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RESEARCH PAPER

A novel pathogenesis-related protein (LcPR4a) from lentil, and its involvement in defence against *Ascochyta lentis*

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Summary. A novel pathogenesis-related protein 4 (PR4) encoding gene, *LcPR4a*, was induced in *Lens culinaris* following *Ascochyta lentis* infection. *LcPR4a* encodes a predicted 146 amino acid protein of 15.8 kDa. The putative LcPR4a protein belongs to the class II PR4 family and has close phylogenetic affinity to PR4 proteins from related species. qPCR analysis revealed differential expression of the *LcPR4a* gene upon *Ascochyta lentis* infection in both resistant and susceptible cultivars. This, combined with preliminary *in vitro* antifungal assays of the recombinant protein expressed in *E. coli*, suggests the potential important role of LcPR4a in the defence response of lentil to *Ascochyta lentis* attack.

Key words: Ascochyta blight, PR4, antifungal activity.

Introduction

Ascochyta blight, caused by *Ascochyta lentis* Vassil, is a major threat to lentil production worldwide, causing yield losses of up to 70% (Gossen and Morrall, 1983; Brouwer *et al*., 1995; Kaiser, 1997). Although a number of fungicides have proven effective in controlling ascochyta blight of lentil, sustainable and profitable control is through the use of resistant cultivars together with cultural practices (Davidson and Kimber, 2007; Taylor *et al*., 2007). To date, many resistance sources have been identified in both cultivated and wild lentil germplasm (Bayaa *et al*., 1994; Ford *et al*., 1999; Nguyen *et al*., 2001), and successfully utilised in lentil breeding programs worldwide (Materne and McNeil, 2007). In Australia, cultivars ILL7537 and ILL358 were reported to be resistant to foliar infection, and ILL5588 (Northfield), ILL7193 and ILL7199 were found to have the lowest level of seed infection (Nasir and Bretag, 1997a, 1997b).

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Due to incomplete resistance, a major lentil breeding focus worldwide remains the search for higher resistance levels. Moreover, the existing resistance in lentil cultivars is simply inherited (Ford *et al*., 1999; Nguyen *et al*., 2001) and, therefore, subject to possible breakdown by new and highly virulent isolates (Banniza and Vandenberg, 2006). Indeed, resistance in the Canadian cultivar Laird was compromised in only fifteen years (Ahmed *et al*., 1996). Similarly, in Australia, due to the likely high genetic variation in the *A. lentis* population (Ford *et al*., 2000) and also the presence of both mating types (Galloway *et al*., 2004), potential evolution of virulent isolates capable of overcoming host resistance is regarded as a major threat (Salam *et al*., 2011) . Hence, an essential strategy in breeding lentil for ascochyta blight resistance is to breed for more durable resistance by pyramiding multiple resistance genes into one cultivar. This strategy is subject to identification of the functional genes and signalling pathways conferring their expression.

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Knowledge of the underlying genes controlling ascochyta blight resistance in lentil was lacking until 2009, when Mustafa *et al.* studied the functional role of many genes involved. This was achieved using a cDNA microarray which was exploited to profile early gene expression changes in resistant (ILL7537) and susceptible (ILL6002) genotypes in response to a highly virulent *A. lentis* isolate (AL4). This led to the identification of several differentially expressed genes encoding pathogenesis-related (PR) proteins (Mustafa *et al.*, 2009).

Although generally encoded at low levels, PR proteins are induced and accumulate in plants upon infection, and for some of them direct anti-pathogenic activities have been reported (Van Loon, 1997). Of the 17 groups of PR proteins (Van Loon *et al.*, 2006), three are involved in the lentil-*A. lentis* pathosystem (Mustafa *et al.*, 2009). Genes encoding a PR4 protein (DY396388), three PR10 proteins (DY396377, DY396347 and DY 396344) and a β-1,3-glucanase (CV793598) (PR2) were up-regulated in the resistant genotype (ILL7537) in response to *A. lentis,* while not differentially expressed or down-regulated in the susceptible genotype (ILL6002) (Mustafa *et al.*, 2009).

The PR4 family consists of low molecular weight chitin-binding proteins sharing a common C-terminal Barwin domain (Svensson *et al.*, 1992), some of which possess antifungal properties (Hejgaard *et al.*, 1992; Ponstein *et al.*, 1994; Caruso *et al.*, 1996). While the mode of action of many PR4 proteins remains unknown, some inhibit fungal growth due to chitinase activity (Ponstein *et al.*, 1994; Lu *et al.*, 2012). Many PR4 proteins also possess ribonuclease and DNase activities (Caporale *et al.*, 2004; Bertini *et al.*, 2009; Guevara- Li *et al.*, 2010; Morato *et al.*, 2010; Lu *et al.*, 2012).

PR4 proteins are divided into two classes (Neuhaus *et al.*, 1996) based on the presence (class I) or absence (class II) of a Hevein domain, *i.e.*, a conserved N-terminal cysteine-rich chitin-binding domain corresponding to a small antifungal protein isolated from the rubber plant (*Hevea brasiliensis*) (van Parijs *et al.*, 1991). In this paper, we report the characterisation of a new class II PR4 encoding gene, *LcPR4a*, from lentil. The differential expression of *LcPR4a* is investigated in both compatible and incompatible lentil-*A. lentis* interactions. Furthermore, antifungal activity of the recombinant protein against *A. lentis* was examined *in vitro.*

Materials and methods

Plant and fungal material

Seed of highly resistant (ILL7537) and susceptible (ILL6002) lentil genotypes as well as a highly virulent *A. lentis* isolate (AL4) were provided by the Victorian Department of Primary Industries (Horsham, Australia). All plants were grown in 12 cm diameter pots, filled with lentil soil mix (one part dolomite in nine parts commercial potting mix) in a growth room at 22 ± 2°C and 14/10 h light/dark photoperiod. After 10 days, seedlings at the eight-leaf stage were used for various treatments. Fungal material was cultured on PDA plates and grown at 22°C and 12/12 h NUV light/dark photoperiod before use.

Sequence analysis and molecular modelling

The full length nucleotide sequence of *LcPR4a* was retrieved from GenBank (accession number JX273653) and the sequence of the putative protein, LcPR4a, was predicted using SIB ExPASy Bioinformatics Resource Portal (Artimo *et al.*, 2012). The nucleotide and deduced amino acid sequences were subject to BLAST searches to identify transcripts with high similarity.

To determine the relationship of LcPR4a with PR4 proteins from other plants, a phylogenetic analysis was performed with the predicted LcPR4a and amino acid sequences of the proteins retrieved from the BLASTP search. Alignments were obtained with ClustalW through the Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura *et al.*, 2011), which was then used to generate a phylogeny tree using the maximum parsimony method and SPR algorithm (Nei and Kumar, 2000).

The ScanProsite web tool (De Castro *et al.*, 2006) and SignalP 4.0 server (Petersen *et al.*, 2011) were used to identify the conserved domains and putative signal peptides within the LcPR4a sequence, respectively. The NetNes 1.1 web server (La Cour *et al.*, 2004) was used to predict leucine-rich nuclear export signals (NES). A three-dimensional model of LcPR4a was created using the MODWEB server (Pieper *et al.*, 2006) based on the available NMR coordinates of the homologous protein Barwin (PDB 1bw3) and figures were visualised using the Jmol program (http://www.jmol.org/) and the STRIDE web server (Heinig *et al.*, 2004).

Gene expression analysis

The differential induction of *LcPR4a* in both compatible and incompatible interactions of lentil and *A. lentis* was investigated through gene expression analysis on highly resistant (ILL7537) and susceptible (ILL6002) lentil genotypes. Inoculation was conducted at the eight-leaf stage with a spore suspension of 1 × 10^6 spore mL^{-1}, containing 0.02% (v/v) Tween 20. Control plants were sprayed with water containing 0.02% (v/v) Tween 20. After spraying, pots were covered with two layers of opaque plastic bags to provide high humidity and darkness for spore germination, and only opened for sample collection. Total seedling foliage (leaves and shoots) from four plants was harvested at two, six, 12 and 48 hours post inoculation (hpi), and was flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. Four samples were harvested at each time point to achieve statistically valid conclusions, assuming a Coefficient of Variation (CV) of 50% as well as a 50% range of confidence interval (Karlen *et al.,* 2007).

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Melbourne, Australia). On-column digestion of residual DNA was performed using the RNase-Free DNase Set (Qiagen). The integrity of the extracted RNA was checked on 1% agarose gel and quantified using the Qubit™ RNA BR Assay Kit (Invitrogen, Melbbourne, Australia). One microgram of RNA was immediately reverse transcribed using an oligo(dT)$_{20}$ primer and the iScript Select cDNA Synthesis Kit (Bio-Rad, Australia). In order to confirm the absence of residual genomic DNA, the synthesized cDNA was used in a traditional PCR with actin primers (Forward: 5′- GTTCCACAATGTTCCCTG-GT-3′ and reverse 5′- ATTCTGCCTTTGCAATC-CAC-3′). Since the size of the actin gene fragment amplified from gDNA (~400 bp) was different from cDNA (<200 bp), due to the presence of an intron, any gDNA contamination could be detected by this method and contaminated samples were retreated with DNase and reassessed.

Amplification efficiency

Percentile Amplification Efficiency (PAE) for the reference (actin) and target (*LcPR4a*) genes was validated through calibration. The regression model proposed by Yuan *et al.* (2006) and SAS version 1.0 package were used to investigate the equality of the target and reference gene efficiencies. The iQ™5 optical system software (Bio-Rad, Ausrtalia) was used to calculate the PAE and R^2 values.

Reference gene validation

The stability of the reference gene expression was investigated under the experimental conditions (high humidity and darkness) as well as treatment (*A. lentis* inoculation) using the method proposed by Schmittgen and Zakrajsek (2000) except that a non-parametric test (Kruskal-Wallis) was employed to investigate the association between the treatment and level of actin gene expression, since the assumption of normality of data was violated (n = 4).

qPCR

Triplicate qPCRs were carried out for each sample in a Bio-Rad iQ5 Real-Time machine (Bio-Rad). Reactions were set up in a total of 25 μL volume containing 13.5 μL SYBR Green Supermix (Bio-Rad), 0.2 μM of each primer (*LcPR4a*: forward: 5′- TAC-CTGGGATGCTAACCAGCCTTT -3′, and reverse 5′- ATTTGCCGCAAGAATCTCTGCCTG -3′) and 4 μL of 10-times diluted cDNA. The PCR cycle was: 3 min at 95ºC, followed by 41 cycles of 30 s at 95°C, 30s at 57ºC, and 30 s at 72ºC. For statistical analysis of the qPCR data, a Wilcoxon two-group test was performed using the SAS program developed by Yuan *et al.* (2006). This yielded an estimation of the -ΔΔCq as well as the *p* value, indicating whether the calculated fold changes in the treatments were significantly different to the untreated controls. Also, the proposed SAS Macro (Yuan *et al.,* 2006) was used to derive the confidence levels for the calculated fold changes as a reliable measure of variability according to the MIQE guideline (Minimum Information for publication of Quantitative real-time PCR Experiments) (Bustin *et al.,* 2009).

Antifungal activity of LcPR4a

Protein expression and purification

The synthetic gene, encoding the deduced LcPR4a protein, was cloned into a His-tagged expression vector, pOPIN-NHis, and transformed into *E. coli* strain BL21 (DE3) pLysS for expression in the Australian Protein Expression Facility at the University of Queensland as follows: A single *E. coli* colony harbouring the pOPIN-NHis-LcPR4-a was selectively grown in TB media containing ampicillin (50 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}), and incubated at

30°C overnight. This culture was used to inoculate one litre of TB media (using the same antibiotics) at a 1:100 dilution and grown at 30°C until the OD_{600} reached 0.5 at which point 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of LcPR4a. The culture was further incubated for 4 h at 25°C before harvest. An expression and solubility analysis was performed by lysing in BugBuster (Novagen, Qld, Australia) to obtain the total fraction, followed by centrifugation at 15,000 × *g* for 15 min to obtain the soluble fraction. Total and soluble fractions were analysed via SDS-PAGE and coomassie staining, as well as an anti-His western blot by ChemiDoc MP System (Bio-Rad), using Novex Sharp pre-stained molecular weight ladder (data not shown).

The expressed protein was purified using Immobalized Metal Ion Chromatography (IMAC). The *E. coli* cell pellet was resuspended in 200 mL lysis buffer (20 mM Sodium Phosphate, 500 mM NaCl, 20 mM Imidazole, 1% Triton X-100, 2 mM $MgCl_2$, 100 U Benzonase, Complete protease inhibitor cocktail [Roche], pH 7.2) and sonicated on ice three times for 25 s with 2 min intervals, after which the lysate was incubated on an orbital wheel for one hour at room temperature to degrade DNA. Total lysate was then centrifuged at 18,500 *g* for 30 min at 4°C to isolate the soluble fraction. The sample was further clarified by filtration through 1.2 µm and 0.45 µm filters, prior to loading onto an IMAC column using the AKTA Explorer FPLC system (GE). Unbound protein was removed with 25 column volumes (CVs) of wash buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.2) before a multi-step elution, whereby increasing concentrations (16, 46, 56, 66, 76, and 100%) of elution buffer (EB; 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.2) were used to elute LcPR4a. Each concentration was held until baseline absorbance. All chromatography steps were performed at a flow rate of 1 mL min^{-1} at room temperature.

Antifungal assay

To test the inhibitory activity of LcPR4a against *A. lentis*, a bioassay was designed based on the method proposed by Broekaert *et al.* (1990). This method enables monitoring fungal growth by culturing the fungus in a microplate followed by density recording at absorbance of 595 nm as an indicator of fungal biomass (growth). The highly virulent *A. lentis* isolate AL4 was used to prepare a spore suspension of 4 × 10^5 spore mL^{-1} in potato dextrose broth (PDB). Twenty microliters of the spore suspension was added to each well in a microtiter plate. Appropriate volumes of EB, containing the purified recombinant LcPR4a, were added to the treatment wells to concentrations of 10, 20, 40, and 50 μg mL^{-1} LcPR4a in a total volume of 200 µL. Equal amounts of EB without the LcPR4a served as negative control. A second control consisted of only the spore suspension and PDB to record fungal growth in the absence of both EB and LcPR4a. The same ratios of PDB, buffer and sterile water mixtures were used as respective blanks. The plates were shaken for 30 min and incubated at 20°C at a 12/12 h NUV light/dark photoperiod. Absorbance readings at 595 nm were recorded every four hours for the first 16 hours, and then every 12 h from 32 until 92 h post treatment (hpt) using a micro-plate reader. All treatments and controls were performed in five technical replications. The entire experiment was conducted in two biological replications. Due to the small sample size at each time point (n = 5), the non-parametric Mann-Whitney test was conducted to compare the A_{595} of the treatments and controls at each time point using SPSS version 18.0.

Results

Bioinformatics and molecular modelling

The full length *LcPR4a* cDNA contained an Open Reading Frame (ORF) of 441 bp, and two non-coding regions of 39 bp and 157 bp at each of the 5' and 3' ends, respectively. The predicted ORF encoded a predicted protein of 146 amino acids in length, with an N-terminal methionine and a C-terminal cysteine, and molecular weight of 15.8 kDa.

The protein domain search revealed the presence of the conserved Barwin domain (Svensson *et al.*, 1992) spanning from positions 27 to 146 in LcPR4a. A signal peptide was detected at the 1–26 amino acid position, with the residues at the 10–18 position predicted to participate in a nuclear export signal, indicating extracellular localisation of LcPR4a. Lack of an N-terminal chitin-binding Hevein domain indicated LcPR4a to be a class II PR4 (Neuhaus *et al.*, 1996). A conserved characteristic of the Barwin family is the presence of six cysteines involved in formation of disulphide bonds, also found in the putative LcPR4a sequence (Figure 1).

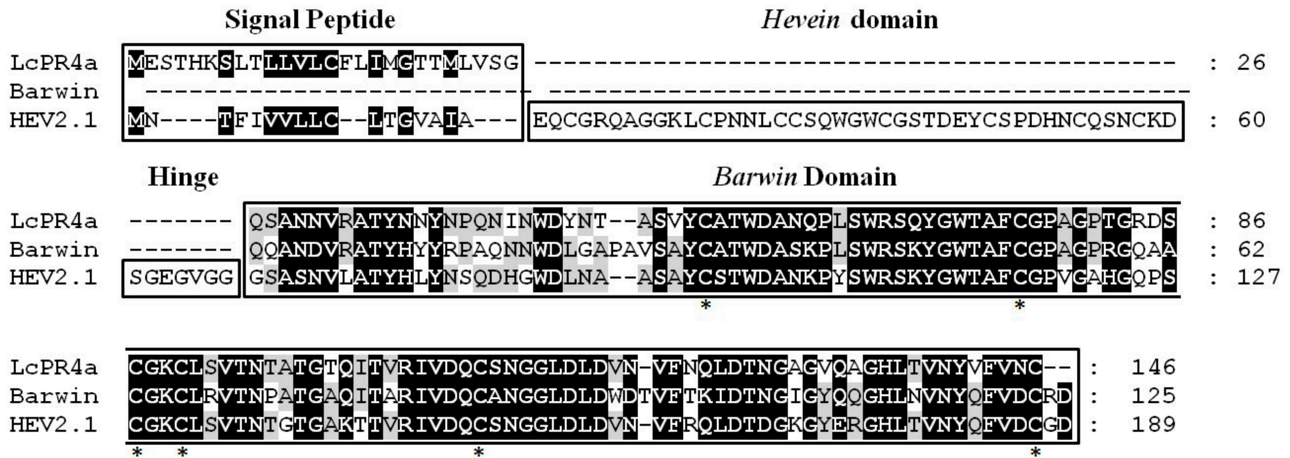

Figure 1. Alignment of the amino acid sequence of deduced LcPR4a with barwin (P28814) and HEV2.1 (AAO63573) showing the presence of Barwin but lack of Hevein domain in LcPR4a. Numbers correspond to the last amino acid position. Black and shaded backgrounds indicate identical and similar amino acids. The six cysteines conserved in the Barwin family of proteins are marked with asterisks. Sequences were aligned in ClustalW and visualised in BoxShade.

Database searches revealed high similarity of the deduced amino acid sequence of LcPR4a with multiple PR4 proteins from related species. Phylogenetic analysis revealed that LcPR4a had closest affinity to class II PR4 proteins from *Pisum sativum*, *Medicago truncatula* and *Cicer arientinum* (Figure 2).

The three-dimensional modelling of LcPR4a showed a structure comparable to the NMR determined 3D structure of Barwin protein (1bw3) from barley (Figure 3). The dominating features were the presence of a well-defined four-stranded antiparallel β-sheet, two parallel β-sheet and four short helices (Ludvigsen and Poulsen, 1992). The remaining sequence formed loops and coils with no regular secondary structural elements (Figures 4). Considering the high similarity of LcPR4a structure to other PR4 proteins with known antifungal activity, it is plausible to hypothesize similar function and role for LcPR4a against fungal pathogens.

Gene expression analysis

The three replicated calibration curves produced for each gene showed high efficiencies with very high linearity (R^2>0.99) and not statistically different from one. The precision and reproducibility of assays was confirmed by low intra-assay variation (CV<10%) and the small standard error from three different experimental runs (supplementary data I).

Statistical analysis of the actin gene expression in the control and AL4-inoculated samples showed no significant relationship between the treatments and reference gene expression, which validated the use of the actin gene as an internal control for this study (supplementary data II).

The normalized *LcPR4a* expression in response to treatments relative to the transcript levels in the untreated controls at each time point is presented in Figure 5. Although the *LcPR4a* expression was found to be induced upon infection in both resistant and susceptible cultivars, the magnitude and pattern of upregulation varied. *A. lentis* infection did not cause a significant change in the *LcPR4a* gene expression at either 2 or 6 hpi. At 12 hpi, a significant induction of *LcPR4a* expression was detected in response to *A. lentis* in both compatible and incompatible interactions of lentil-*A. lentis*, and the magnitude of *LcPR4a* expression was higher in the susceptible cultivar. However, the expression of *LcPR4a* continued to increase only in the resistant genotype past this time point.

Antifungal activity of LcPR4a

When grown in the presence of 40 μg mL^{-1} of recombinant LcPR4a, a significant reduction was observed onwards from 32 hpt in the fungal biomass compared to the negative control (P=0.009). EB had no significant effect on fungal growth (P=0.142). This

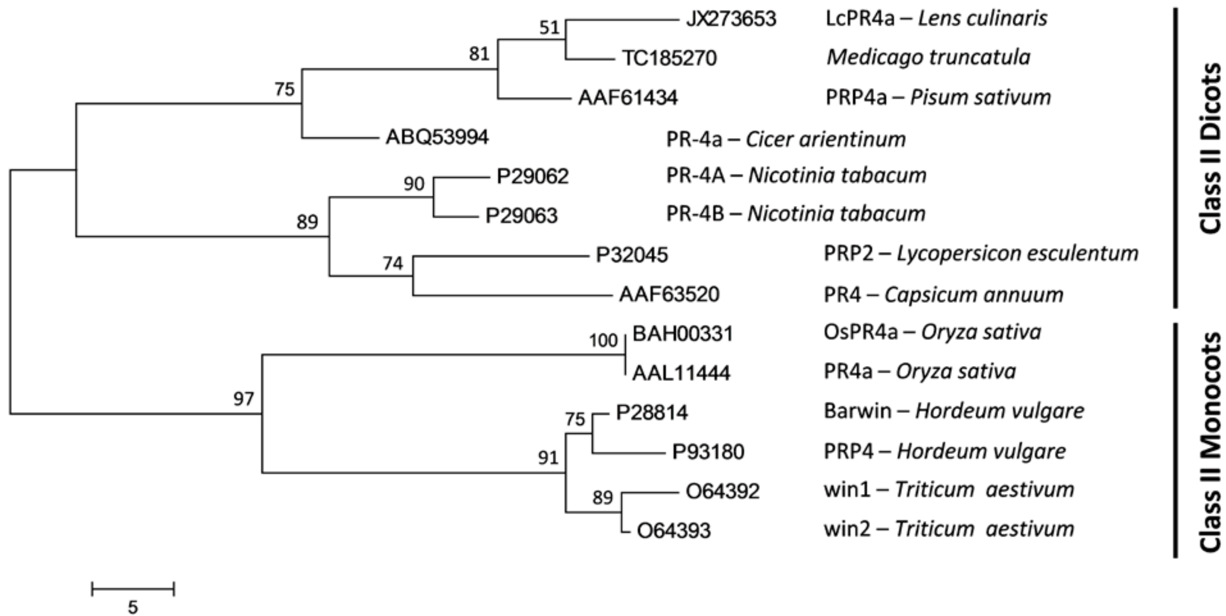

Figure 2. Phylogenetic relationship of class II PR4 proteins, showing close affinity of LcPR4a to PR4 proteins of other legumes. GenBank accession numbers for proteins are indicated, except for *Medicago truncatula* putative PR4 protein (TC185270) which was sourced from DFCI *Medicago truncatula* Gene Index (MtGI). The bootstrap consensus tree was constructed using Maximum Parsimony method and SPR algorithm. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. Branches with less than 50% bootstrap replicates were collapsed.

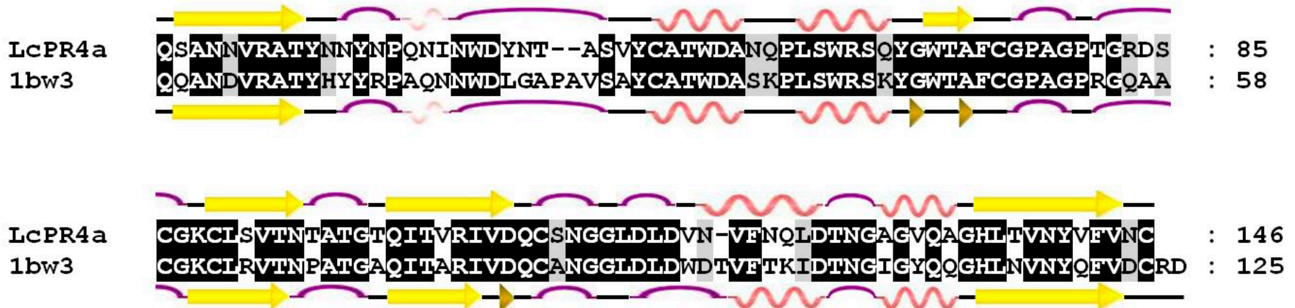

Figure 3. Structure-based sequence alignment of Barwin protein (PDB 1bw3) and predicted amino acid sequence of LcPR4a after cleavage of the putative signal peptide, depicting the secondary structure elements corresponding to helices (), beta strands (), isolated beta bridges (), turns () and coils (–) assigned by STRIDE (Heinig *et al*., 2004).

represented a 36% reduction in growth at 32 hpt, with increasing effect of 54% reduction at 92 hpt (Figure 6). This implied that LcPR4a has an IC_{50} of less than 40 μg mL^{-1} under the experimental conditions used. No significant inhibition of fungal growth was found when 10 or 20 μg mL^{-1} LcPR4a was used.

Discussion

In this study, we characterised a novel PR4 gene (*LcPR4a*) in lentil, and investigated its involvement in the defence response to *A. lentis*. The differential expression of *LcPR4a* against *A. lentis* was studied and

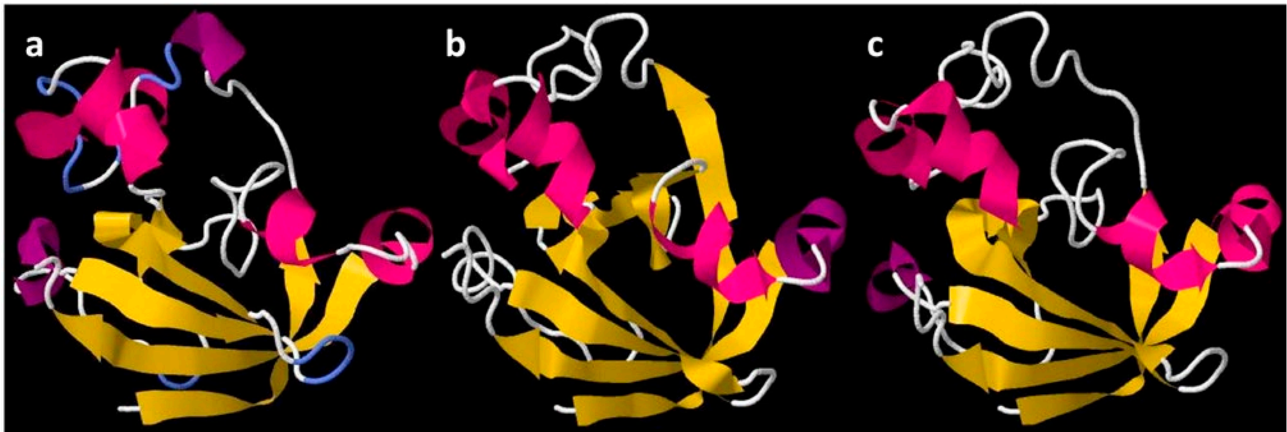

Figure 4. Three dimensional structural model of the a. deduced LcPR4a, b. Barwin (PDB 1bw3) and c. Wheatwin1 (PDB 1C2Z) proteins. The structure of LcPR4a was predicted through homology modelling using Barwin as template.

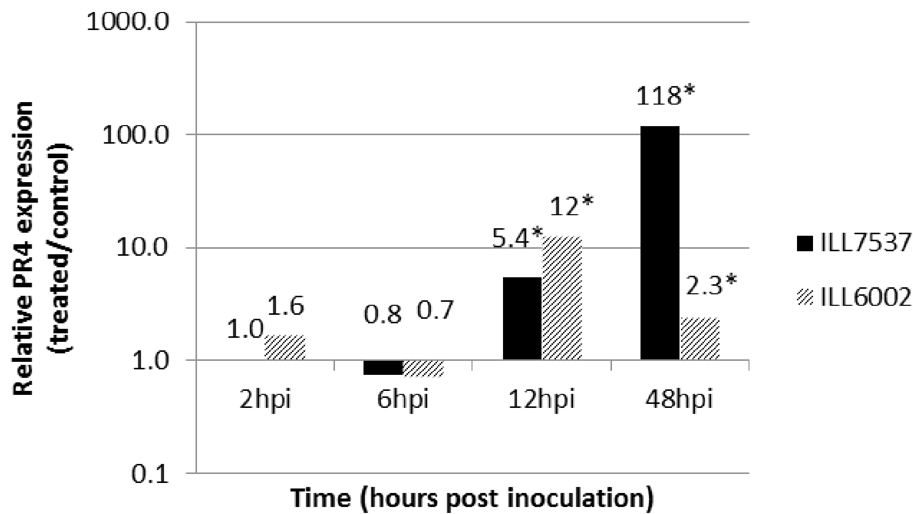

Figure 5. Fold changes in relative transcript level of PR4 gene in the resistant (ILL7537) and susceptible (ILL6002) lentil genotypes. Columns represent average data from four independent samples at each time point. The numbers on each bar show the fold change in PR4 transcript level caused by *A. lentis* inoculation relative to those in untreated controls at the same time point. The asterisk indicates that the observed fold change was significantly different from the control samples (based on the Wilcoxon two-group test).

the fungal inhibitory activity of the encoded protein was demonstrated *in vitro*. Based on the presence of the Barwin domain and also lack of a chitin-binding domain in the N-terminus of the predicted mature protein, LcPR4a was deemed to be a class II PR4 protein, the first PR encoding gene characterised from lentil.

PR4 proteins have been isolated from a wide range of plant species and some have proven chitinase, RNase and DNase activity. LcPR4a showed a close phylogenetic affinity to the class II PR4 proteins. It has a molecular weight of 15.8 KDa, which is similar to most PR4 proteins characterised to date (Zhu *et al.*, 2006; Li *et al.*, 2009). Sequence and structural similarity of LcPR4a with Barwin as well as other PR4 proteins with known antifungal activities suggested a potential role in disease defence.

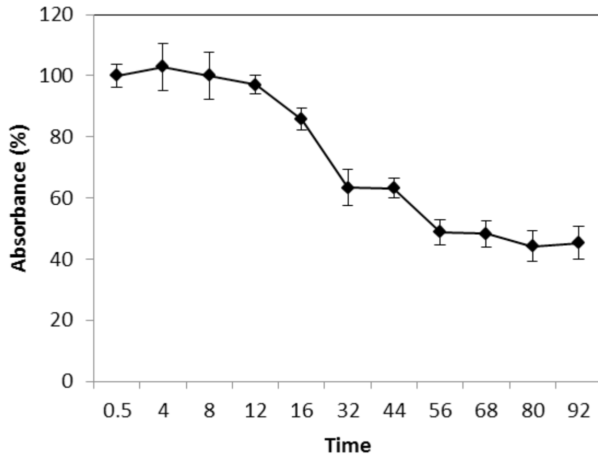

Figure 6. Inhibitory effect of 40 μg mL^{-1} LcPR4a on *A. lentis* growth. Significant reduction in fungal biomass was observed at 32 hours post treatment (hpt) (*P* 0.009). Each time point indicates relative fungal biomass in the presence of 40 μg mL^{-1} LcPR4a, expressed as percentage absorbance of the control cultures. Bars indicate standard errors (n=5).

The time course qPCR study of the *LcPR4a* expression following *A. lentis* inoculation further suggested a role in the defence response to *A. lentis*. While there was no significant difference in the expression of *LcPR4a* at 2 and 6 hpi relative to the untreated controls, a 5-fold upregulation was observed at 12 hpi in the resistant cultivar, which built up to 114 fold at 48 hpi. Likewise, Mustafa *et al.* (2009) found no differential expression of this PR4 gene in ILL7537 at 6 hpi but detected an increase in the transcript level at 24 hpi. Induction of *LcPR4a* in response to *A. lentis* occurred in both resistant and susceptible genotypes at 12 hpi; however, the increasing expression level was sustained only in the resistant one.

Morphological studies show that *A. lentis* spore germination occurs in the initial hours of infection (2–6 hpi) and appressoria are formed within 10 hours (Roundhill *et al.*, 1995; Sambasivam, 2011). The length of the germ tubes before forming appressoria on the resistant genotype are significantly shorter than on the susceptible, suggesting an effective pathogen recognition and activation of host defence responses very early in the infection process (Sambasivam, 2011). Likewise, transcriptional profiling of the lentil-*A. lentis* pathosystem (Mustafa *et al.*, 2009; Sambasivam, 2011) detected an elevated expression of many early defence-related genes. This includes *LcPR4a*, which appears to be switched on in the very early interaction and sustained only in the resistant genotype, indicating its importance in early defence. Extension of the time-course study of the *LcPR4a* transcription during the host-pathogen interaction and among other genotypes will elucidate the relationship further.

Previous studies have reported inhibitory effects of PR4 proteins on hyphal growth of several phytopathogenic fungi (Van Parijs *et al.*, 1991; Ponstein *et al.*, 1994; Caporale *et al.*, 2004; Zhu *et al.*, 2006; Li *et al.*, 2010). Different studies have reported the effect of PR4 proteins to be dosage-dependent (Zhu *et al.*, 2006) or independent (Li *et al.*, 2010). We found that LcPR4a was inhibitory toward *A. lentis* at 40 μg mL^{-1}. The 54% reduction in fungal growth at 92 hpi indicated that under the experimental conditions, the LcPR4a had an IC_{50} of about 40 μg mL^{-1}, similar to some other PR4 proteins (Caporale *et al.*, 2004).

The mechanism whereby PR4 proteins inhibit fungal growth occurs through chitinase (Ponstein *et al.*, 1994; Lu *et al.*, 2012) as well as RNase and DNase (Caporale *et al.*, 2004; Bertini *et al.*, 2009; Guevara-Morato *et al.*, 2010; Li *et al.*, 2010; Lu *et al.*, 2012) activities. Although the purified recombinant LcPR4a inhibited *A. lentis* growth, its mode of action remains unknown and will be the subject of future studies. Since chitin-binding proteins act synergistically with other chitinases (Hejgaard *et al.*, 1992) and β-glucanases (Ponstein *et al.*, 1994), it is plausible that *LcPR4a* in coordination with the β-1,3-glucanase reported from lentil (Mustafa *et al.*, 2009) plays a role in lentil basal defence response against a range of fungal pathogens. Further studies are required for detailed characterisation of *LcPR4a* and investigation of antifungal activity of the native LcPR4a against *A. lentis* as well as other fungal pathogens.

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