

# Biochemical and Molecular Biological Studies on Infection (*Ascochyta rabiei*)-Induced Thaumatin-Like Proteins from Chickpea Plants (*Cicer arietinum* L.)\*

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Cultivar Specific Expression, PR-Proteins, Thaumatin-Like Protein

A pathogenesis-related protein induced by infection with *Ascochyta rabiei* was purified from intercellular washing fluid of chickpea (*Cicer arietinum* L.) leaves. Amino-terminal sequencing identified the protein, named PR-5a, as a thaumatin-like protein. The isoelectric point was determined with 6.5 and the molecular mass is 16 kDa. Therefore, chickpea PR-5a is the first dicot member of a TLP subgroup containing small TLPs with a molecular weight between 15 and 18 kDa. PR-5a shows no antifungal activity towards *A. rabiei*. Screening of a chickpea cDNA library led to the isolation of a cDNA clone (*p5a-241*) for this protein. A second cDNA clone (*ELR112*) encoding a TLP was isolated using differential hybridisation of cDNA libraries obtained from elicited and water treated cell suspension cultures of chickpea. The deduced protein (PR-5b) has a molecular mass of 22 kDa. PR-5b is postulated to be located in the vacuole due to the presence of a respective N-terminal signal peptide and a carboxy-terminal extension. Southern blot analyses showed that *ELR112* and *p5a-241* represent single copy genes. During fungal infection of chickpea plants expression of both genes proceeds much faster in an *A. rabiei* resistant cultivar than in a susceptible one.

## Introduction

After recognition of a microbial pathogen plants react with an array of induced defence mechanisms. Besides the establishment of a hypersensitive reaction, lignification and protein cross-linking of cell walls, and the synthesis of phytoalexins, the formation of pathogenesis-related proteins are well known responses (Bol *et al.*, 1990; Cutt and Klessig 1992; Otte and Barz, 1996).

The known PR-proteins are divided into 14 families (van Loon and van Strien, 1999). In the last years main interest focused on chitinases (PR-3, PR-8, PR-11 families) and  $\beta$ -1,3-glucanases (PR-2) because of their role in degrading the cell walls of invading fungal pathogens (Legrand *et al.*, 1987; Mauch *et al.*, 1988).

Members of the PR-5 family show sequence homology to thaumatin, a sweet tasting protein iso-

lated from fruits of *Thaumatococcus daniellii* (Edens *et al.*, 1982) Therefore, these proteins were named thaumatin-like proteins (TLPs). Although TLPs have been isolated from a wide range of plant species the function of these proteins is still not clarified. Some thaumatin-like proteins, together with other antimicrobial proteins, are constitutively expressed in seeds of maize, *T. daniellii*, and barley for example (Vigers *et al.*, 1991; Edens *et al.*, 1982; Hejgaard *et al.*, 1991). TLPs accumulate during osmotic stress (Singh *et al.*, 1987; King *et al.*, 1988), but the role of TLPs during water deficit and in salt stress adaption remains to be elucidated. Recently, a gene encoding a transmembrane receptor protein kinase (PR5K) carrying an extracellular amino-terminal domain structurally related to TLPs, was isolated from *Arabidopsis thaliana* (Wang *et al.*, 1996). For many plants induction of TLPs during pathogenesis has been demonstrated (Bryngelsson and Green 1989; Heitz *et al.*, 1994; Lin *et al.*, 1996). *In vitro* antifungal activity of thaumatin-like proteins has been described in several cases (Woloshuk *et al.*, 1991;

Abbreviations: TLP, Thaumatin-like protein.

\* Sequence data were submitted to EMBL as accession number AJ010501 (PR-5b) and AJ010502 (PR-5a).

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Vigers *et al.*, 1992; Abad *et al.*, 1996). In addition, constitutive overexpression of genes encoding TLPs can result in an increase of resistance towards fungal pathogens (Liu *et al.*, 1994; Datta *et al.*, 1999). The mechanism of TLP antifungal activity remains to be elucidated (Velazhahan *et al.*, 1999).

With regard to their molecular weight the TLPs are divided into two sub-groups (Velazhahan *et al.*, 1999). The majority of TLPs possesses a molecular weight between 21 and 26 kDa. These "large" TLPs are located in the apoplastic space as well as in vacuoles and have been described for both dicots and monocots. Members of the second sub-group are smaller with a molecular weight of 15–18 kDa due to an intramolecular deletion. These small TLPs are hitherto described only for monocots and all members of this sub-group occur as extracellular proteins.

*Ascochyta rabiei* (Pass.) Labr. is the causal agent of *Ascochyta*-blight on chickpea (*Cicer arietinum* L.). Fungal growth stops in the apoplastic space of the resistant cultivar after the pathogen has invaded the leaf tissue via direct cuticle penetration whereas the fungus spreads unretardedly in the apoplastic space of the susceptible plants (Höhl *et al.*, 1990; Köhler *et al.*, 1995). During the infection several chitinases and  $\beta$ -1,3-glucanases accumulate in the vacuole and the apoplast of chickpea leaves, but no significant cultivar-specific differences, neither in activity nor in isoenzyme pattern of these hydrolytic enzymes, have been observed (Vogelsang and Barz 1993; Mackenbrock *et al.*, 1992; T. Hanselle and W. Barz, unpublished).

Here we report the purification and characterization of PR-5a, a 16 kDa thaumatin-like protein isolated from the apoplastic space of *A. rabiei*-infected chickpea leaves, and the cloning of the corresponding cDNA. Furthermore, the isolation of a cDNA encoding a second thaumatin-like protein (PR-5b) and expression studies on both genes in *A. rabiei*-infected and mock inoculated chickpea plants are described.

## Materials and Methods

### Plant and fungal materials

For all experiments plants of the chickpea (*C. arietinum* L.) cultivars ILC3279, resistant to *A. rabiei*, and ILC1929, susceptible to *A. rabiei*, and the

*A. rabiei* strain 21 (ATCC 76502, mating type 2) were used. *A. rabiei* was cultivated on potato dextrose agar and reisolated from susceptible plants every 4 to 6 months. Plants (7 per pot) were grown in a mixture of potting soil and white sand (3:2 ratio) in a growth chamber at  $20 \pm 1$  °C and a cycle of 15 h of light and 9 h of darkness. 14 days old plants were placed in glass boxes and inoculated by spraying with an aqueous spore suspension of *A. rabiei* strain 21 (106 spores ml<sup>-1</sup>, 0.5 ml per plant). Control plants were treated with equal volumes of water. The glass boxes were covered with transparent plastic foil for two days to maintain 100% relative humidity.

### Isolation and analysis of intercellular washing fluids

The second to fourth fully developed leaves were harvested. For purification of PR-5a whole leaves were used whereas for time course experiments the rhachis was cut off with a razor blade, in addition. IWF was obtained by vacuum infiltration of distilled water (4 °C) for two minutes, followed by centrifugation at 2,000  $\times g$  for 10 min.

Proteins were subjected to vertical sodium dodecylsulfate polyacrylamide gel electrophoresis using 15 % acrylamide in the separation gel (Laemmli 1970). Following electrophoresis proteins were stained with Coomassie Blue R250 or silver. Immuno blot analysis was carried out after semi-dry transfer of the separated proteins on polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corporation, Richmond, USA) using a Multiphor II Nova Blot system (LKB Pharmacia, Uppsala, Sweden) according to Westermeier (1990). Immunodetection was performed according to Legrand *et al.* (1987) using anti rabbit IgG alkaline phosphatase conjugate from Sigma Bio Science (St. Louis, USA) as secondary antibody.

Isoelectric focussing was performed at 4 °C in rehydrated gels according to Westermeier (1990). Rehydration was carried out in 10 % (w/v) sorbitol containing 2.5 % (w/v) Pharmalyte pH-range 3–10. The factual pH-gradient was determined by cutting 1 cm strips of the gel into 0.5 cm pieces. After incubation of each piece for 5 min in 1 ml sonified, distilled water (4 °C) the pH was measured.

### Purification of PR-5a

36 ml IWF isolated from 100 g chickpea leaves harvested 7 days after plant inoculation with *A. rabiei* were subjected to a stepwise ammonium sulfate precipitation. The precipitate of the 50 - 75 % fraction was suspended in 25 mM bis-Tris/iminodiacetic acid, pH 7.1 (buffer A), desalted using Sephadex G-25 with buffer A as eluent (pre-packed Sephadex G-25-columns, as well as the Mono P 5/5, the Mono Q 5/5 and the Phenyl Superose 5/5 column were purchased from Pharmacia, Uppsala, Sweden). The elut was loaded on a Mono P column using buffer A as start buffer. The flow-through solution was titrated to pH 8.6 with 1 M Tris, concentrated with Centriprep (Amicon, Beverly, USA) and loaded on a Mono Q column equilibrated with 25 mM Tris/HCl, pH 8.6 (buffer B). Proteins were eluted using a linear gradient of 0-20 % Buffer B with 1 M NaCl as an eluent. Fractions containing PR-5a were pooled, desalted by passage through Sephadex G-25 columns using 20 mM potassium acetate, pH 5.2 (buffer C) as an eluent. An equal volume of buffer C containing 2.66 M ammonium sulfate was added and the sample was loaded on a Phenyl Superose column equilibrated with buffer C containing 1.33 M ammonium sulfate. Proteins were eluted with a linear gradient of 0-100 % buffer C. Fractions containing PR-5a were pooled and desalted using Sepharose-25-columns and bidistilled water as eluent.

N-terminal aminoacid sequencing of purified PR-5a was performed with an Applied Biosystems 477A protein sequencer (Foster City, USA) at the Institute of Physiological Chemistry, Münster University.

Antifungal activity was tested using the filter disc system according to Vogelsang and Barz (1993) and inhibition of spore germination was performed as described for *Cladosporium fulvum* by Joosten *et al.* (1995).

### Construction of cDNA-libraries and isolation of TLP cDNA

The cDNA-libraries from chickpea plants (ILC3279) four days after inoculation with *A. rabiei* and control plants of the same age were constructed in the phagemid vector lambda-ZAP II

(Stratagene, La Jolla, USA) according to the manufacturer.

Partial cDNA for PR-5a was recovered from the cDNA library of infected chickpea plants by PCR using the forward primer tlp1-1 (5'-TTYGAY-ATHGTNAAYCARTG-3'), derived from the amino-terminal sequence of PR-5a, and the reverse primer tlp-con-1 (5'-ACGGGNCTYCG-NAT-3'), derived from a conserved region in TLPs.

The PCR-product was purified by gel-electrophoresis and subsequent elution from the gel using the Jetsorb Gel Extraction Kit (Genomed, Bad Oeynhausen, Germany), and cloned into pMos blue using the pMos-blue T-vector kit (Amersham, Buckinghamshire, UK).

The PCR-fragment was DIG-labelled with the non radioactive labeling and detection-kit (Boehringer Mannheim, Mannheim, Germany) and used as a probe in screening the cDNA library for full length clones. Positive plaques were subjected to *in vivo* excision and pBluescript II SK(-) phagemids were isolated with the Plasmid Midi-Kit from Qiagen (Hilden, Germany).

cDNA inserts were sequenced by the dideoxy sequencing method using the T7T3 -sequencing kit (Pharmacia, Uppsala, Sweden) and  $\alpha$ 35S-dATP or the Thermo sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham International, Little Chalfont, UK) and the automated DNA sequencer Li-Cor 4000 (MWG-Biotech, Ebersberg, Germany).

### Southern and northern blot analyses

Genomic DNA was isolated from 3 to 4 week old uninfected leaves of ILC3279 following the method of Doyle and Doyle (1991). 20  $\mu$ g of genomic DNA at a time were digested with different restriction enzymes, fragments were separated by electrophoresis on a 0.6 % (w/v) agarose gel, transferred in three hours to a nylon membrane (Nytran plus, Schleicher und Schüll, Dassel, Germany) following the method of Chomczynski (1992). *Eco*RI/*Xba*I inserts of *ELR112* and *p5a-241* were labelled using the DNA labeling kit from MBI Fermentas (Vilnius, Letvia) and 32P-dCTP. After hybridisation blots were washed three times in 2x SSC, 0.1 % SDS and once in 0.2x SSC, 0.1 % SDS each for 15 min and 55 °C, and once in 0.2x SSC,

0.1 % SDS for 30 min and 55 °C. After these washing steps the membranes were exposed for 7 to 14 days with intensifying screen at -70 °C.

Total RNA was extracted from chickpea leaves according to Chomczynski and Sacchi (1987). 16 µg of total RNA of each time point were separated under denaturing conditions on 2% (w/v) formaldehyde agarose gels. After upward transfer (Maniatis *et al.*, 1982) to a nylon membrane (Nytran, Schleicher und Schüll, Dassel, Germany). After hybridization, the membrane was washed twice in 2x SSC, 0.1 % SDS at 68 °C for 15 min each, and twice in 0.1x SSC, 0.1 % SDS at 68 °C for 20 min each, and exposed to photographic film.

DIG-labelled anti sense RNA was synthesized using the RNA Transcription Kit from Stratagene (La Jolla, USA) and the DIG RNA Labelling Mix from Boehringer Mannheim (Mannheim, Germany).

## Results

### Purification and characterization of PR-5a

A method for isolation of intercellular washing fluid (IWF) from chickpea leaves was established. The contamination of intracellular components in the IWF measured by determination of malate dehydrogenase and glucose-6-phosphate dehydrogenase activities was below 0.1 % in comparison to the activity of both enzymes in protein extracts obtained from leaf material after isolation of IWF (data not shown).

Due to the infection of chickpea with *A. rabiei* a set of proteins accumulated in the IWF of leaves (Fig. 1.A). Seven of them were identified as chitinases, one as  $\beta$ -1,3-glucanase (T. Hanselle and W. Barz, unpublished). Beside these hydrolases a further protein with a molecular weight of about 15 kDa, measured by SDS-PAGE, increased during infection (Fig. 1.A). Purification of this protein with FPLC as described in material and methods yielded a homogeneous preparation (Fig. 1.B). The isoelectric point of this protein was determined as 6.5 using native isoelectric focusing (Fig. 1.C). To obtain amino acid sequence data, the purified protein was submitted to N-terminal sequencing. 29 amino acids were determined (Fig. 2) and protein sequence alignment with BLAST and FASTA (Altschul *et al.*, 1997; Pearson and Lipman, 1988) identified the protein as a thaumatin-

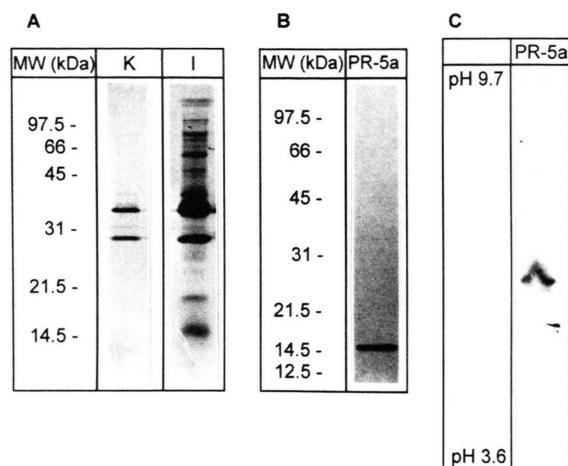


Fig. 1. SDS-PAGE and western blot analysis on IWF isolated from chickpea leaves and purified PR-5a. The proteins were analysed on 15% SDS-polyacrylamide gels. (A) IWF, corresponding to 50 mg leaf material, isolated from control plants (K) (ILC3279) and IWF isolated from infected plants (I) (ILC3279), 6 days post inoculation with *A. rabiei*; proteins were silver stained. M: Molecular mass standard. (B) Purified chickpea PR-5a stained with Coomassie blue. M: Molecular mass standard plus Cytochrome c. (C) Isoelectric focussing of purified PR-5a in a rehydrated polyacrylamide gel. After electrophoresis the gel was silver-stained. The pH gradient, determined as described in material and methods, was linear from pH 9.7 to pH 3.6.

like protein. The lack of a signal in cycle nine of automated Edman degradation indicated, that a postulated cystein residue is part of an intramolecular disulfide bridge as described for thaumatin and zeamatin (De Vos *et al.*, 1985; Batalia *et al.*, 1996). Sequencing of cDNA clones encoding the 15 kDa protein showed that the ninth amino acid of the mature protein is in fact a cystein (Fig. 2). According to van Loon *et al.* (1994) this PR-protein was named PR-5a, because it is the first TLP described for chickpea.

Antifungal activity of PR-5a against *A. rabiei* strain 21 was tested *in vitro* using the filter disc system as well as a microtiter plate test, but neither rupture of hyphal tips nor an inhibitory effect on germinating spores was observed even at high concentrations of purified PR-5a (25 µg per disc in the plate test, 100 µg per ml in the microtiter test).

Immuno-blot analyses of IWF were used to show the time course of induction of PR-5a in chickpea leaves inoculated with *A. rabiei* (Fig. 3). We applied antiserum raised against PR protein S

Avesa	----- <u>MATSSTVL</u> FLLAVFAASASAATFTI TNNCGYTVWPAAI PVGGQQQL	=
Orysa	MASPATSSAVLVVVLVATLAAGGANAATFTI TNRCSTVWPAATPVGGGVQL	
Horvu	----- <u>MSTSAVL</u> FLLAVFAAGASAATFN1KNNCGSTIWPAGIPVGGGFEL	
PR-5a	----- <u>MSLIKICL</u> SMLAFC <u>LI</u> SAQGARFDIVNQCSYNVWPAAI PSGGRQL	
PR-5b	----- <u>SILTI</u> TC <u>L</u> SL <u>F</u> LLTPSQAANFEIVNNCPYTVWAASp-GGGRRL	
Arath	----- <u>MANLLV</u> STFIF <u>S</u> ALLLISTATAATFEI LNQCSYTVWAASp-GGGRRL	
Lyces	----- <u>AFV</u> TYT <u>Y</u> ATF <u>EV</u> RNNCPYTVWAASp-PIGGRRRL	
Nicta	----- <u>MGNL</u> RSSFVF <u>FL</u> LLALV <u>TY</u> ATIEV <u>RNN</u> CPYTVWAASp-PIGGRRRL	
	= = = = = = = =	
Avesa	DQGQTWTLNVPAGTNSGRIWGRGCSFNG-GSGSCQTGDCAGALSCTLSQ-	
Orysa	SPGQTWTINVPA <u>G</u> TSSGRVWGRGCSFDGSGRGSCATGDCAGALSCTLSQK	
Horvu	GSGQTSSINVPA <u>G</u> TQAGRIWARTGCSFNG-GSGSCQTGDCGGQLSCSLSQP	
PR-5a	NP <u>RE</u> TWGLDIPAGTQSARIWGRGCSFDGSGRGSCQTGDCGGDL <u>S</u> CHLSQP	
PR-5b	DRGQTWNLWVNAGTSMARIWGRGCSFDGSGRGRC <u>E</u> TG <u>D</u> CTGG <u>L</u> QCTGWGP	
Arath	DAGQSWR <u>L</u> DVAAGTKMARIWGR <u>T</u> NCNF <u>D</u> SSGRGRC <u>Q</u> TGDCGG <u>L</u> QCTGWGP	
Lyces	DRGQTWVINAPRGT <u>K</u> MARIWGR <u>T</u> NCNF <u>D</u> GAGR <u>G</u> SC <u>Q</u> TGDCGG <u>V</u> LQCTGWGP	
Nicta	DRGQTWVINAPRGT <u>K</u> MARVWGR <u>T</u> NCNFNAAGR <u>G</u> TC <u>Q</u> TGDCGG <u>V</u> LQCTGWGP	
	= = = = = = = = = =	
Avesa	PATLAEFSIGGE--HDYYDISVIDVYNLAMDFS-----CSTGD-----	
Orysa	PLT <u>LAE</u> FTIGGS--QDFYDLSVIDGYNVAMSFS-----CSSGV-----	
Horvu	PATLAEFTIGGGSTQDFYDISVIDGFNLAMDFS-----CSTGD-----	
PR-5a	PTT <u>LAE</u> FSLN <u>G</u> GNQDYFDISVIDGFNIPM <u>Q</u> FSSTT---NNCNRL-----	
PR-5b	PNTLAE <u>P</u> ALNQYGN <u>L</u> DFYDISLVDGFNIPMDF <u>FF</u> PIN---GGCHKIMCTADIN	
Arath	PNTLAEYALNQFN <u>L</u> DFYDISLVDGFNIPME <u>F</u> SP <u>T</u> TS---SN <u>CH</u> R <u>I</u> L <u>C</u> TADIN	
Lyces	PNTLAEYALDQFSN <u>L</u> DFW <u>D</u> ISLVDGFNIP <u>M</u> TF <u>F</u> APTNP <u>S</u> GGK <u>C</u> HA <u>I</u> HCTANIN	
Nicta	PNTLAEYALDQFS <u>G</u> LD <u>F</u> W <u>D</u> ISLVDGFNIP <u>M</u> TF <u>F</u> APTNP <u>S</u> GGK <u>C</u> HA <u>I</u> HCTANIN	
	= ===== = = = = = = = =	
Avesa	-----AL-QCRDSS-----CP--DA	
Orysa	-----TV-TCRDSR-----CP--DA	
Horvu	-----AL-QCRDPS-----CP <u>PP</u> QA	
PR-5a	-----TCKDSS-----CP--DA	
PR-5b	GQCPN <u>E</u> LA <u>Q</u> GGC <u>NN</u> P <u>C</u> TV <u>K</u> TNE <u>C</u> CTNG <u>Q</u> GSCGPT <u>F</u> STFFKDRCH--DA	
Arath	GQCPN <u>V</u> LR <u>A</u> PGG <u>NN</u> P <u>C</u> TV <u>F</u> QT <u>N</u> Q <u>C</u> CTNG <u>Q</u> GCS <u>D</u> TV <u>S</u> RFFKQRCP--DA	
Lyces	GEC <u>P</u> GLR <u>V</u> PGG <u>NN</u> P <u>C</u> TT <u>F</u> GG <u>Q</u> Y <u>C</u> CT--QG <u>P</u> CG <u>P</u> TL <u>S</u> RFFKQRCP--DA	
Nicta	GEC <u>P</u> RE <u>L</u> R <u>V</u> PGG <u>NN</u> P <u>C</u> TT <u>F</u> GG <u>Q</u> Y <u>C</u> CT--QG <u>P</u> CG <u>P</u> FF <u>S</u> KFFKQRCP--DA	
	= = =	
Avesa	YHQPDD--PKTHSCNTNS-NYQITFCP	
Orysa	YLFPED-NTKTHACSGNS-NYQVVFCP	
Horvu	YQHPND--VATHACSGN-NNYQITFCP	
PR-5a	YHQPND--VKT <u>V</u> SCP <u>GG</u> -TNYRIVFCP	
PR-5b	YSY <u>P</u> QDDPTST <u>F</u> CP <u>A</u> GS-NYKVVFC <u>P</u> L <u>G</u> PHI <u>E</u> MLIQTNVY	
Arath	YSY <u>P</u> QDDPTST <u>F</u> CT <u>N</u> --TNYR <u>V</u> VFC <u>P</u> RS <u>R</u> L <u>G</u> AT <u>G</u> SH <u>Q</u> L <u>P</u> I <u>K</u> M <u>V</u> TEEN	
Lyces	YSY <u>P</u> QDDPTST <u>F</u> CP <u>G</u> ST <u>N</u> YR <u>V</u> VFC <u>P</u> NG <u>V</u> TF <u>P</u> LE <u>M</u> SS <u>D</u> E <u>E</u> AK	
Nicta	YSY <u>P</u> QDDPTST <u>F</u> CP <u>GG</u> ST <u>N</u> YR <u>V</u> I <u>F</u> CP <u>NG</u> QAH <u>P</u> N <u>F</u> LE <u>M</u> PG <u>S</u> DE <u>V</u> AK	
	= = = = = = =	

Fig. 2. Sequence alignment of the deduced amino acid sequence of *p5a-241* (PR-5a) and *ELR112* (PR-5b) with six thaumatin-like proteins from different plant species. The first four sequences represent the sub-group of small TLPs and sequences five to eight represent the sub-group of the large TLPs. The amino-terminal leader sequences and the carboxyterminal extensions are underlined. Amino acids conserved in all eight TLPs are indicated by signs of equality. To optimise the alignment hyphens are introduced into sequences. The 29 amino acids of chickpea PR-5a, determined by Edmann degradation of the purified protein, are marked by a dotted line. Avesa: thaumatin-like PR-protein 4 from *Avena sativa* (Lin *et al.*, 1996, SwissProt-number: P50698). Orysa: TLP from *Oryza sativa* (Reimann and Dudler 1993, P31110). Horvu: PR-protein 1A/1B from *Hordeum vulgaris* (Hahn *et al.*, 1993, P32937). Arath: Osmotin-like protein from *Arabidopsis thaliana* (Capelli *et al.*, 1997, P50700). Lyces: PR-protein P23 from *Lycopersicum esculentum* (Ruiz-Medrano *et al.*, 1992, Q01591). Nicta: Osmotin from *Nicotiana tabacum* (Melchers *et al.*, 1993, P14170).



Fig. 3. Time course of PR-5a accumulation in chickpea leaves. Intercellular washing fluid was isolated at various times (indicated in days above the blots) from plants inoculated with *A. rabiei* (I) or control plants treated with water (K). IWF isolated from 50 mg leaf material of the susceptible (ILC1929) or the resistant chickpea cultivar (ILC3279) was loaded on each lane of a 15% SDS-polyacrylamide gel. Blots taken from 15% SDS-polyacrylamide gels were immunopropbed with antiserum raised against the tobacco thaumatin-like protein S. PR-5a was the only chickpea protein cross-reacting with this antiserum.

from tobacco (Kauffmann *et al.*, 1990). Preceding investigations led to the observation that PR-5a was the only chickpea protein cross-reacting with this antiserum (data not shown). In leaves from uninfected control plants of the resistant (ILC 3279) as well as the susceptible cultivar (ILC 1929) only a slight increase of PR-5a was detectable starting at day 4. In contrast, in the resistant cultivar PR-5a increased from day 2 to day 6 post inoculation whereas in the susceptible chickpea cultivar infected with *A. rabiei* no induction of PR-5a was observed during the first four days, but strong signals, even stronger than in the resistant cultivar at the same time, appeared at day 5 and 6. On western blots of protein extracts of leaves from which IWF had been isolated no detectable reaction was measured (data not shown), indicating that PR-5a accumulation is restricted to the intercellular space.

#### Isolation and sequencing of *ELR112*

In differential hybridization studies of cDNA libraries from elicited and water-treated heterotrophic cell suspension cultures of the chickpea cultivar ILC3279, one cDNA (named *ELR112*) was isolated which showed sequence similarity to genes encoding thaumatin-like proteins (accession number AJ010501; Ichinose *et al.*, 2000). The 909 bp insert of *ELR112* is nearly full length. No start codon was detected due to loss of a few nucleotides in the 5' coding region. The stop codon at

position 720 is followed by a 165 bp 3' non coding region harboring two potential cleavage/polyadenylation signals at position 754 and 850. The putative mature protein is expected to be located in the vacuole because of the presence of an amino-terminal leader sequence (< 1 to 21) and a carboxy-terminal extension (223 to 239). After identification in plants the encoded protein will be named PR-5b. In western blot analyses with protein extracts from infected and control plants using antisera raised against protein S and osmotin from tobacco, respectively, no significant signal was detected (data not shown). The calculated molecular weight of the 202 amino acid protein is 21.9 kDa, the calculated isoelectric point is 5.0. The homologies of PR-5b to known TLPs are up to 78% based on identical amino acids and up to 86% if similar amino acids are included (Cornelissen *et al.*, 1986; Capelli *et al.*, 1997).

#### Isolation of cDNAs encoding PR-5a

Because the cDNA clone *ELR112* derived from differential hybridisation does not encode PR-5a, synthesis of partial PR-5a genes by PCR was used as a strategy to obtain a specific probe for isolation of full length cDNAs encoding PR-5a. Two guessmer oligonucleotides were used for PCR: the forward primer corresponded to a part of the N-terminus from PR-5a (FDIVNQC) and the reverse primer was derived from one of the highly conserved regions of TLPs (CPDAY). In the PCR experiment a 390 bp fragment of the expected size was amplified and cloned in pMos blue (data not shown). The derived amino acid sequence was in full agreement with the result of the N-terminal sequencing of purified PR-5a (Fig. 2). Thus the 390 bp fragment was used as a probe for screening a cDNA library of *A. rabiei* infected chickpea plants to isolate full length clones. 300,000 plaques of the cDNA library were screened and 17 positive plaques were isolated. After *in vivo* excision three randomly chosen clones of the expected size (*p5a-241*, *p5a-131*, *p5a-411*) were sequenced. These clones show differences only in the length of the 5' non-coding region. The amino acid sequence of PR-5a derived from the sequence of *p5a-241* (accession number AJ010502) is presented in Figure 2. The 525 bp open reading frame of *p5a-241* encodes an 175 amino acid precursor

from which a 20 amino acid leader sequence is cleaved resulting in a mature protein of 155 amino acids.

Homology of PR-5a with other small TLPs is up to 63% identity and 76% similarity (Lin *et al.*, 1996). The homology to known large dicot TLPs is up to 48% identity and 59% similarity, but if the region of the deletion in the small TLPs is excluded from the alignment the homology is up to 63% identity and therefore as high as for the small TLPs from monocots (Graham *et al.*, 1992). The total homology between PR-5a and PR-5b is only 44% identity and 57% similarity; but it is 56% identity and 65% similarity if the region of the deletion in PR-5a is not considered. Such levels of amino acid divergence between TLPs of the same plant species is not the exception and has been described for several plant species including maize (Malehorn *et al.*, 1994; Frendo *et al.*, 1992), and tobacco (Singh *et al.*, 1989; van Kan *et al.*, 1989). The calculated molecular weight for PR-5a of about 16.6 kDa shows only slight differences in comparison to that obtained with SDS-PAGE (15 kDa). Based on the derived amino acid sequence, an isoelectric point of 5.1 was calculated in contrast to 6.5 determined with isoelectric focusing.

#### *Southern and northern analyses*

Genomic DNA was digested with different endonucleases. After separation of fragments, transfer and hybridisation with the *p5a-241* insert as a probe one single restriction fragment only hybridised to the probe in each digest (Fig. 4). We therefore conclude that PR-5a is encoded by a single-copy gene. If genomic DNA was digested by *Hind*III or *Xba*I one strong band in each digest appeared in Southern blots using the insert of *ELR112* as a probe (Fig. 4). These results led to the suggestion that *ELR112* also is a single-copy gene. However, in the *Eco*RI digest two dominant bands appear instead of one as expected. It is likely that the gene encoding PR-5b has an intron with an additional *Eco*RI cleavage site which would be responsible for the additional positive band or that two alleles of the gene are detected. The weak bands appearing in all digests could represent genes encoding closely related thaumatin-like proteins. In this context it is of interest that

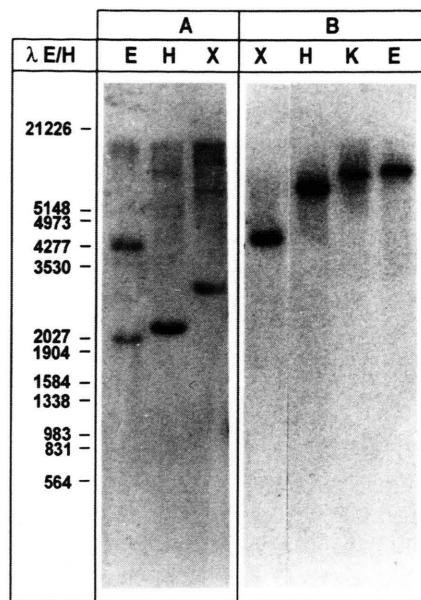


Fig. 4. Southern blot of genomic DNA from chickpea cultivar ILC3279. Genomic DNA (20 µg / lane) was digested either with *Eco*RI (E), *Hind*III (H), *Kpn*I (K), or *Xba*I (X). After electrophoresis on 0.6 % agarose gels, blotting on nylon membranes, hybridization with the insert of *ELR112* (A) or the insert of *p5a-241* was performed (B). After washing the autoradiographic exposure was for seven days at -70 °C using intensifying screen. The insert of *ELR112* and the insert of *p5a-241* were used as positive controls, respectively.  $\lambda$ -DNA digested with *Eco*RI and *Hind*III ( $\lambda$  E/H) was used as molecular size marker.

recently an extracellular protein with a molecular mass of about 21 kDa was identified in chickpea as a thaumatin-like protein by amino-terminal sequencing and serological relationship (Hanselle and Barz, unpublished results). The amino-terminal sequence was different from that of PR-5a and PR-5b. Therefore this third chickpea TLP was named PR-5c.

In order to determine whether there are differences in the expression pattern of *ELR112* and *p5a-241*, northern analyses were performed. Chickpea plants of the resistant and susceptible cultivar were inoculated with *A. rabiei* or mock inoculated with sterile water. Only small amounts of transcript were detectable in control plants of ILC3279 using the insert of *p5a-241* as a probe (Fig. 5). During infection with the fungal pathogen a transient expression starting 12 h post inoculation and reaching a maximum at 36 h to 72 h was

observed in ILC3279. In control plants of ILC1929 the gene encoding PR-5a was weakly induced starting at time point 96 h. The high amount of transcript in the 24 h control sample (Fig. 5) was unusually high and not observed in subsequent kinetics. At time point 120 and 144 h very high amounts of transcript were detectable in samples of the infected plants of the susceptible cultivar whereas no effect of inoculation was observed up to 96 h. The northern analysis with *p5a-241* also corroborated the differential induction of PR-5a in infected plants of the resistant and the susceptible cultivar of chickpea and suggested that the induction of PR-5a is regulated at the transcriptional level.

Only small amounts of *ELRI12* mRNA were detectable in control plants of the susceptible or the resistant cultivar. However striking differences in the expression of *ELRI12* appeared after inocu-

lation with *A. rabiei*. Two phases of transcript accumulation were observed in ILC3279. The first accumulation was transient and started 12 h after inoculation. After increasing up to 48 h a decline to levels slightly higher than in mock treated plants of the same time point was detectable. The second phase started 120 h after inoculation but was less distinct. This pattern of two phases of transcript accumulation was repeatedly observed in independent experiments. In ILC1929 large amounts of the transcript were detectable 120 h and 144 h post inoculation but in contrast to ILC3279 no significant differences appeared between the amounts of transcript in samples of infected and control plants during the first 96 h.

## Discussion

*A. rabiei* invades the leaf tissue of both chickpea cultivars via direct penetration through the cuticle.

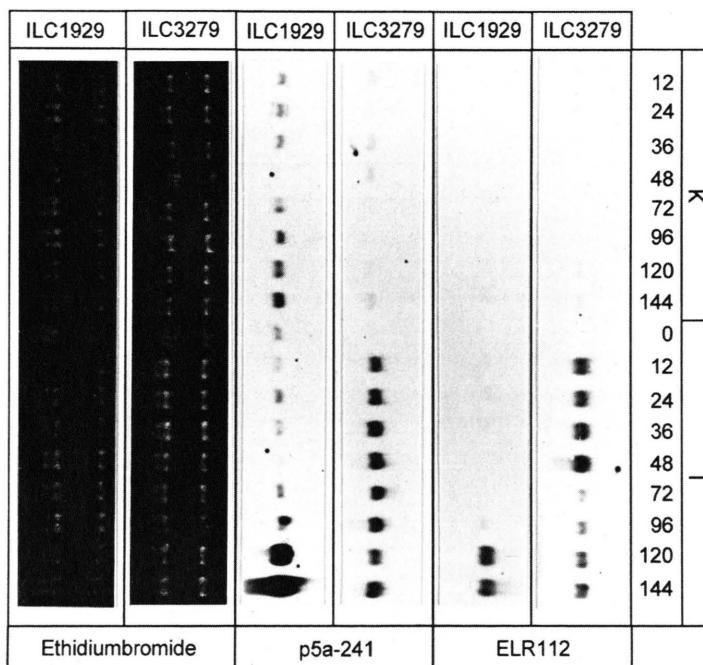


Fig. 5. Effect of inoculation with *A. rabiei* on the accumulation of mRNAs encoding thaumatin-like proteins in the resistant (ILC3279) and susceptible (ILC1929) chickpea cultivars. DIG labelled antisense RNA probes were synthesized using *ELRI12*- and *p5a-241*-cDNA as template. Total RNA (16 µg / lane) isolated from leaves of six chickpea plants at various times after inoculation with *A. rabiei* spores (I, indicated in hours above the blots) or from control plants (K) treated with water. Gel loading was controlled by staining the gel with ethidiumbromide after separation of total RNA as described in material and methods.

In the resistant cultivar of chickpea (ILC3279) growth of *A. rabiei* stops in the apoplastic space whereas the fungal hyphae rapidly spread in the apoplastic space of the susceptible cultivar (ILC1929) (Höhl *et al.*, 1990; Köhler *et al.*, 1995). Therefore, we concentrated our investigations on the soluble components of the apoplastic space. For the latter compartment we successfully established a method to isolate IWF from chickpea leaves. The importance of the apoplastic space in plant-microbe interactions has been described by several authors (de Wit *et al.*, 1985; Hahn *et al.*, 1993).

After inoculation of the aerial parts of chickpea plants with *A. rabiei* a set of PR-proteins accumulated in the apoplastic space. Six of these proteins were identified as acidic chitinases, including the acidic chitinase previously purified from chickpea cell cultures (Vogelsang and Barz, 1993), and a protein determined to be a  $\beta$ -1,3-glucanase (T. Hanselle and W. Barz, unpublished).

A 15 kDa acidic protein also purified from IWF of infected chickpea plants of the resistant cultivar, was identified as a thaumatin-like protein based on its N-terminal amino acid sequence. According to van Loon *et al.* (1994) this protein was named PR-5a, because it is the first TLP described for chickpea. Purified PR-5a showed cross reactivity with antiserum raised against protein S, an acidic, extracellular protein from tobacco, but no significant signals appeared on immuno-blots using antiserum to osmotin, a basic TLP of tobacco stored in the vacuole. Furthermore, no significant cross reactivity with any other protein was observed in IWF and protein extracts of chickpea leaves, indicating that there is no serological relationship to chickpea PR-5b and PR-5c using the mentioned antisera. Weak serological relationship has been described for PR-protein S and osmotin using anti-osmotin or anti-zeamatin antiserum, even both proteins show strong signals using anti-PR-protein S (Vigers *et al.*, 1992; Heitz *et al.*, 1994). Heterologous expression of *ELR112* in *E. coli* and production of antiserum against the expressed protein will be useful tools to detect PR-5b *in planta*. This work is in progress.

TLPs are divided into two subgroups depending to their size, namely large TLPs with a molecular weight between 21 and 25 kDa on the one hand, and small TLPs between 15 and 18 kDa on the other (Velazhahan *et al.*, 1999). With a molecular

mass of 21.9 kDa the putative PR-5b precursor encoded by *ELR112* is a member of the large subgroup of TLPs. PR-5b contains an incomplete amino-terminal signal peptide responsible for the transport through the secretory pathway and a carboxy-terminal extension most likely targets the protein to the vacuole (Neuhaus *et al.*, 1991a; Chrispeels and Raikhel 1992).

TLPs with a molecular weight below 20 kDa are hitherto described only for monocots (Velazhahan, 1999), and therefore chickpea PR-5a is the first dicot member of this sub-group. Even for tobacco, potato and tomato species in which PR-proteins are well studied no small TLP is described. In all small TLPs of monocots the loss of one fourth of the amino acids in relation to the big TLPs is due to an internal deletion near the C-terminus of the proteins (Hahn *et al.*, 1993; Lin *et al.*, 1996; Reimann and Dudler 1993). With regard to the low molecular weight of chickpea PR-5a alignment of the deduced amino acid sequence with TLPs from other species showed that the deletion is located in the same region as in the small TLPs from monocots (Fig. 2).

In agreement with the observation that the precursor protein obviously contains no carboxy-terminal extension, responsible for targeting PR-proteins into the vacuole (Shinshi *et al.*, 1988; Neuhaus *et al.*, 1991a), the PR-5a is located in the apoplastic space. Southern analysis of chickpea genomic DNA pointed out that both, PR-5a and PR-5b, from chickpea are encoded by a single copy gene. Weak bands appearing in the Southern-blots using *ELR112* as a probe leads to the suggestion that further genes encoding closely related TLPs might exist in the chickpea genome. This result fits to our recent studies in which a 22 kDa protein isolated from IWF was identified by amino-terminal sequencing and serological relationship as a further TLP. The amino-terminal sequence of this TLP (PR-5c) differs from those of PR-5a and PR-5b, indicating that at least three isoforms of PR-5 are expressed in chickpea. The presence of several TLP-genes seems to be a common feature and was reported for several plant species including monocots and dicots (Heitz *et al.*, 1994; Lin *et al.*, 1996; Malehorn *et al.*, 1994).

Immunoblot analysis using an antiserum raised against protein S, an extracellular protein from tobacco (Kauffmann *et al.*, 1990), showed different

expression patterns of PR-5a in the resistant and the susceptible chickpea cultivars used in our investigations. A three days delay of PR-5a expression in plants of the susceptible cultivar in comparison to the resistant plants was observed at the protein level, and this agrees with the results obtained with northern analyses (Fig. 5). This means when *A. rabiei* enters the apoplast after direct penetration, the penetration hyphae can be attacked by PR-5a alone or in synergism with other compounds already expressed into the apoplast of the resistant cultivar. The apoplast of the susceptible cultivar contains no or only small amounts of PR-5a at this stage of infection. Further investigations will have to show whether the early cultivar specific expression of PR-5a represents an essential factor for resistance of chickpea towards *A. rabiei* or whether the expression of this protein only correlates with resistance. A cultivar specific induction pattern is described for the PRHv-1a gene, encoding a small extracellular TLP from *Hordeum vulgare* (Hahn *et al.*, 1993). *ELR112* encoding the putative PR-5b showed a similar time course in northern analyses as *p5a-24I* (Fig. 5). The induction of these two TLPs (PR-5a and PR-5b) differs from the induction pattern of chitinases and  $\beta$ -1,3-glucanases in chickpea. For these hydrolases no significant differences in expression of *A. rabiei* infected plants of the resistant and susceptible cultivars were observed in western-blot analyses in a six days time study (Vogelsang and Barz 1993; T. Hanselle and W. Barz, unpublished). The high expression levels of the PR-5a gene in the susceptible cultivar, even higher than in the resistant cultivar, at a later stage of the infection might be the result of at present unknown elicitation factors. These could be water stress, compounds set free as a result of the tissue desintegration during necrotrophic growth of *A. rabiei* or phytotoxic compounds excreted by the fungus (Höhl *et al.*, 1990; Benning and Barz, 1995). To analyse the mode of differential induction of hydrolases on the one and TLPs on the other side further investigations are required.

Antifungal activity of PR-proteins including members of the PR-5 family, when measured *in vitro*, has been described by several authors (Mauch *et al.*, 1988; Woloshuk *et al.*, 1991; Vigers *et al.*, 1991 and 1992; Malehorn *et al.*, 1994). However antifungal activity is not a general property

of PR-proteins and some (phytopathogenic) fungi show resistance. For example, the basic isoforms of chitinases and  $\beta$ -1,3-glucanases isolated from tomato strongly inhibit the non-pathogenic fungus *Trichoderma viride* whereas *Cladosporium fulvum*, the fungal pathogen causing tomato leaf mould, is not sensitive to these hydrolases *in vitro* (Joosten *et al.*, 1995). A strict specificity of antifungal proteins including osmotin for the target cell was demonstrated by Yun *et al.* (1997). In our investigations we used two different systems to assay antifungal activity of chickpea PR-5a *in vitro*. In both cases we found no inhibitory effect of the purified protein on growth of *A. rabiei*. One observed effect of TLPs on fungi is the desintegration of the cytoplasma membrane, which leads to an efflux of intracellular compounds (Vigers *et al.*, 1992; Woloshuk *et al.*, 1991). The influence of growth conditions on fungal cell walls and membrane structure and composition is well studied for the penetration structures of biotrophic pathogens forming haustoria (Chong *et al.*, 1985; Mendgen *et al.*, 1996; Pain *et al.*, 1994; Hahn *et al.*, 1997). In axenic cultures sensitivity of *A. rabiei* to chitinases depends on the composition of the medium (G. Hanselle and W. Barz, unpublished results). In general data from *in vitro* studies should not be taken for a final proof for exclusion of biological activity; under *in planta* condition antifungal activity could still be expressed. Different recent studies exclude that TLPs act as unspecific, pore forming proteins and confirm the thesis of the existence of a receptor or a facilitator located in or at the fungal plasmamembrane (Batalia *et al.*, 1996; Yun *et al.*, 1997). A participation of the cell wall of resistant or sensitive fungi in the decrease or facilitation of antifungal activity of TLPs, respectively, was discussed (Yun *et al.*, 1997). Taking all these observations together, the fact that no antifungal activity of chickpea PR-5a was detectable *in vitro*, does not exclude that there is antifungal activity of PR-5a *in planta*, either alone or in synergism with other defense mechanisms, for example other PR-proteins. On the other hand it is also possible that *A. rabiei* is insensitive against PR-5a because there is no target site for PR-5a in *A. rabiei* or because PR-5a is inhibited or degraded by fungal proteins.

Further investigation on chickpea TLPs will concentrate on the regulation of the expression of

the three genes encoding thaumatin-like proteins, in comparison to the expression of the chitinases and  $\beta$ -1,3-glucanases.

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