conserved residues, respectively.

Protein	N-Terminal Sequence
PR-p16.5c	– G I F T F E D E S T S I V A P A R L Y K
H4	M G I F T F E D E T T S P V A P A T L Y K
DRR49c	M G V F N F E E E A T S I V A P A T L H K
PvPR1	– G V F T F E D Q T T S P V A P A T L Y K
STH-21	M G V T S Y T L E T T T P V A P T R L F K
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showing that it accumulated both locally and systemically. Treatment of the leaves with the abrasive Carborundum and inoculation with sterile water had no effect on the polypeptide pattern of the leaf (Fig. 2).

The M_r 16,500 band also seems to be detected in lupin leaves 24 h after inoculation with a virus (CMV), by chemical treatment with salicylic acid, or by treatment with UV light (Fig. 3). By that time, plants inoculated with CMV or treated with salicylic acid showed no macroscopic symptoms. UV-treated plants showed a few white dots on the leaves. Not being a specific response to the infecting pathogen, this polypeptide band is probably part of the general plant defense mechanism to adverse environmental conditions. According to the nomenclature proposed by van

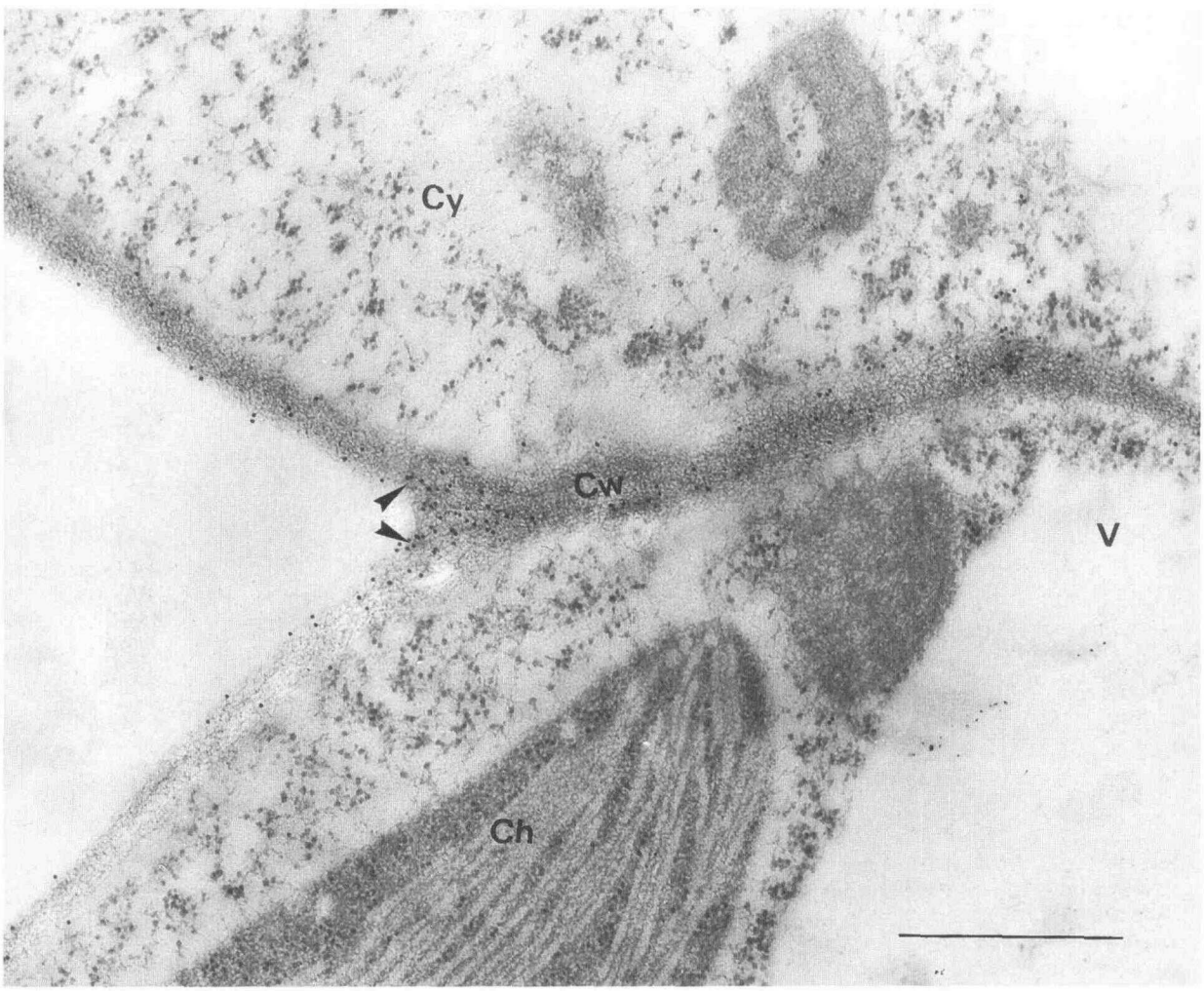

Figure 7. Gold immunolabeling of PR-p16.5 in leaf cross-sections of *L. albus* plants infected with *C. gloeosporioides*. Sections were treated with anti-PR-p16.5 antiserum. Labeling is localized in the cell wall (arrowheads). Cy, Cytoplasm; Cw, cell wall; Ch, chloroplast; V, vacuole. Bar = 1.5 μm.

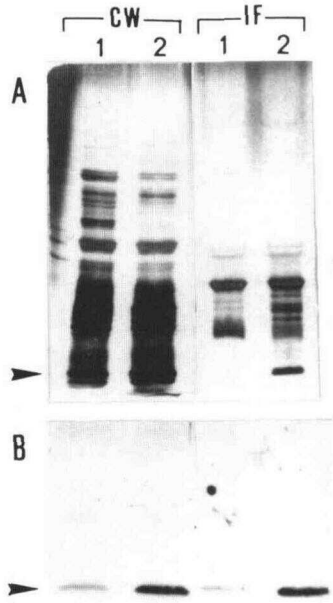

Figure 8. SDS-PAGE analysis of cell wall and intercellular fluid polypeptides of *L. albus* leaves. A, Total proteins stained with silver; B, western blots probed with anti-PR-p16.5 antiserum. CW, Cell wall. Lane 1, Noninfected leaves; lane 2, leaves infected with *C. gloeosporioides*. IF, Intercellular fluid. Lane 1, Extracted with water; lane 2, extracted with 0.1 M KCl. Arrowhead indicates PR-p16.5.

Loon et al. (1994) for unidentified PR proteins, the M_r 16,500 polypeptide band will be designated by PR-p16.5.

Production of Polyclonal Antibodies against PR-p16.5

Polyclonal antibodies against the eluted SDS-polyacrylamide gel band were raised in rats. The antibodies recognized only one polypeptide on western blots of polypeptides separated by SDS-PAGE, indicating specific binding to the antigen (Fig. 4A). When western blots of proteins separated on native gels were probed with the anti-PR-p16.5 serum, three acidic proteins were detected: PR-p16.5a, PR-p16.5b, and PR-p16.5c (Fig. 4, B and C). Affinity-purified antibodies against either protein c or proteins a plus b were used to analyze the immunological relationship among them. Irrespective of the antiserum used, proteins a, b, and c were always detected, indicating cross-reactivity (Fig. 5).

N-Terminal Amino Acid Sequencing

Preparations of partially purified PR-p16.5, containing the proteins a, b, and c, were separated on anodic native gels and electroblotted onto a polyvinylidene difluoride membrane (Sigma). Blots were stained with Ponceau S, and two bands containing protein c and proteins a plus b, respectively, were excised and used for N-terminal amino acid sequencing. A sequence of 20 amino acid residues was determined for PR-p16.5c (Table I). The presence of only one amino acid peak in each cycle shows that the excised band containing protein c was not contaminated with other proteins. Analysis of the blot band containing both PR-p16.5a and PR-p16.5b also showed the presence of only one N terminus, identical to PR-p16.5c, which confirms that the three proteins are related. Since these proteins have slightly different pI values (Fig. 6), they are probably charge isomers of the same protein. Although the anti-PR-p16.5 antibodies could also recognize a fourth, minor band, the reaction does not seem significant when compared to the other three detected bands (Fig. 6).

Localization of PR-p16.5

Electron microscopic observations of immunogold-labeled infected leaves revealed gold particles predominantly in the cell wall, although a few gold particles could be found in the cytoplasm (Fig. 7). No binding of gold particles was observed when sections were incubated with preimmune serum, which gives a good indication that the observed labeling in infected leaves, although not particularly strong, is significant. These observations seem to be supported by results obtained with SDS gels and western blots of cell-wall and intercellular fluid proteins. The PR-p16.5 is detected in the cell-wall fraction and in 0.1 M KCl-extracted intercellular fluid but is less intense in water-extracted intercellular fluid (Fig. 8).

To obtain information on the localization of PR-p16.5 within the different leaf tissues, tissue printing experiments were performed. Although of low anatomic resolution, tissue printing is a recently developed technique (Cassab and Varner, 1987) that provides a rapid and reliable approach to the immunolocalization of antigens in plant tissues, since the native antigen in the nitrocellulose membrane is readily accessible to the antibodies. Tissue prints of infected and noninfected tissues were performed side by side on the same nitrocellulose membrane, so that there were no differences due to time of exposure. In noninfected leaves, staining was located above the xylem vessels and on the epidermis of midvein cross-section prints (Fig. 9, A and C). In infected leaves the staining was greatly intensified above the xylem vessels and was also detected throughout the parenchyma of the midvein (Fig. 9B). As in the ultrastructural experiments, no labeling was observed in tissue prints treated with preimmune serum (data not shown).

DISCUSSION

The PR-p16.5 proteins have electrophoretic mobility and acidic properties similar to those of the tobacco PR-1 class. However, when the N-terminal sequence of PR-p16.5c was used to scan a protein sequence data bank (Pc-Gene version 6.8 package, Intelligenetics, Inc., Mountain View, CA) with the Swiss-Prot 28 data bank (Bairoch and Boeckmann, 1991), no similarity to the tobacco proteins was found. Instead, high similarities were detected with members of the defense-related PR-10 proteins (Table I). The highest similarities (>77%) were found with the stress-related product of the soybean gene H4 (Crowell et al., 1992) and with the disease-resistance-related protein Drr4G49C from pea (Chiang and Hadwiger, 1990) (Table I). Significant similarity (40%) with the PR proteins from bean, PvPR1

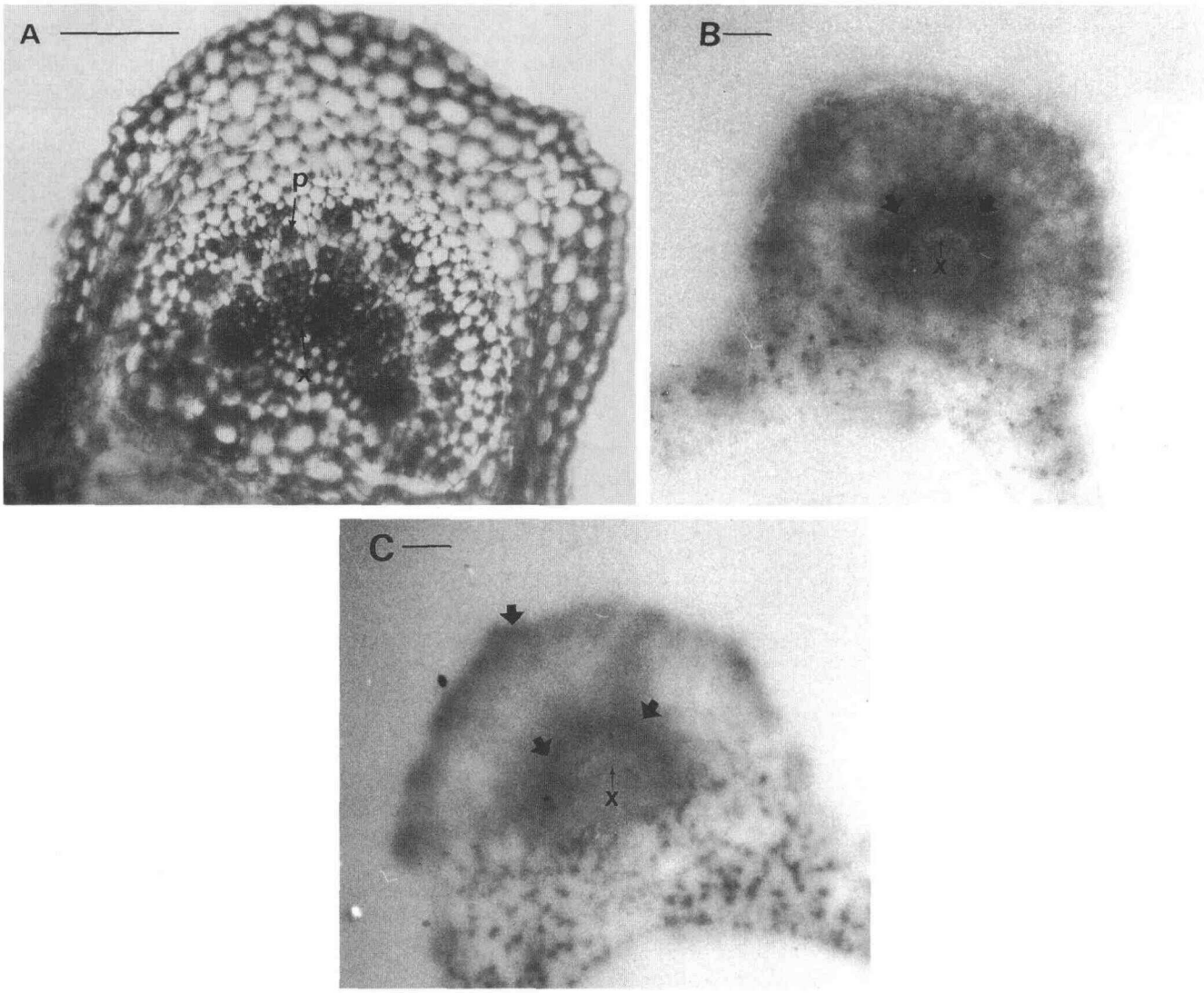

Figure 9. Anatomy of leaf midvein cross-sections of infected *L. albus* plants (A) and localization of PR-p16.5 on tissue prints of infected plants (B) and noninfected plants (C). For the anatomy studies, handmade sections were stained with methylene blue. In the tissue prints, immunolabeling was most abundant above xylem vessels (thick arrows). p, Phloem; x, xylem. Bars = 50 μm. The black dots on the chlorophyllous parenchyma are due to chloroplasts.

(Walter et al., 1990), and from potato, PR-10b, formerly pSTH21 (Matton et al., 1990), was also observed. These results suggest that the PR-p16.5 proteins are members of the PR-10 family, although the N-terminal sequence of PR-p16.5c has only 35% similarity with the Betv-1 pollen allergen (Breiteneder et al., 1989). Other species that express proteins of the PR-10 family in response to some form of stress are parsley, with PcPR1–1 (Somssich et al., 1988), and *Asparagus officinalis*, with AoPR1 (Warner et al., 1992). However, they did not show any similarity with the N-terminal sequence of 20 amino acid residues obtained for PR-p16.5c.

With the exception of the monocotyledonous plant *A. officinalis*, in which only one such protein was discovered so far, in all other species, including *L. albus*, there are at least two highly identical (>80%) members of the PR-10 family coordinately induced by the stress factor. It is apparent that these similar proteins are encoded by a multigene family, showing a gene complexity close to or greater than that of the tobacco PR-1 class.

Tissue-printing experiments suggested association of PR-p16.5 proteins primarily with the vascular system. In tobacco plants, it was found that during the systemic response to tobacco mosaic virus infection, the distribution of PR-1 proteins in the noninoculated leaves was limited to the extracellular spaces surrounding vascular bundles as well as in vacuoles of crystal idioblasts (Dixon et al., 1991). More recently, studies of developmental expression of the *A. officinalis* AoPR1 gene in transgenic tobacco plants have shown that the strongest expression was in the expanding secondary xylem of the stems as they became lignified (Warner et al., 1994).

To our knowledge, no ultrastructural studies on the localization of PR-10 proteins have been made so far, but it has been proposed that these proteins act intracellularly, since no signal sequences typical of extracellular proteins are encoded by the genes (Somssich et al., 1988). However, the lupin proteins seem to be accumulated in the cell wall, as are the tobacco PR-1 proteins. The extracellular localization of tobacco PR-1 proteins is in accordance with the fact that the PR-1 genes encode a 30-amino acid signal peptide that is cleaved from the polypeptide to produce the mature protein (Carr et al., 1985; Cornelissen et al., 1986).

Further information on the properties of PR-p16.5 proteins will come from studies on gene structure and expression.

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