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Study of the Intercellular Fluid of Healthy *Lupinus albus* Organs[1]

Presence of a Chitinase and a Thaumatin-Like Protein

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Proteins in the intercellular fluid (IF) of healthy *Lupinus albus* leaves were characterized. Silver staining of the proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed more than 30 polypeptides, with the major ones having a molecular mass lower than 36 kD. After amino-terminal amino acid sequence analysis, one of the major polypeptides, IF4, was shown to have no identity with any of the proteins present in the data bases. Two others, IF1 and IF3, showed identity with previously reported pathogenesis-related proteins, IF1 with an antifungal protein from *Hordeum vulgare* that belongs to the thaumatin family (PR-5 family), and IF3 with class III chitinase-lysozymes. IF3 was also present in the IF of stem and root and it represents the major polypeptide in the medium of *L. albus* cell-suspension cultures. The ubiquitous presence of this enzyme in healthy, nonstressed tissues of *L. albus* cannot be explained.

The whole plant can be viewed as consisting of two compartments separated by a continuous plasma membrane: (a) an intracellular compartment, known as the symplast, which is made up of the total protoplast community, including the sieve tubes of the phloem bounded by the combined plasma membranes of these living cells; and (b) an extracellular compartment, known as the apoplast, composed of all of the cell walls, the intercellular spaces, the empty dead cells of xylem tubes, and the water contained therein.

Although the apoplast occupies only 5% or less of the tissue volume of aerial organs and the root cortex (Grignon and Sentenac, 1991), it is of great importance in several processes of plant biology. This is particularly true in phytopathological interactions, because it constitutes a physical barrier to infection, being viewed as part of a preformed, constitutive defense mechanism. Furthermore, in response to pathogen attack, plants utilize a large arsenal of inducible mechanisms to prevent colonization, several of which are also expressed in the apoplast. These mechanisms involve the induction of numerous plant genes encoding defense proteins that comprise (a) extracellular matrix proteins that contribute to the confinement of the pathogen (Keller, 1993), (b) enzymes of secondary metabolism, e.g. of phytoalexin biosynthesis (Darvill and Albersheim, 1984), and (c) PR proteins that represent the major quantitative changes in soluble proteins during the defense response (Stintzi et al., 1993).

The PR proteins were first reported in tobacco cultivars reacting hypersensitively to tobacco mosaic virus (Van Loon and Van Kammen, 1970). Based on serological properties, molecular mass, and sequence data, tobacco PR proteins have been classified into five major families. Subsequent studies have led to the identification of the same or similar proteins in several other plant species, including both monocotyledons and dicotyledons (Bol et al., 1990). PR proteins not related to one of the established PR families have been taken as members of new families (Van Loon et al., 1994). Since the apoplast represents an important cellular barrier to pathogen attack, we were concerned about the constitutive presence of defense proteins in the apoplast from healthy *Lupinus albus* plants. We were particularly interested in the soluble or ionically bound proteins that were obtained by preparing IF. *L. albus* is an important grain legume crop (Hill, 1977) well adapted to the Mediterranean climate and soils and is considered to be resistant to pathogen attack. Surprisingly, we found that PR proteins were present in healthy *L. albus* IF.

MATERIALS AND METHODS

Plant Material

Lupin plants (*Lupinus albus* L. cv Rio Maior and cv Ultra) were grown for 14 to 20 d in a growth room under a 12-h photoperiod supplied by white fluorescent lights (160 μE m^{-2} s^{-1}) at 24/18°C (light/dark). The plants were grown either on a sandy soil from a *Lupinus* growing region, watered with tap water, or on a coarse quartz sand, watered with a nutritive solution (macro elements from Shea et al. [1968] and micro elements from Johnson et al. [1957]). In addition, plants originated from surface-sterilized seeds (0.1% [w/v] $HgCl_2$ and 0.02% [w/v] Tween 20) were

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Abbreviations: IF, intercellular fluid; PR, pathogenesis-related; TBS, Tris-buffered saline.

grown on sterilized soil under the same growth-room conditions. *L. albus* L. cv Rio Maior and *Lupinus mutabilis* Sweet cv Inti grown under natural field conditions were also used. Salicylic acid treatment was carried out as described by Van Huijsduijnen et al. (1986).

In Vitro Cultures

Cell suspensions were obtained from 1-month-old callus formed by culturing 1-week-old hypocotyl or root segments on Murashige and Skoog (1962) medium containing 0.15 mg/L 2,4-D, 0.3 mg/L kinetin, 3% (w/v) Suc, and 0.8% (w/v) agar. Small pieces of callus were suspended in liquid medium with the same composition and maintained on a gyratory shaker (120 rpm) at 24 to 26°C under a 14-h photoperiod.

IF, Leaf Homogenates, and Acidic Protein Extraction

The IF was isolated by the method of Parent and Asselin (1984). In leaves the main vein was eliminated and in stems and roots the cortex was separated from the stele. The effect of composition of the infiltration solution on IF leaf extraction was tested by comparing the following solutions: (a) distilled water; (b) 1 mM PMSF, 1 mM DTT, and 1 mM EDTA in 25 mM Tris, pH 7.8; (c) 0.5 M KCl; (d) 0.5 M KCl in solution b; (e) 1 M KCl; and (f) 1 M KCl in solution b. IF samples were desalted and concentrated using a microconcentrator (Centricon 10 [Amicon, Beverly, MA], 10,000 cutoff). Stems and roots were always infiltrated in the presence of KCl (solution d above).

Leaf homogenates were obtained by grinding leaves frozen in liquid nitrogen (after removal of the main vein) in a mortar with solution b above. The homogenate was centrifuged at 10,000*g* for 20 min and the supernatant was subjected to centrifugation at 100,000*g* for 30 min. The proteins in the supernatant were precipitated overnight at −20°C with 0.1 M ammonium acetate in methanol. The pellet was washed twice with cold acetone, and the proteins were resuspended in electrophoresis sample buffer (200 mM DTT, 4% [w/v] SDS, 0.012% [w/v] bromphenol blue, and 30% [v/v] glycerol in 100 mM Tris-HCl, pH 8.6).

For acidic protein extraction, 1 g of leaf lamina was ground in 2 mL of either buffer A (84 mM citric acid, 32 mM Na_2HPO_4, 14 mM 2-mercaptoethanol, and 6 mM L-ascorbic acid at a final pH of 2.8; Antoniw and Pierpoint, 1978) or buffer B (15 mM 2-mercaptoethanol in 0.5 M sodium acetate pH 5.2; Jung et al., 1993). The crude extracts were centrifuged at 13,000*g* for 15 min at 4°C and the supernatant was desalted on a Sephadex G-25 column (Pharmacia) equilibrated in 100 mM Tris-HCl, pH 6.8, containing 15 mM 2-mercaptoethanol. The proteins were concentrated using a Centricon 10 cartridge.

Recovery of Proteins from the Media of Suspension Cultures

The culture medium from 1-month-old suspension cultures was filtered through a cellulose acetate filter (pore size 0.22 μm), and the proteins were precipitated by the addition of 2.5 volumes of cold acetone. The resulting acetone-insoluble residue obtained by centrifugation (15,000*g* for 10 min at 4°C) was resuspended in the electrophoresis sample buffer.

PAGE and Staining

Electrophoresis under denaturing conditions was performed on slab gels using the SDS-discontinuous system of Laemmli (1970) with a 4% stacking gel and a 12% resolving gel. 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. Gels were stained for 30 min with 0.1% (w/v) Coommassie blue R in fixative (40% [v/v] methanol, 10% [v/v] acetic acid) and destained overnight in the same fixative. Alternatively, gels were silver stained by the method of Blum et al. (1987).

Detection of Con A-Binding Glycoproteins

After electrophoresis, the proteins were transferred (80 V for 60 min at 4°C) onto polyvinylidene difluoride membranes with the electrode buffer of 192 mM Gly and 10% (v/v) methanol in 25 mM Tris, pH 8.3. After transfer, the proteins were fixed with acetic acid:isopropyl alcohol:water (10:25:65, v/v) and rinsed several times with distilled water and then with TBS (500 mM NaCl in 20 mM Tris-HCl, pH 7.4). The basic procedure of Faye and Chrispeels (1985) was used for glycoprotein detection except that Tween 20 replaced gelatin in the blocking and remaining solutions.

Immunological Methods

IF3 was purified by SDS-PAGE and the fragmented band cut from the gel was administered (100 μg/inoculation) to New Zealand rabbits by intradermal injections. Ten days after the second boost, immunization serum was collected and clarified by centrifugation. For immunodetection the blots were soaked for 2 h at room temperature in TBS containing 0.2% (w/v) Tween 20 and then incubated for 60 min at room temperature in rabbit antiserum, anti-IF3, or anti-PR-Q from tobacco, diluted 1:10,000 or 1:1,000, respectively, with TBS containing 0.1% (w/v) Tween 20. The blots were washed, incubated for 60 min at room temperature with phosphatase-conjugated anti-rabbit IgG (Sigma) diluted in the same buffer, washed again, and developed according to Harlow and Lane (1988).

Amino-Terminal Amino Acid Sequence Analysis of the Electroblotted Proteins

The IF proteins from leaves were electrophoretically separated under denaturing conditions as described above but with some improvements (according to Applied Biosystems User Bulletin, issue No. 25, 1986). Amino-terminal amino acid sequence determination of the blotted proteins was accomplished by the automated Edman degradation method, carried out in a 477A Applied Biosystems gas-phase protein sequencer, and the phenylthiohydantoin amino acid derivatives were automatically identified with a 120A Applied Biosystems analyzer used on-line with the sequencer.

RESULTS

Effect of the Infiltration Solution and Efficiency of the IF Extraction Method

SDS-PAGE was performed to analyze the electrophoretic patterns of IF proteins from healthy leaves of *L. albus* extracted with different infiltration solutions (see "Materials and Methods"). Several polypeptides could be detected, but the major ones were less than 36 kD (Fig. 1). No qualitative changes in the electrophoretic patterns were observed when water or a buffer solution were used; however, the presence of KCl (0.5 M or 1 M) led to the additional extraction of a set of high-molecular mass Con A-binding glycoproteins (data not shown). These results suggest that, except for the glycoproteins extracted with KCl, proteins present in IF samples are not ionically bound to apoplastic structures.

To reduce IF contamination from sieve tube proteins, the main veins of the leaves were excised prior to IF extraction. Two controls were prepared, consisting of homogenates from leaf lamina portions either previously subjected or not to IF extraction. The polypeptide patterns of IF proteins were systematically very different from those of intracellular proteins (Fig. 1), irrespective of the control used. This means that the IF proteins represent a small fraction of the whole tissue proteins and that IF samples are not significantly contaminated with intracellular proteins.

Homology of IF Proteins to Known Proteins

We sequenced the amino termini of the four most prominent polypeptides (IF1, IF2, IF3, and IF4; Fig. 1) from the IF of healthy *L. albus* leaves. From IF2 only seven nonconsecutive amino acid residues were detected. To establish possible identity between the amino termini of IF1, IF3, and IF4 and proteins of known sequence, a search was carried out using PC Gene version 6.8 (Intelligenetics, Mountain View, CA) and the protein sequence data bank SWISS-PROT version 29 (October 1994). No homology was found between the amino-terminal amino acid sequence from IF4 (V-D-Y-T-V-T-N-N-A-L-N-X-D-G-G-V-X-F-X-D) and sequences present in the data base. However, 15 and 16

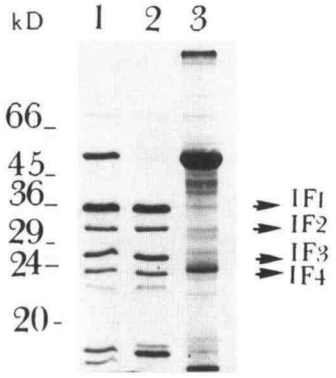

Figure 1. Comparison of IF and leaf homogenate SDS-PAGE patterns stained with Coomassie blue R. Lane 1, IF extracted without KCl; lane 2, IF extracted with 0.5 M KCl; lane 3, leaf homogenate. Each lane contains 15 μg of protein.

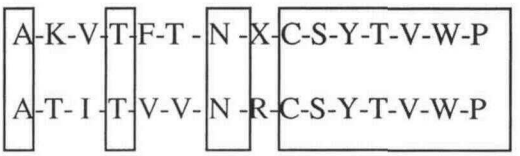

Figure 2. Comparison of the amino-terminal amino acid residues of IF1 with those of an antifungal protein from barley (BP-R) (Hejgaard et al., 1991). X, No amino acid derivative was detected by HPLC. Identical amino acids are boxed.

amino-terminal amino acid residues of IF1 and IF3, respectively, were identical to those of previously reported PR proteins. IF1 (Fig. 2) had 70% identical (80% similar) amino acid residues to an antifungal protein (BP-R) from *Hordeum vulgare* (Hejgaard et al., 1991), which belongs to the thaumatin family (PR-5 family). The 16 amino-terminal amino acid residues from IF3 also had strong identity to those of previously reported class III chitinase-lysozymes, as shown in Figure 3.

It is important to stress that IF3 was specifically immunodetected with a serum raised against the acidic tobacco chitinase PR-Q (Legrand et al., 1987) (results not shown). According to the classification of chitinases (Jekel et al., 1991), PR-Q is a class II chitinase. IF3 is identical to class III chitinases, but it must have some internal antigenic sites common to PR-Q. Previously reported results suggest that chitinases from different plant families are serologically closely related (Legrand et al., 1987; Joosten et al., 1989).

Effect of Growing Conditions and Salicylic Acid on the Electrophoretic Pattern of IF Proteins

Several constitutively expressed polypeptides were present in the IF, with at least two of them having similarity to PR proteins. Although plants were raised in a growth room with no added inducer (biotic or abiotic), the IF polypeptides could have resulted from a stress imposed by the growth conditions or by some cryptic infection. Recently, it has been reported that untreated sunflower plants have PR proteins at a level strongly dependent on their growth conditions (Jung et al., 1993). Using healthy, field-grown lupin plants, we detected the same IF polypeptides not only in *L. albus* cv Rio Maior (Fig. 4, lane 1), but also in a different lupin species, *L. mutabilis* (results not shown); however, growth-room conditions intensified these polypeptides (Fig. 4, lanes 2 and 5). To exclude the action of a cryptic microorganism, lupin plants originating from surfaced-sterilized seeds were grown on sterilized soil. No differences were detected on the IF polypeptide patterns (data not shown). We observed a further increase in the IF polypeptides by chemical treatment with salicylic acid (Fig. 4, lane 3), a chemical inducer of PR proteins that is implicated in signal transaction of resistance responses. Since *L. albus* cv Rio Maior is a high-alkaloid variety, we decided to analyze also a *L. albus* low-alkaloid variety (cv Ultra); the protein pattern for this variety was similar to that for *L. albus* cv Rio Maior (Fig. 4, lane 4). In addition, all of the

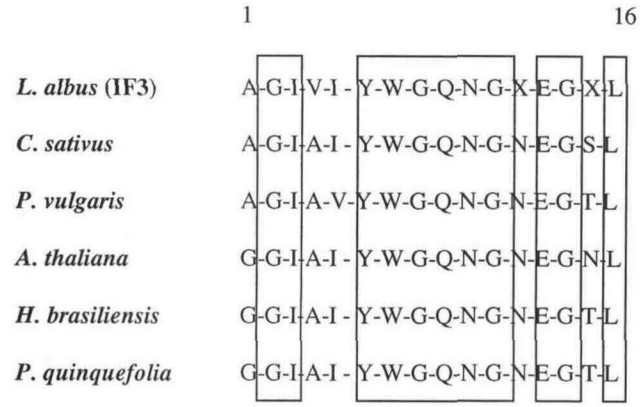

Figure 3. Comparison of the amino acid sequence of IF3 with those of lysozyme/chitinases from *Parthenocissus quinquefolia* (Bernasconi et al., 1987), *Cucumis sativus* chitinase (Metraux et al., 1989), *Arabidopsis thaliana* chitinase (Samac et al., 1990), lysozyme/chitinase from *Hevea brasiliensis* (Jekel et al., 1991), and *Phaseolus vulgaris* chitinase (Margis-Pinheiro et al., 1993). X, No amino acid derivative was detected by HPLC. Identical amino acids are boxed.

most prominent IF polypeptides of lupin leaves were detected in total leaf homogenates prepared at low pH (data not shown), the major polypeptides being preferentially extracted at pH 2.8 (one of the characteristics shared by the PR proteins).

Identification of IF3 in the IF of Stem and Root and in the Culture Medium of Lupin Cell-Suspension Cultures

By immunoblotting analysis using an antiserum raised against purified IF3, we showed that this polypeptide is also present in the IF of stem and root and that it represents the major polypeptide of the culture medium of lupin cell-suspension cultures from root and shoot explants (Fig. 5, A and C). This chitinase is not a glycopolypeptide rec-

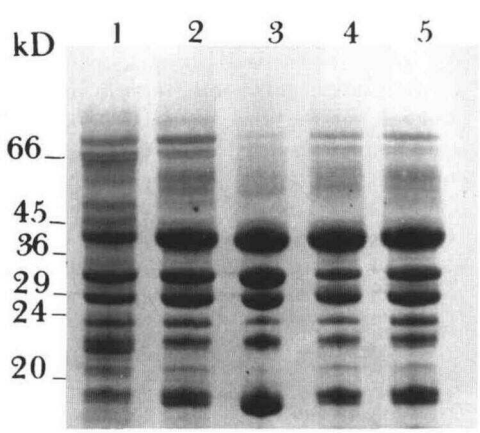

Figure 4. Effect of growing conditions and salicylic acid treatment on the IF leaf proteins from *L. albus* cv Rio Maior (lanes 1–3 and 5) or cv Ultra (lane 4). Lane 1, Field-grown plants; lanes 2 and 4, growth-room conditions, plants on soil; lane 3, growth-room conditions, plants on soil and treated with salicylic acid; lane 5, growth-room conditions, plants on coarse sand fed with nutrient solution. Samples were subjected to SDS-PAGE and were stained with Coomassie blue R. Each lane contains 20 μg of protein.

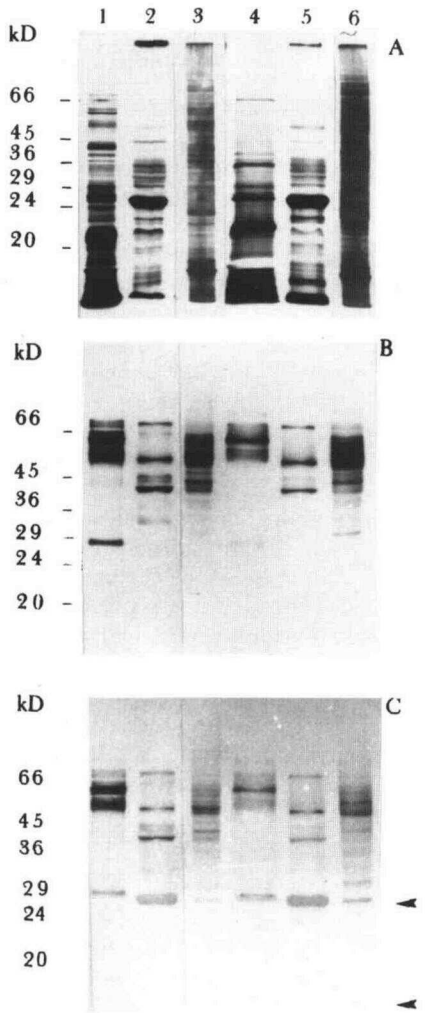

Figure 5. SDS-PAGE separation of IF polypeptides from different organs or from cultured cells. A, Polypeptides stained with silver; B, Con A-binding glycopeptides; C, immunoblots developed with anti-IF3 serum after Con A-binding glycopeptide detection. IF from stems (lane 1) or from roots (lane 4), growth media of cell-suspension cultures from hypocotyls (lane 2) or from roots (lane 5), and soluble extracts of the corresponding cultured cells from hypocotyls (lane 3) or from roots (lane 6). Each lane contains 8 μg of protein. The immunoreactive bands are indicated by arrowheads.

ognized by Con A (Fig. 5B). Two small immunoreactive bands could also be detected among the intracellular proteins of the cultured cells, one with the same molecular mass as IF3 and the other with lower molecular mass (Fig. 5C).

After basic native PAGE we detected only one acidic form of IF3 in both root and stem IF samples, but we detected three forms in the leaf, two of which were very faintly immunoreactive (results not shown).

DISCUSSION

Although it has been known for a long time that many extracellular enzymes are present in plant tissues (Cassab and Varner, 1988), some studies of IF composition were

unable to detect extracellular proteins in healthy leaves by means of SDS-PAGE (Parent and Asselin, 1984). On the contrary, our results suggest the presence of several polypeptides in the IF of healthy lupin leaves and other organs. Although significant quantitative differences were observed between the electrophoretic patterns from healthy plants grown in the field and those grown under controlled room conditions or treated with salicylic acid, qualitatively they were identical, irrespective of the cultivar (including high- and low-alkaloid cultivars), species, or culture conditions studied. It was surprising that some of the prominent IF polypeptides had identity to PR proteins, which have been reported in healthy plant tissues, but only at specific physiological stages such as flowering and senescence (Legrand et al., 1987). These observations lead us to conclude that several proteins are constitutively present in the IF of healthy lupin tissues (leaves, stems, and roots) including PR-like proteins (at least a chitinase and a thaumatin-like protein) in addition to the expected enzymes such as the peroxidases already reported for this plant (Jackson and Ricardo, 1994).

The widespread occurrence of chitinase is particularly intriguing, since this enzyme appears to lack endogenous substrates in higher plants (Boller et al., 1983). However, since chitin is a major component of the cell walls of many fungi (Bartnicki-Garcia, 1968), it has been hypothesized that this enzyme may be involved in plant defense reactions. In support of this hypothesis, it has been shown that the purified enzyme restricts fungal growth in vitro (Broekaert et al., 1988; Mauch et al., 1988; Verberg and Huynh, 1991), although several of the isolated chitinases do not possess antifungal activity (Woloshuk et al., 1991; Sela-Buurlage et al., 1993), and in some cases transgenic plants transformed with chitinase genes did not show increased resistance against pathogens (Neuhaus et al., 1991; Nielsen et al., 1993). These observations and the facts that some chitinase genes are expressed in the absence of pathogens and that their expression may be controlled in an organ-specific and developmental manner (Kombrink et al., 1993) raise the question of their possible participation in other cellular processes. In fact, it has been suggested that a 32-kD glycosylated acidic endochitinase is responsible for the observed rescue of a carrot somatic embryo mutant arrested at the globular stage (De Jong et al., 1992). Additionally, recent data indicate a further possible role for chitinases constitutively present in legume roots, i.e. the hydrolysis and inactivation of nodulation-inducing factors contributing to the host specificity of rhizobia (Staehelin et al., 1994).

Furthermore, chitinases have been reported to be produced by some cultured plant cells (Esaka et al., 1993). It was interesting to find that the most prominent polypeptide present in the liquid medium of cultured lupin cells was the chitinase present in the leaf IF. The marked increase in chitinase activity and protein during callus formation from pumpkin fruit tissues has been attributed to wounding and not to differentiation of the tissues (Esaka et al., 1993). In cultured lupin cells we cannot attribute the presence of chitinase to wounding, although we cannot rule out the hypothesis that it is due to some other stress. Although the ubiquitous presence of this enzyme in lupin cannot be explained at the moment, it is possible that the plant has recruited a PR protein for use as a constitutive, preformed defense against fungal pathogens.

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