Planta (2000) 210: 543–550

Planta

The *Lupinus albus* class-III chitinase gene, *IF3*, is constitutively expressed in vegetative organs and developing seeds

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Received: 15 March 1999 / Accepted: 12 July 1999

Abstract. A cDNA fragment encoding a *Lupinus albus*. L. class-III chitinase, IF3, was isolated, using a cDNA probe from *Cucumis sativus* L., by in-situ plaque hybridization from a cDNA library constructed in the Uni-ZAP XR vector, with mRNAs isolated from mature lupin leaves. The cDNA had a coding sequence of 293 amino acids including a 27-residue N-terminal signal peptide. A class-III chitinase gene was detected by Southern analysis in the *L. albus* genome. Western blotting experiments showed that the IF3 protein was constitutively present during seed development and in all the studied vegetative lupin organs (i.e., roots, hypocotyls and leaves) at two growth stages (7- and 20-d-old plants). Accumulation of both the IF3 mRNA and IF3 protein was triggered by salicylic acid treatment as well as by abiotic (UV-C light and wounding) and biotic stress conditions (*Colletotrichum gloeosporioides* infection). In necrotic leaves, IF3 chitinase mRNA was present at a higher level than that of another mRNA encoding a pathogenesis-related (PR) protein from *L. albus* (a PR-10) and that of the rRNAs. We suggest that one role of the IF3 chitinase could be in the defense of the plant against fungal infection, though our results do not exclude other functions for this protein.

Key words: Chitinase – Gene expression (chitinase, *IF3*) – *Lupinus* (chitinase) – Pathogenesis-related protein

Introduction

Plants in their natural environment are continuously exposed to several stress factors including pathogen attack. The plant cell is capable of defending itself by means of a combination of constitutive and induced defenses. The typical preformed constitutive defense mechanisms are the complex structural barriers represented by the cell walls, which are further reinforced upon pathogen attack. The most frequently observed and the best-characterized induced defenses include: rapid localized cell death (hypersensitive response; Levine et al. 1996); synthesis of phytoalexins (Darvill and Albersheim 1984); reinforcement of cell walls by rapid oxidative cross-linking of pre-existing cell wall structural proteins (Brisson et al. 1994) and by deposition of structural components like callose (Bowles 1990), lignin (Vance et al. 1980) and related wall-bound phenolics (Matern and Kneusel 1988), as well as glycoproteins (Showalter 1993); and synthesis of pathogenesis-related (PR) proteins (Stintzi et al. 1993).

Chitinases (EC 3.2.1.14) are members of several families of PR proteins, six classes being proposed on the basis of the amino-acid sequence information from cloned genes or purified proteins (Neuhaus et al. 1996). However, not all known chitinases fall into one of the six categories (Graham and Sticklen 1994).

In most studied plants, chitinases occur in several isoforms (Trudel et al. 1989; Darnetty et al. 1993; Ludwig-Müller et al. 1994) and some chitinases appear in certain organs only during specific stages of the plant's life (Neale et al. 1990; Lawton et al. 1994). Some of them, expressing lysozyme activity, have been implicated in the defense reactions of plants against fungal and bacterial infection. Direct antifungal activity of chitinases has been demonstrated in vitro (Schlumbaum et al. 1986; Broekaert et al. 1988; Jacobsen et al. 1990) and in vivo (Benhamou et al. 1993). However, not all chitinases have antifungal activity (Sela-Buurlage et al. 1993; Joosten et al. 1995). Apart from a role in defense, chitinases have also been implicated in mechanisms of plant development and reproduction (Leung 1992; de Jong 1994; van Hengel 1998), in the host specificity of rhizobia (Staehelin et al. 1994), in nodule development (Goormachting et al. 1998) and in the process of cold acclimatation in freezing-tolerant plants (Hon et al. 1995).

The IF3 cDNA sequence has been submitted to the GenBank data base under the accession number Y16415

Abbreviations: dpa = days post-anthesis; PCR = polymerase chain reaction; PR = pathogenesis-related

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Chitinase gene expression is induced, in addition to pathogen attack by various factors including elicitors, wounding, salicylic acid, inorganic salts, ethylene, auxin, cytokinin, ozone and UV light (Ernst et al. 1992; Margis-Pinheiro et al. 1993; Graham and Sticklen 1994).

We have previously detected the presence of an extracellular class III chitinase (IF3 protein) in the apoplast of healthy lupin (*Lupinus albus* L.) leaves (Regalado and Ricardo 1996). In this paper we describe the cloning and characterization of an IF3 cDNA fragment and we compare its predicted amino-acid sequence with related enzymes available in databanks. We show that the *IF3* gene is constitutively expressed in developing and mature seeds and in all vegetative organs (roots, hypocotyls and leaves) from lupin plants at two growth stages, and is highly induced as a response to stress.

Materials and methods

Plant material. Seeds of lupin plants (*Lupinus albus* L. cv. Rio Maior and cv. Ultra), kindly supplied by J. Neves Martins (Instituto Superior de Agronomia, Lisboa), were surface-sterilized [0.1% (w/v) $HgCl_2$ and 0.02% (w/v) Tween-20] prior to aseptic germination. The plants were grown on sterilised soil (from a *Lupinus* growing region) or coarse quartz sand, in a growth room under a 12-h photoperiod supplied by white fluorescent lights (160 μmol m^{-2} s^{-1}) at 24/18 °C (light/dark) and watered with sterilized tap water. *Lupinus albus* cv. Ultra was used in the construction of the cDNA library and in the Southern blotting analysis. Gene expression studies were carried out with cv. Rio Maior.

Stress conditions and salicylic acid treatment. Plants (*L. albus* cv. Rio Maior) were subjected to the following treatments: spraying with 5 mM salicylic acid (van Huijsduijnen et al. 1986) 7 d before harvesting; wounding with carborundum 7 d before harvesting; UV-C light (254 nm; 5 W m^{-2}) irradiation, for 30 min, 24 h before harvesting; inoculation with *Colletotrichum gloesporioides* (10^6 spores mL^{-1}) 5 d before harvesting. Infected and control (water sprayed) plants were incubated for 24 h in a moist chamber in the dark after inoculation. Plants from all treatments were harvested 24 d after germination.

Construction and screening of the cDNA library. Total RNA was prepared from mature leaves of 24-d-old plants (*L. albus* cv. Ultra) using the guanidinum isothiocyanate method (Chomoczynski and Sacchi 1987). The poly(A)$^+$RNA was isolated using the Poly(A) Quik mRNA purification kit (Stratagene).

The cDNA library was constructed in the Uni-ZAP XR vector system included in the ZAP-cDNA synthesis kit (Stratagene), using 5 μg of poly(A)$^+$RNA according to the manufacturer's protocols. The constructed λ cDNA plaques were packaged using Gigapack II Gold packaging extracts (Stratagene).

The cDNA library was screened by in-situ plaque hybridization using the ^{32}P-labelled *Cucumis sativus* class-III chitinase as a probe (Métraux et al. 1989). Isolated plaques coinciding with the positive signals on the X-ray exposed film were picked up, soaked overnight at 4 °C in SM buffer [100 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), 0.01% (w/v) gelatine], titrated and reselected by polymerase chain reaction (PCR) analysis using two degenerated 23-bp oligonucleotides as primers (chit1: 5′GGGGNCA$^A/_G$AA$^T/_C$ GGNAA$^T/_C$GA$^A/_G$GG3′; chit2: 5′CANGGNGG$^A/_G$TT$^A/_G$TT$^A/_G$TA$^A/_G$AA$^T/_C$TG3′) corresponding, respectively, to the N-terminal amino-acid sequence (W-G-Q-N-G-N-E-G) and to an internal amino-acid sequence (Q-F-Y-N-N-P-P-C) that are conserved between cucumber and *Arabidopsis* class-III chitinases (Samac et al. 1990). From each phage suspension 1×10^6 plaque-forming units were diluted in 10 μL of deionised water, heated at 70 °C for 10 min, chilled on ice and submitted to PCR amplification in a reaction mixture composed of 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 1 pmol μL^{-1} each primer and 0.02 U μL^{-1} *Taq* DNA polymerase (Pharmacia) in 1 × concentrated buffer solution supplied with the enzyme. The reactions were carried out in a Trio-Thermoblock (Biometra) thermal reactor, first 5 cycles of 30 s at 92 °C, 1 min 30 s at 45 °C and 30 s at 72 °C, then 35 cycles of 30 s at 92 °C, 30 s at 50 °C and 30 s at 72 °C.

Positive phages were excised in-vivo with the Ex-Assist helper phage (Stratagene) and recircularized to form phagemid subclones in the pBluescript SK (-) vector.

Southern, Northern and Western blotting analysis. Genomic *L. albus* DNA was isolated as described by Jofuku and Goldberg (1988). Samples of 10 μg were digested with restriction enzymes under the conditions suggested by the supplier, electrophoresed on a 0.8% (w/v) agarose gel, and transferred to Hybond-N+ nylon membranes (Amersham) using the alkaline transfer procedure.

The filters were hybridized with the *L. albus* IF3 cDNA that had been ^{32}P-labelled by random priming (Megaprime DNA Labelling Systems; Amersham) in 6 × saline sodium citrate buffer (SSC; 1 × SSC = 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0), 5 × Denhart's solution, 0.5% (w/v) SDS and 25 μg mL^{-1} sonicated salmon sperm DNA, at 65 °C (high-stringency conditions) or at 55 °C (low-stringency conditions). For low-stringency conditions the filter was washed twice, 15 min each, in the following sequence of washing solutions, at 55 °C: (i) 2 × SSC/0.5% (w/v) SDS; (ii) 1 × SSC/0.5% (w/v) SDS. When high-stringency conditions were used the blot was washed at 65 °C in a sequence of SSC/SDS solutions with the last wash in 0.1 × SSC/0.5% (w/v) SDS.

Total RNAs were prepared using the isothiocyanate method (Chomoczynski and Sacchi 1987). The RNAs were denatured and electrophoresed in 2% (w/v) formaldehyde-agarose gels. The gels were blotted to Protran BA nitrocellulose membranes (Schleicher & Schuell) in 20 × SSC according to the manufacture's instructions. Equal loading and intactness of the preparations were always confirmed by ethidium bromide staining of RNA prior to blotting.

The filters were hybridized with ^{32}P-labelled *L. albus* class-III chitinase and PR-10 cDNAs in the solution above described for Southern blotting at 60 °C for 12–16 h. Blots were washed at 60 °C in the following sequence of SSC/SDS solutions, 10 min each: (i) 2 × SSC/0.1% (w/v) SDS; (ii) 1 × SSC/0.1% (w/v) SDS; (iii) 0.1 × SSC/0.1% (w/v) SDS.

Protein samples were prepared by grinding leaves, hypocotyls and roots, frozen in liquid nitrogen, in a mortar with the following buffer solution: 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). Developing seeds were also ground in liquid nitrogen with pestle and mortar. Two millilitres of 50 mM phosphate buffer, (pH 7.5) containing 10% NaCl, 10 mM DTT, 5 mM EDTA, 1.5 μM Pepstatin A, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride and 1 mM $MgCl_2$, was added to 1 g of ground seeds. The crude protein 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 (Amicon).

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 immunological blotting was performed as described previously, using a polyclonal antibody against IF3 protein from *L. albus* cv. Rio Maior prepared as reported before (Regalado and Ricardo 1996).

Sequencing of cDNA and data analysis. The cDNA sequence was determined in the pBluescript vector on both strands by dideoxynucleotide sequencing (Sanger et al. 1977) with the Thermo Sequenase Cycle Sequencing Kit according to the supplier's instructions (Amersham). Specific oligonucleotides were used as primers to fill the gaps (primer walking).

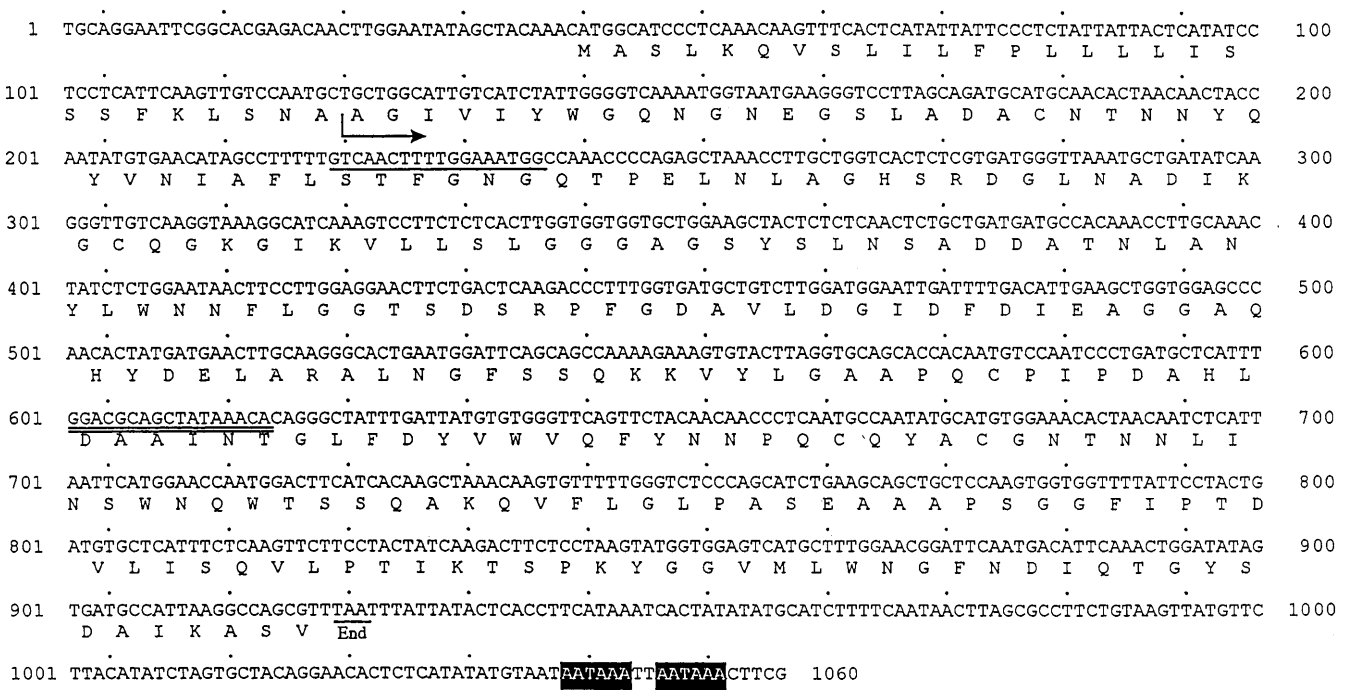

Fig. 1. Nucleotide and deduced amino-acid sequences of IF3 cDNA. The N-terminus of the mature protein is indicated by an *arrow*. The stop codon is *underlined* and indicated by *End*. The sequence of oligonucleotides N (*underlined*) and C (*doubly underlined*) is indicated. The two probable polyadenylation signals are *shaded*

The FASTA and BLAST searches were conducted using the GenBank, SwissProt, DDBJ and EMBL sequence data bases. The determination of the degree of identity between *L. albus* IF3 chitinase and class-III chitinases from other species was conducted using the GAP program. The alignments of the sequences created using the PILEUP program were used for the determination of pairwise genetic distances by the DISTANCES program and the resulting distance matrix was used for the construction of the phylogenetic tree by the GROWTREE program. All four computer programs are part of the Wisconsin Sequence Analyse Package Version 8.

Results

Cloning and sequence analysis of IF3 chitinase cDNA from L. albus. A cDNA library was prepared using poly(A)$^+$RNA isolated from mature leaves of healthy plants. Previously, we had detected the presence of an extracellular protein (IF3) from healthy leaves of *L. albus* showing strong identity to reported class-III chitinase/lysozymes (Regalado and Ricardo 1996). This observation prompted us to use the class-III cucumber chitinase cDNA (Métraux et al. 1989) as a probe to screen the lupin cDNA library.

About 100 000 recombinants were screened with a 1-kb restriction fragment generated from the cucumber chitinase cDNA by *Eco*RI digestion. The positive plaques were then screened by PCR analysis using two degenerate oligonucleotides as primers, corresponding to two conserved regions of the cucumber and *Arabidopsis* cDNA (see *Materials and methods*). A cDNA clone of IF3 was obtained and sequenced.

The 1060-bp cDNA insert (Fig. 1) has an open reading frame starting with an ATG initiation codon 44 nucleotides downstream of the first nucleotide and ending at nucleotide 922 before the TAA stop codon. The 3′ untranslated region contains two putative polyadenylation signals (AATAAA) located at positions 1042 and 1050.

The open reading frame encodes a polypeptide of 293 amino-acid residues that includes, in addition to the mature protein of 266 residues, a putative N-terminal signal peptide that was not found in the purified IF3 protein by Edman degradation. This peptide most probably constitutes the signal sequence involved in the secretion of the IF3 protein to the apoplast (Regalado and Ricardo 1996). The deduced amino-acid sequence (Fig. 1) shows at the NH_2-terminus, after the signal peptide, a sequence completely identical to that obtained by Edman degradation of the mature IF3 protein (Regalado and Ricardo 1996).

Southern blotting of genomic DNA was carried out to test for the presence of related class-III chitinase genes. Using the ^{32}P-labelled IF3 cDNA as a probe, under high- and low-stringency conditions (see *Materials and methods*), single strong hybridization bands were obtained for each restriction digest (Fig. 2). Additional hybridization bands were not detected even after long exposures. This result seems to indicate that only one *IF3* chitinase gene is present in the *L. albus* genome.

Sequence comparisons. The predicted amino-acid sequence of the IF3 cDNA fragment was compared with sequences present in the database. The IF3 protein is closely related to class-III chitinases from various plant species, thus allowing IF3 protein to be considered as a class-III chitinase. Indeed, the highest identity is shared

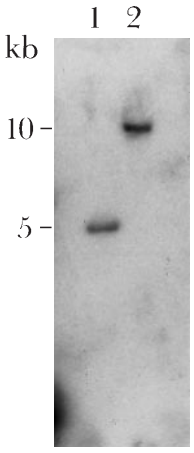

Fig. 2. Southern blot analysis of *IF3* in *L. albus*. The DNA was digested with *Eco*RI (*lane 1*) and *Xba*I (*lane 2*) restriction endonucleases and hybridization was performed under stringent conditions as described in *Materials and methods*, using the IF3 cDNA as a probe. The approximate sizes of the hybridizing fragments are indicated in kb

with class-III chitinases from legumes: *Cicer arietinum* (79%; GenBank accession number P36098), *Vigna angularis* (75%; GenBank accession number P29024) and *Psophocarpus tetragonolobus* (74.7%; GenBank accession number D49953). Identity to the remaining class-III chitinases so far studied ranges from 58.0% (*Nicotiana tabacum*; basic chitinase; GenBank accession number P29061) to 68.1% (*Nicotiana tabacum*; acidic chitinase; GenBank accession number P29060). The alignment and comparison of the deduced amino-acid sequence of IF3 chitinase from *L. albus* with class-III chitinases from other species revealed different conserved regions including four cysteine residues in positions 51, 98, 193 and 222, the function of which has not yet been determined (Fig. 3). The signal peptide is highly divergent among all the chitinases compared. The phylogenetic tree built using the amino-acid sequences of the *L. albus* IF3 chitinase and class-III chitinases from other species shows the clustering of legume chitinases (Fig. 4).

Expression of IF3 chitinase gene. In order to understand the biological role of chitinase genes in uninfected plants, *L. albus IF3* chitinase gene expression was analysed. Protein samples were extracted from developing seeds and from roots, hypocotyls and leaves of *L. albus* plants at two growth stages: 7-d-old (young) and 20-d-old (mature) plants. Western blotting analysis revealed that IF3 was present in all vegetative organs at both growth stages although the protein amounts were different (Fig. 5A). The highest amounts were detected in roots of 7- and 20-d-old plants and in mature leaves of 20-d-old plants increasing during leaf growth (Fig. 5A). Furthermore, this chitinase was also present in developing seeds, being detectable at 20 d post-anthesis (dpa) and from then onwards until seed maturity (Fig. 5B).

Anthracnose (*Colletotrichum gloeosporioides* infection) is one of the most important diseases of white lupin (*L. albus*) in several countries, particulary during the rainy season, causing severe seed yield reduction in affected fields. In order to understand the reaction of lupin to this pathogen, infection was induced in greenhouse conditions as described in *Materials and methods* and the levels of IF3 protein and their corresponding mRNAs were analysed by Western and Northern blotting, respectively. Protein and total RNA samples were extracted from leaves of *Colletotrichum*-infected plants, showing symptoms (young leaves) or not (mature leaves). Western blot analysis showed that the levels of leaf IF3 chitinase were strikingly increased upon infection (Fig. 6A). An enhancement was also observed in the level of the corresponding mRNAs as revealed by Northern blot analysis (Fig. 6B). In young necrotic leaves the level of IF3 chitinase mRNA was higher than that of PR-10, studied under the same experimental conditions (Fig. 6C), and that of rRNAs (Fig. 6D). The PR-10 proteins were previously shown to be induced after infection of *L. albus* with *C. gloeosporioides* (Pinto and Ricardo 1995).

The effects of salicylic acid, a chemical inducer of PR proteins that is implicated in signal transduction for resistance, and of abiotic stress conditions (wounding and UV-C irradiation), on the expression of the *IF3* chitinase gene were analysed in mature leaves from 24-d-old plants (Fig. 7). The levels of lupin IF3 protein were determined by Western blotting. As shown in Fig. 7A, the amount of chitinase increased in plants treated with salicylic acid or submitted to any of the stress conditions studied (see legend to Fig. 7). In addition we determined the corresponding levels of IF3 mRNA by Northern blot analysis. As illustrated in Fig. 7B, there was a dramatic increase in the level of chitinase mRNA compared with control plants (compare lane 1 with lanes 2, 3 and 4). It is likely then that the *IF3* gene is induced as a general response to stress.

Discussion

Based on their primary structure, plant chitinases have been classified into six classes (Meins et al. 1994; Neuhaus et al. 1996). Most of the class-III chitinases are extracellular proteins, two of them showing lysozyme activity (Bernasconi et al. 1987; Jekel et al. 1991). Taking into account results recently published (Yeboah et al. 1998), class-III chitinases could be further classified into one of the two following classes: (i) class Chib1, according to the previously recommended nomenclature (Neuhaus et al. 1996), and (ii) a new class, Chib2, represented by a soybean chitinase, specifically expressed in the developing seeds, which exhibits 51–53% identity to those of other class-III homologues and to a *Sesbania* homologue (Yeboah et al. 1998). The Chib1 chitinases have relatively short C-terminal regions. According to the results presented in Fig. 3, *L. albus* IF3 chitinase belongs to the Chib1 class. When considering the identity of the amino-acid sequences between IF3 chitinase and Chib1 chitinases from other species the highest scores were obtained for legume chitinases (74.7–79.1%). As has been shown for some other plants, we

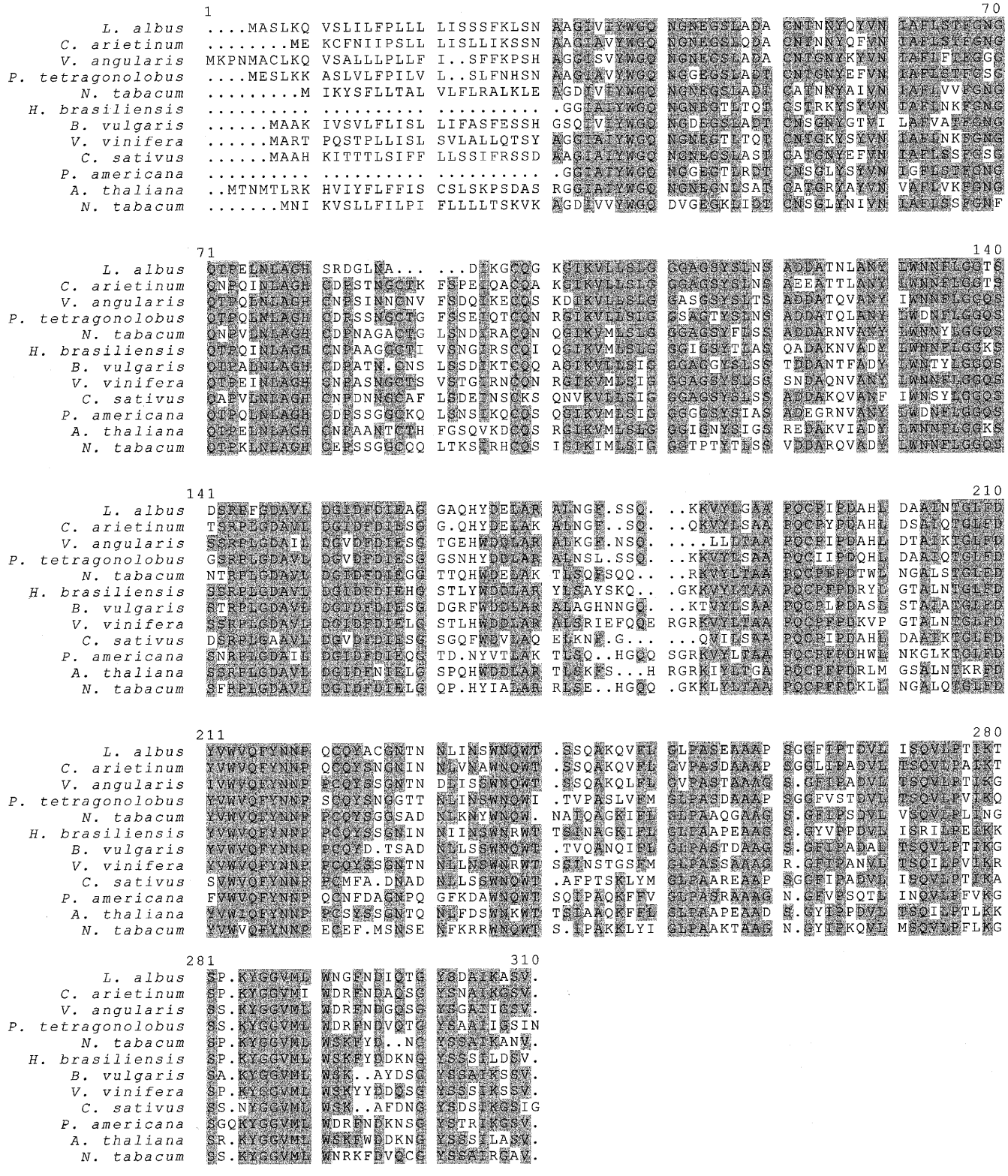

Fig. 3. Alignment of the predicted *L. albus* IF3 chitinase and class-III chitinases from *Cicer arietinum* (Vogelsang and Barz 1993; GenBank accession number P36098), *Vigna angularis* (GenBank accession number P29024), *Psophocarpus tetragonolobus* (GenBank accession number D49953), *Nicotiana tabacum* (acidic chitinase; Lawton et al. 1992; GenBank accession number P29060), *Hevea brasiliensis* (Jekel et al. 1991; GenBank accession number P23472), *Beta vulgaris* (Nielsen et al. 1993; GenBank accession number P36910), *Vitis vinifera* (Busam et al. 1997; GenBank accession number Z68123), *Cucumis sativus* (Métraux et al. 1989; GenBank accession number P17541), *Phytolacca americana* (Tanigawa et al. 1995; GenBank accession number JC4053), *Arabidopsis thaliana* (Samac et al. 1990; GenBank accession number P19172) and *Nicotiana tabacum* (basic chitinase; Lawton et al. 1992; GenBank accession number P29061). Regions of homology (identity) are *shaded*. Gaps, which were introduced to optimize the alignment, are indicated by *dots*

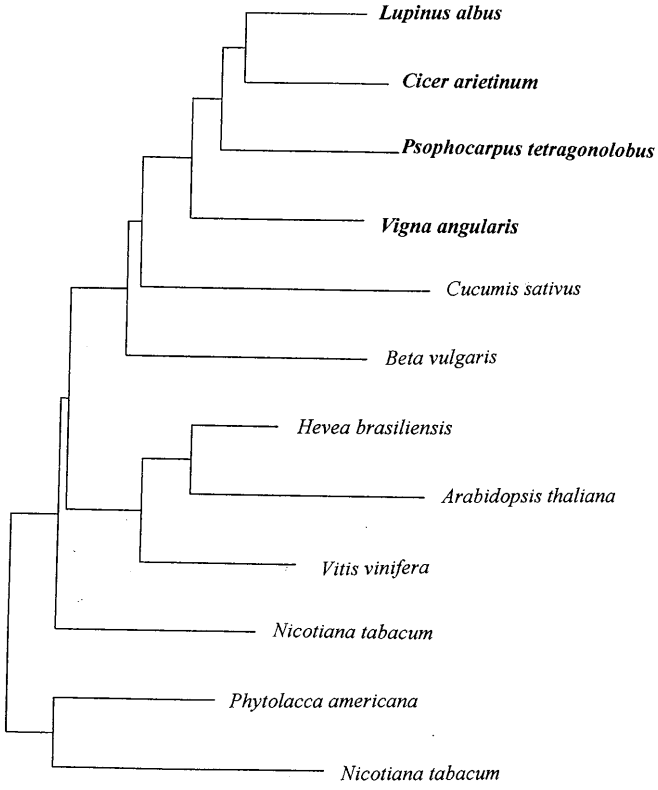

Fig. 4. Phylogenetic tree built from the amino-acid sequences of IF3 chitinase from *L. albus* and class-III chitinases from various plant species. Legume chitinases are represented in *bold*

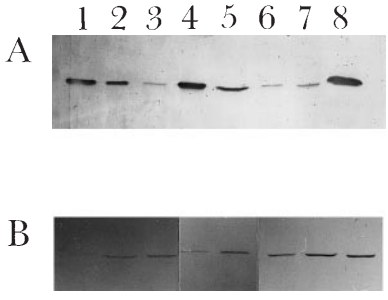

Fig. 5A,B. **E**xpression of the *IF3* gene in vegetative organs of uninfected *L. albus* plants and in developing seeds. **A** Immunoblotting analysis in roots (*lane 1*), hypocotyls (*lane 2*) and leaf primordia (*lane 3*) from 7-d-old plants, and in roots (*lane 4*), hypocotyls (*lane 5*), leaf primordia (*lane 6*), young leaves (*lane 7*) and mature leaves (*lane 8*) from 20-d-old plants. **B** Immunoblotting analysis at different stages of seed development: 20 dpa (*lane 1*), 30 dpa (*lane 2*), 40 dpa (*lane 3*), 50 dpa (*lane 4*), 60 dpa (*lane 5*), 70 dpa (*lane 6*), 80 dpa (*lane 7*) and 90 dpa (*lane 8*). Protein samples (10 μg) were separated by SDS-PAGE, transferred to polyvinylidenedifluoride membrane and immunodetected with the sera raised against IF3 protein as indicated in *Material and methods*

only detected one chitinase class-III gene in the *L. albus* genome (Métraux et al. 1989; Samac et al. 1990; Margis-Pinheiro et al. 1993; Nielsen et al. 1993). We have indeed performed Southern blot analysis using different stringency conditions and never found more than one hybridizing band. However, we cannot exclude the

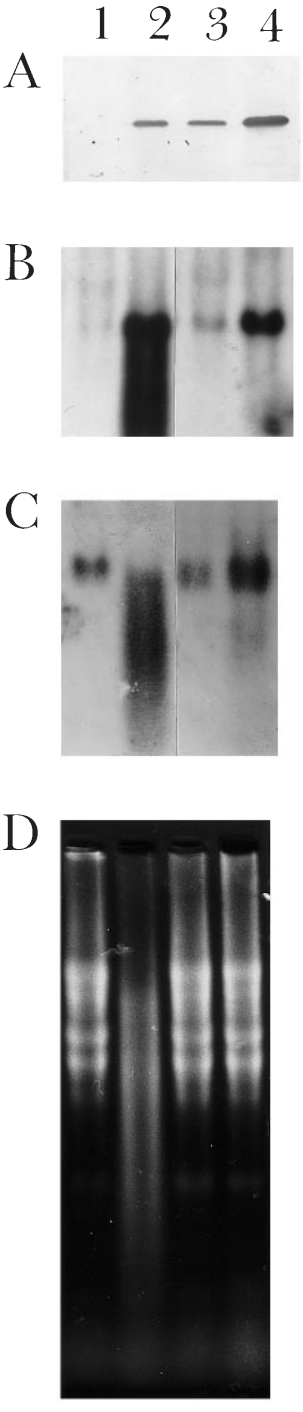

Fig. 6A–D. **E**xpression of the *IF3* and *PR-10* genes in leaves from 24-d-old *L. albus* infected with *C. gloesosporioides*. Plants were treated and harvested as indicated in *Materials and methods*. **A** Immunoblotting analysis in young (*lane 1*) and mature (*lane 3*) leaves from control plants, or young (*lane 2*) and mature (*lane 4*) leaves from infected plants. Protein samples (8 μg) were separated and immunodetected with the serum against IF3 as indicated in *Materials and methods*. **B** Equivalent amounts of total RNA (20 μg) from young (*lane 1*) and mature (*lane 3*) leaves from control plants, or young (*lane 2*) and mature (*lane 4*) leaves of infected plants were hybridized with IF3 cDNA under high-stringency conditions as indicated in *Materials and methods*. **C** The membrane from the experiment shown in **B** was re-hybridized using a PR-10 cDNA from *L. albus* as probe. **D** Total RNA samples (10 μg) were run in formaldehyde-agarose gel and stained with ethidium bromide

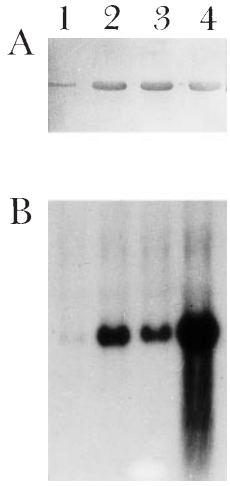

Fig. 7A,B. Expression of the *IF3* gene in mature leaves from 24-d-old *L. albus* plants treated with salicylic acid or under stress conditions. Plants were treated and harvested as described in *Materials and methods*. **A** Immunoblot analysis of control plants (*lane 1*), plants treated with salicylic acid (*lane 2*), wounded plants (*lane 3*) and plants irradiated with UV-C light (*lane 4*). Protein samples (10 μg) were separated by SDS-PAGE, transferred to polyvinylidenedifluoride membrane and immunodetected with the serum raised against IF3 protein as described in *Materials and methods*. **B** RNA blot analysis of control plants (*lane 1*), plants treated with salicylic acid (*lane 2*), wounded plants (*lane 3*) and plants irradiated with UV-C light (*lane 4*). Equivalent amounts of total RNA (20 μg) were hybridized with IF3 cDNA under high-stringency conditions as indicated in *Materials and methods*

possibility of the presence of related genes in the *L. albus* genome.

Not many studies have been carried out in order to clarify the constitutive expression of chitinase genes. As far as we know the most complete studies on the expression of class-III chitinase genes have been performed with *Phaseolus vulgaris* (Margis-Pinheiro et al. 1993), C*ucumis sativus* (Lawton et al. 1994) and G*lycine max* (Yeboah et al. 1998). In *P. vulgaris*, no hybridization signal was detected from any of the studied organs (roots, stems, primary and secondary-trilobate leaves from 10- to 12-d-old plants) using Northern blotting experiments (Margis-Pinheiro et al. 1993). In *C. sativus*, chitinase mRNA was detected by Northern blotting at low levels in roots and cotyledons from 7- to 10-d-old plants but no signal was present in leaves and stems, while in 3- to 5-week-old plants the chitinase message was present in older and mature, fully expanded leaves but not in young, expanding leaves or stems (Lawton et al. 1994). In *G. max* the class-III chitinase mRNA was detected by Northern blotting in developing seeds and extremely faint reverse transcriptase-PCR products were observed from the leaves and stems (Yeboah et al. 1998). We therefore examined IF3 gene expression in developing seeds and in vegetative organs of uninfected *L. albus* plants. To our knowledge this is the first reported class-III chitinase to be constitutively expressed not only in all vegetative organs but also in developing and mature seeds.

Inducing agents for class-III chitinases include both biotic (Métraux et al. 1989; Lawton et al. 1992; Margis-Pinheiro et al. 1993; Nielsen et al. 1993; Vogelsang and Barz 1993) and abiotic agents (Margis-Pinheiro et al. 1993; Lawton et al. 1994). Here, we have shown that *L. albus* IF3 chitinase is induced by *Colletotrichum gloeosporioides* infection as well as by abiotic factors like UV-light and wounding. It might therefore be concluded that in *L. albus* the *IF3* gene is induced as a general response to stress.

Given the general constitutive presence of the *L. albus* IF3 chitinase, it can be suggested that it is a component of a constitutive plant defense mechanism. Our results, however, do not exclude the possibility of its potential participation in other biological processes. In fact, Goormachting et al. (1998) have recently demonstrated that the protein encoded by the cDNA clone *Srchi 13* (with homology to acidic class-III chitinase genes, corresponding to an early nodulin gene) exhibit Nod-factor degradation activity. Within this context, we are currently isolating the IF3 chitinase from *L. albus* leaves in order to test for antifungal as well as Nod-factor degradation activities in vitro. In-situ expression studies are also being carried out.

We thank Dr. J. Ryals (Ciba-Geigi, Basel, Switzerland) for providing cucumber chitinase cDNA. Evguenia Bekman (Instituto Gulbenkian de Ciência, Oeiras) and Dr. Adriano Henriques (Instituto de Tecnologia Química e Biológica, Oeiras) for helpful discussions. We also thank Dr. Paula Pinto (Escola Superior Agrária de Santarém, Santarém, Portugal) for providing the PR-10 probe and Dr. Ana Neves (Escola Superior Agrária de Santarém, Santarém) for helping in the construction of the cDNA library. This research received financial support from Praxis/2/2.1/BIO/1146/95. A.P.R. thanks Praxis XXI for the fellowship BD/2708/94.

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