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# Functional analysis of elicitins and identification of cell wall proteins in *Phytophthora cinnamomi*

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ABSTRACT

*Phytophthora cinnamomi* Rands, a soil-borne oomycete plant pathogen, is a major threat to natural vegetation in many countries, especially Australia. The disease that it causes also has economic impacts on several agricultural and forestry systems worldwide. To investigate the virulence mechanisms of *P. cinnamomi* the roles of elicitins and elicitin-like proteins were examined using a susceptible model species, *Lupinus angustifolius*. Elicitins are 10 kDa proteins secreted by many *Phytophthora* species. Here, elicitins were isolated from *P. cinnamomi* liquid culture and separated according to their isoelectric point. A β-cinnamomin elicitin was then purified and used to develop an immunoaffinity purified antibody as a tool to examine virulence. The specificity of the β-cinnamomin antiserum was confirmed through Western-blot analysis. β-cinnamomin was found to be produced at different *P. cinnamomi* life stages and in inoculated plant roots by using immuno detection via confocal laser scanning microscopy. Pre-treatment of *P. cinnamomi* zoospores with β-cinnamomin antiserum and then inoculation of susceptible *L. angustifolius* roots revealed a partial loss of virulence of the pathogen. In addition, the expression of the β-cinnamomin gene at an early infection stage in susceptible *L. angustifolius* suggested an intrinsic role of the elicitin. Furthermore, the identification of covalently bound cell wall proteins in *P. cinnamomi* mycelia in this study has opened up the opportunity to investigate their role in virulence.

## 1. Introduction

The oomycete genus *Phytophthora* contains numerous plant pathogens which are known to cause crop production losses and are causative agents of forest declines and dieback [1,2]. For over 90 years in Australia, the soil-borne pathogen *Phytophthora cinnamomi*, an important species of this genus, has been responsible for damage and destruction of native vegetation. This pathogen has also caused forest decline in Europe [2,3], the Americas [2,4] and Asia [5] and is considered a major threat to biodiversity [6–8]. Therefore, a better understanding of the biology and ecology of *P. cinnamomi* is vital for improving management practices currently utilised in forest ecosystems.

*Phytophthora* spp. release many proteins, including effectors and elicitors, during interactions with both compatible and incompatible hosts. Effectors act as suppressors of plant defence and facilitate further infection [9] whereas elicitors elicit plant defence [10]. Elicitins are considered to be a subgroup of elicitors and have been found in oomycetes. *Phytophthora* elicitins are a major group of small acidic or basic peptides, which are known to be released into the host during infection [11]. Elicitins are 10 kDa holoproteins, which contain 98 amino acids, have three conserved disulphide bonds and have high sequence similarity. Elicitins may have a number of functions including sterol binding and transport between membranes [12]. Sterols are known to control membrane fluidity and permeability in all eukaryotic organisms [13]. It has been hypothesized that elicitins are secreted by *Phytophthora* species to act as sterol carriers because *Phytophthora* species are not able to synthesize sterols [14]. The sterol binding capacity of elicitins has been strongly suggested to be effective in inducing biological responses in plants [15]. In a recent review [10], it was emphasized that elicitins act primarily as plant resistance inducing

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factors. Additionally, a cell surface receptor from a wild *Solanum* species, that has specificity to a large range of elicitins, has been described [10].

In various *Phytophthora* species, elicitins are encoded by complex gene families and grouped into elicitin (ELI) and elicitin-like (ELL) genes [16,17]. Phylogenetic analysis of elicitins reproduced seventeen clades that are reported to be secreted into liquid culture. However, there is uncertainty as to their involvement in inducing the hypersensitive response (HR) [17,18]. The biological significance of the elicitin-like proteins is still largely unknown.

The earlier studies about the role of elicitins as avirulence factors were limited to their interaction with *Nicotiana* species [19,20]. However, more recently other plant species have also been studied. Following the earlier, seminal studies of *P. nicotianae* and *P. infestans* with *Nicotiana tobacum* and *N. benthamiana* [19–25] other host species including *Quercus robur*, *Q. suber*, and *Fagus sylvatica* [16,25–27] have now been examined for their interactions with various elicitins and associated virulence has been reported [25]. Elicitin gene clusters have been described in several *Phytophthora* species such as *P. cryptogea*, *P. cinnamomi* and *P. cambivora* (recently re-named as *P. xcambivora* according to a recent description [5]). These clusters are conserved and contain four open reading frames [26]. In *P. cinnamomi*, the clusters are arranged in tandem pairs and were reported to be within ELI clade − 1. In these clusters, two genes showed homology to basic and acidic elicitins, whereas two others showed homology to highly acidic elicitins [17,28]. The induction of all elicitin genes in *P. cinnamomi* during infection of *Q. suber* roots indicated their association in the infection process. Moreover, silencing of the β-cinnamomin gene (ELI-1 clade) in *P. cinnamomi* affected the induction of the other three elicitin genes and the silenced mutants showed reduced *in planta* colonization in *Q. suber* [26]. Additionally, β-cinnamomin silenced mutants were less virulent compared to the wild type and were unable to effectively penetrate *Q. suber* root tissue. Therefore, this elicitin was considered to be associated with virulence in this specific host-pathogen interaction [27]. However, the biological function of *P. cinnamomi* elicitins is yet to be studied for other susceptible *P. cinnamomi* host interactions. A recent study reported that an elicitin-like gene, identified by RNA-seq analysis, was expressed in zoospores and germinating cysts thus indicating a role in the pre-infection stage in *Persea americana* [29]. However, there is no information on additional *P. cinnamomi* elicitin-like proteins and their functions.

The present study was undertaken to identify elicitins, cell wall associated elicitins or proteins in *P. cinnamomi,* and to explore the functions of elicitins through immunodepletion of β-cinnamomin using a susceptible model species, *Lupinus angustifolius*.

## 2. Materials and methods

### 2.1. Isolation of P. cinnamomi elicitins and characterization on Tris/Tricine gels

Elicitins were isolated from *Phytophthora cinnamomi* grown in M1 liquid medium [30]. The pathogen was grown on 10% CV8 agar medium for 5 days at 24 °C and then eight mycelial plugs were transferred into 250 mL conical flasks containing 150 mL liquid M1 medium. The conical flasks (total eight) were incubated in an orbital mixer incubator (Ratek) at 24 °C and 90 rpm for eight days and then the liquid was filtered through a non-sterile filter paper (185 mm, Toyo Roshi Kaisha Ltd.) into a funnel. The mycelia was then frozen in liquid nitrogen and frozen mycelia was used to extract cell wall associated protein. The filtered liquid medium was subjected to ammonium sulfate precipitation (70.7 g/100 mL solution) at 4 °C overnight. The solution was centrifuged at 3900 × *g* for 5 min and the resulting pellet was dissolved in distilled water sufficient for complete solubilisation of the pellet. The concentrated solution was dialyzed against 20 mM phosphate buffer (pH 7.2) overnight at 4 °C in a dialysis tube with a molecular weight cut off of 6–8 kDa (Spectra/PorR 1, Roth, Germany). The dialyzed solution was then centrifuged at 3900 × *g* for 5 min and the resulting supernatant was freeze-dried (Christ alpha 1–2, Martin Christ). Finally, the powder was dissolved in a small amount of distilled water sufficient for complete solubilisation and the total protein concentration was measured using the BCA method.

The dissolved proteins were analysed on a 10% Tris/Tricine gel [31] using a vertical electrophoresis cell (Mini-protein® tetra, BioRad) (supplementary method S1). The activity of elicitins present in M1 extracted proteins was checked in 40-day-old tobacco (*Nicotiana tabacum* L. cv. Samsun) [30]. Tobacco plants were grown in a growth cabinet maintained at 21 °C with a 16/8 h day: night photoperiod. One-half of a mature leaf was infiltrated with 100 µL of the protein solution (M1 extracted protein, 1 µg/mL) into the mesophyll tissue by injection with a syringe without a needle. The injected solution covered approximately one $cm^2$. A similar amount of water was injected into the other half of the same leaf as the control. Images were captured two days after infiltration.

### 2.2. Analysis of elicitins by isoelectric-focusing

The concentrated elicitin solution was further separated with isoelectric focusing (PROTEAN i12 IEF Cell, BIO-RAD, Australia) according to the protocol described by the manufacturer to determine how many elicitin isoforms were released into the culture medium. After the run, the sample-containing strips were dried with filter paper and analysed either using coomassie stain (Bio-Safe™) or a 2nd dimension (2D) protein analysis on Tris/Tricine gel.

### 2.3. Purification of β-cinnamomin

#### 2.3.1. Purification by cation and anion exchange column chromatography

Total proteins were extracted from the 4.5 l M1 medium in which *P. cinnamomi* was grown as described above. To purify β-cinnamomin, protein solution was concentrated first using a protein concentrator spin column (vivaspin 20, 3 kDa MWCO, GE Healthcare UK Limited) to remove proteins smaller than the elicitins (10 kDa). The retained protein solution was passed through a cation exchange column equilibrated with 20 mM Na-acetate buffer adjusted to pH 5.0 with a pH meter. All acidic proteins with an isoelectric point (pI) smaller than pH 5.0 that carried a negative charge did not adhere to the cation exchange column and were collected as the run through. However, all proteins with a pI higher than 5.0 were positively charged, including the basic β-cinnamomin, and adhered to the column. These positively charged proteins, mainly β-cinnamomin, were eluted from the column with 0.5 M NaCl dissolved in 5 mL 20 mM Na-acetate buffer pH 5.0 (Figure S1). Further, the acidic fraction (run through of the cation exchange column) was purified on an anion-exchange column (Bio-Scale Mini Macro-Prep High Q, Bio-Rad, Australia). To do so, the acidic fraction was first equilibrated in 20 mM Tris buffer pH 8.0 using PD-10 columns (GE Healthcare Life Sciences, UK) and then loaded on an anion exchange column which was equilibrated with 20 mM Tris-HCl buffer pH 8.0. The acidic proteins that adhered to the anion exchange column were eluted with 0.5 M NaCl dissolved in 20 mM Tris buffer pH 8.0 (Figure S2).

To obtain a higher amount of purified basic protein, the process described above, which used a cation exchange column, was repeated twice using fresh *P. cinnamomi* cultured M1 medium. The purified basic, acidic and total protein were analysed using a Tris/Tricine gel and isoelectric focusing as described above in section 2.1. The purity of purified basic β-cinnamomin was also confirmed using MALDI-MS analysis. In addition, the basic samples were further passed through vivaspin protein concentrator spin columns (MWCO 30 kDa, GE Healthcare) to remove possible contaminants with molecular mass higher than elicitins.

#### 2.3.2. Confirmation of the purity of β-cinnamomin using MALDI-MS

The molecular mass of intact β-cinnamomin was determined by MALDI-MS in positive linear reflection mode on a MALDI-TOF micro MX (Waters Co., Manchester, UK), equipped with a pulsed nitrogen laser (λ = 337 nm). Prior to the acquisition of spectra, 1 μL of sample protein solution (100 pmol/μL) was mixed with 1 μL of saturated α-cyano-4- 4-hydroxycinnamic acid matrix solution (10 mg/mL in acetonitrile/trifluoroacetic acid 0.1%, 1:1, v:v) and a droplet of the resulting mixture (1 μL) placed on the mass spectrometer's sample target. The droplet was dried at room temperature and the sample loaded into the mass spectrometer and analysed. The instrument was calibrated using a three-point external calibration using a mixture (10 pmol/mL) of insulin, cytochrome c and trypsinogen as standard proteins (Sigma, MO, USA) using a polynomial equation. All spectra were processed and analysed using MassLynx 4.0 software (Waters). The instrument source voltage was set to 12 kV. The pulse and detector voltages were optimized at 1999 V and 5200 V respectively. Measurements were performed with a suppression mass gate set to *m/z* 5000 and an extraction delay of 600 ns. Data were acquired by accumulating and averaging at least 10 spectra randomly acquired over the well surface.

### 2.4. Generation of the antiserum

An affinity purified polyclonal chicken IgY antiserum against β-cinnamomin (1 mg protein $mL^{-1}$) was produced commercially (Davids Biotechnologie, Regensburg, Germany) using 1.0 mg of β-cinnamomin for the chicken immunization and 0.6 mg β-cinnamomin for the affinity purification. β-cinnamomin expressed in *E. coli* (kindly donated by Hofzemahaus and Schallmey, RWTH Aachen University, Germany) that may have contained non-targeted proteins Z and the purified β-cinnamomin that may have contained non-targeted proteins X and Y which were covalently bound to the column along with cinnamomin protein. For the β-cinnamomin protein, a polyclonal antiserum was made containing ~80% of other immunoglobulins (i.e. IgGs). Then, the whole solution was passed through the purification column to concentrate β-cinnamomin antiserum and to remove other antiserum against non-targeted proteins (i.e. X and Y). So, β-cinnamomin antiserum (EA) was covalently bound with the column and contaminants along with additional IgG collected as a run through. Then the bound β-cinnamomin antiserum (highly concentrated and free from non-targeted proteins) was re-eluted from the column and collected separately (Figure S1). This highly purified (90–95%) β-cinnamomin antiserum was used for further experiments in this study.

### 2.5. Isolation of P. cinnamomi cell wall associated proteins

To identify the elicitin or elicitin-like proteins, *P. cinnamomi* mycelial cell wall protein extraction was performed as described by Ref. [32] with modifications. The detailed method is described in supplementary method S1.

### 2.6. Analysis of covalently bound cell wall proteins using high resolution nano LC–Tandem Mass Spectrometry

Chemical fragmentation with cyanogen bromide (CNBr) of cell wall proteins (300 μg) was performed in 70% formic acid as previously described [33]. The sample was dried under vacuum, washed three times with Milli Q $H_2O$ and dissolved in $NH_4HCO_3$. Following reduction with 10 mM dithiothreitol (DTT) (1 h at 56 °C) and alkylation with iodacetamide 7.5 mM (15 min at room temperature in the dark), enzymatic hydrolysis was performed on CNBr reduced and alkylated proteins by adding Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin with an enzyme/substrate (E/S) ratio of 1:50 (w/w). Mixtures were then incubated at 37 °C for 16 h and frozen for subsequent analyses. Peptide mixtures (2 μg) were then analysed by mass spectrometry (LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer, Thermo Fisher Scientific, Bremen, Germany). Peptide separation was performed on a C-18 reverse phase capillary column (75 μm × 10 cm, Thermo Fisher Scientific) at a flow rate of 300 mL/min, by using the following linear gradient of eluent A (0.2% formic acid in 2% acetonitrile) and eluent B (0.2% formic acid in 95% acetonitrile): t = 0 min, 5% solvent B; t = 10 min, 5% solvent B; t = 90 min, 50% solvent B; t = 100 min, 80% solvent B; t = 105 min, 100% solvent B; t = 115 min, 100% solvent B; t = 120 min; 5% solvent B. Peptide analysis was performed using data-dependent acquisition of one MS scan followed by CID and ETD fragmentations of the three most abundant ions. For the MS scans, the scan range was set to 400–1800 *m/z* at a resolution of 60000, and the automatic gain control (AGC) target was set to $1 \times 10^6$. For the MS/MS scans, the resolution was set to 15000, the AGC target was set to $1 \times 10^5$, the precursor isolation width was 2 Da, and the maximum injection time was set to 500 ms. The CID normalized collision energy was 35%; the charge-dependent ETD reaction time was enabled and the ETD AGC target was set to $1 \times 10^5$. Data were acquired by the Xcalibur™ software (Thermo Fisher Scientific). Proteins were identified by a licensed version of Mascot Software (Version 4.2.1). The Mascot search parameters were: NCBInr as database; trypsin and CNBr cleavages specific at one terminal end, allowed number of missed cleavage 3, carbamidomethyl C as fixed modifications, 10 ppm MS tolerance, 0.6 Da MS/MS tolerance, peptide charge from +2 to +3.

### 2.7. Western-blot analysis

To check the specificity of the newly generated anti-β-cinnamomin antibody, a western-blot was performed using an anti-chicken IgY-alkaline phosphatase antibody produced in rabbit (Sigma, Australia) as a secondary antibody. For this analysis, a Trans-Blot® Turbo ™ Blotting system (Bio-Rad) was used according to the manufacturer's instructions. The details of this method are described in supplementary method S1.

### 2.8. Plant growth, inoculation with zoospores and treatment with anti-β-cinnamomin antibody

Seeds of *Lupinus angustifolius* var. wonga (Naracoorte Seeds, South Australia), a species which is susceptible to *P. cinnamomi*, were sown in a soil-free plant growth system (SPS) and placed within temperature-controlled growth cabinets under 200 μmol $m^{-2}$ photosynthetically active radiation provided by sodium lights at 21 °C with a 16/8 h photoperiod [34]. Plants that were eight days old were used for inoculation. The inoculum was prepared as a suspension [35] and diluted to $10^4$ zoospores/mL with different concentrations of anti-β-cinnamomin antibody in sterilized distilled (sd) $H_2O$. Initially, several experiments were performed to optimize the experimental conditions for successful infection and vigorous root growth (data not shown). Finally, zoospores were added into the antibody solution 1 h before the inoculation at a zoospore density of $10^4$/mL and with the desired antibody concentration. To monitor any non-specific effects of antiserum on plants or the pathogen, a pre-immune normal serum without β-cinnamomin antibodies was used for inoculation. A range of concentrations of anti-β-cinnamomin were tested first (data not shown); finally three concentrations (1:100, 1:300 and 1:1000) were selected to test the effect of β-cinnamomin antiserum on infection and on the host plant.

The inoculation method is described fully in supplementary method S1.

### 2.9. Localisation of elicitins in roots of L. angustifolia inoculated with P. cinnamomi

Infected root samples were prepared for laser-scanning microscopy [16] from plants that were grown and inoculated as described above. The harvested roots at different hours post-inoculation (hpi) were first fixed in 3% formaldehyde prepared in PBS (pH 7.2) and then the root sections were incubated two times, firstly with 100 mM glycine in PBS/

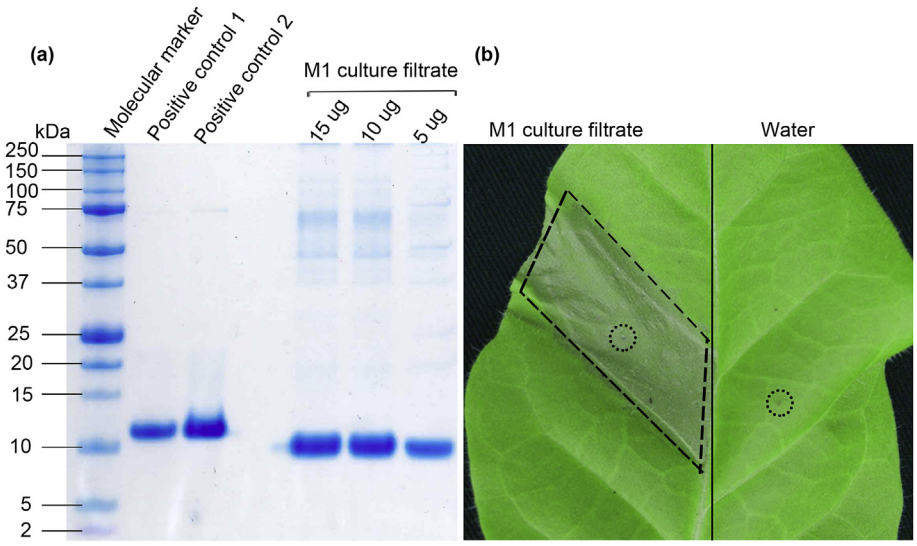



**Fig. 1.** Identification of elicitins by gel electrophoresis and infiltration into a tobacco leaf of *P. cinnamomin* M1 culture filtrate (a) Tris/Tricine gel electrophoresis of protein extracts from P. *cinnamomi* M1-liquid culture filtrate. β-cinnamomin protein expressed in *Escherichia coli* was used as a positive control and shows a band at 11 kDa due to the His-tag. Lanes with different amounts of M1 culture filtrate show a band corresponding to a 10 kDa elicitin protein (b) Necrosis on tobacco leaf infiltrated after 48 h with *P. cinnamomi* M1 culture filtrate (1μg/mL). Broken circle denotes the infiltration area and broken line delineates the necrosis area.

0.2% Tween and secondly with 1% BSA in PBS (pH 7.2) for 30 min to prevent nonspecific antibody binding in the tissue sample. Following this incubation, the first set of root sections harvested at each time point were further incubated for 2 h at 37 °C in a solution containing primary antiserum (1:100 diluted chicken anti-β-cinnamomin serum) and the second set of root sections were incubated with 1:400 diluted commercial rabbit anti-*Phytophthora* spp. polyclonal antiserum (Loewe Diagnostica, Sauerlach, Germany). Three washing steps were then performed for both sets separately, two times with 0.2% Tween in PBS and one time with PBS. The first set of washed root sections were incubated for 60 min at 37 °C with secondary antisera (goat anti-chicken conjugated to Alexa Fluor 633, Invitrogen, Australia) and the second set of root sections were incubated with goat anti-rabbit conjugated to Pacific Blue (Invitrogen, Australia) under similar conditions. The sections were washed again with 0.2% Tween in PBS and with PBS before transferring onto slides for confocal laser-scanning (Leica TCS SP5, Leica Microsystems CMS GmbH, Mannheim, Germany) microscopy. For Alexa Fluor 633 labelling, a 633 laser line (emission between 641 and 655) and for Pacific Blue labelling, a 405 laser line (emission between 430 and 480 nm) was used to capture the image.

### 2.10. Detection of elicitins in P. cinnamomi at different developmental stages

To analyse β-cinnamomin production in zoospore cysts during development of the germ tube and subsequent hyphal growth a preparation of zoospores at a density of $10^4$ zoospores/mL was used. The anti-β-cinnamomin antiserum at a ratio of 1:100 or 1:200 or water as a control was added onto zoospore drops placed on a piece of dialysis tube (one layer, 3 × 1.5 cm) on CV8 agar plates. Germ tube growth was examined first by light microscopy and the dialysis membrane was removed at zero, two, three and 16 h from the surface of the agar and placed in fixative consisting of 3% formaldehyde prepared in a 20 mM PBS pH 7.2 buffer solution. The germinated cysts that were attached to the membrane were then washed and a protein block was performed as described above. Subsequently, the membrane was incubated with both the primary β-cinnamomin antibody and the *Phytophthora* specific antibody for 2 h at 37 °C. After washing the material (two times with 0.2% Tween in PBS and one time with PBS), the membrane was incubated with two different secondary antisera for 2 h at 37 °C. Before examining the membrane with a confocal microscope as described above for localisation of elicitins in roots of *L. angustifolius* inoculated with *P. cinnamomi* the membranes were washed several times with 0.2% Tween in PBS and with PBS.

### 2.11. β-cinnamomin gene expression analysis

To analyse *in planta* expression of the β-cinnamomin gene, *L. angustifolius* plants were grown in the SPS system and inoculated as described in section 2.8. Twenty roots per replicate per time point were harvested at zero, three, six and 12 hpi. The harvested roots were immediately placed in liquid N and stored at −80 °C until used for gene expression analysis. RNA was extracted from harvested root tissue using an RNeasy Plant Mini kit (Qiagen) and relative purity was confirmed using a NanoDrop ND-1000 spectrophotometer. Then a tetro cDNA synthesis kit (Bioline) was used to synthesiz cDNA and quantitative real-time PCR was performed to analyse the induction of the β-cinnamomin gene in inoculated *L. angustifolius* roots. Actin was selected as the internal control and the primer sequence (Table S1) used for both the target and the internal control was previously reported [26]. Primer specificity and melting temperature were checked using conventional PCR. The quantitative real-time PCR was performed using Rotor-Gene SYBR green PCR master mix (Qiagen) on a Rotor-Gene 3000 (Qiagen) as per a previous description [36].

## 3. Results

### 3.1. Identification of P. cinnamomi elicitins and isoelectric-focusing of P. cinnamomi M1-extract proteins

The M1-culture filtrate extract of *P. cinnamomi* showed a clear band at 10 kDa on the gel confirming the presence of elicitins in the extract (Fig. 1a). Tobacco leaves developed necrosis after two days of infiltration with M1-extracts of *P. cinnanmomi* (Fig. 1b). The isoelectric focusing (IEF) analysis of β-cinnamomin protein expressed in *E. coli* (used as a control) resulted in one clear band at isoelectric-point (pI) 7.67 on the IEF strip and on the 2D-gel (Figure S3a-b). Moreover, the isolated M1-extracts showed four different bands on IEF strips at pI 7.75, 5.50, 4.80 and 4.00 (Figure S3c). In addition, six different bands appeared on the 2D-gel using the same sample (Figure S3d). However, the desalted M1-extract resulted in three clear bands on the IEF strip at pI 7.61, 4.45 and 3.16 or 3.19, although some faint banding was observed. The faint bands may be artifacts and due to overloading of the gel or smearing of the clear band (Figure S3e).

### 3.2. Purification of β-cinnamomin and specificity of the β-cinnamomin antiserum

The IEF analysis produced one clear band at approximately pI 7.61

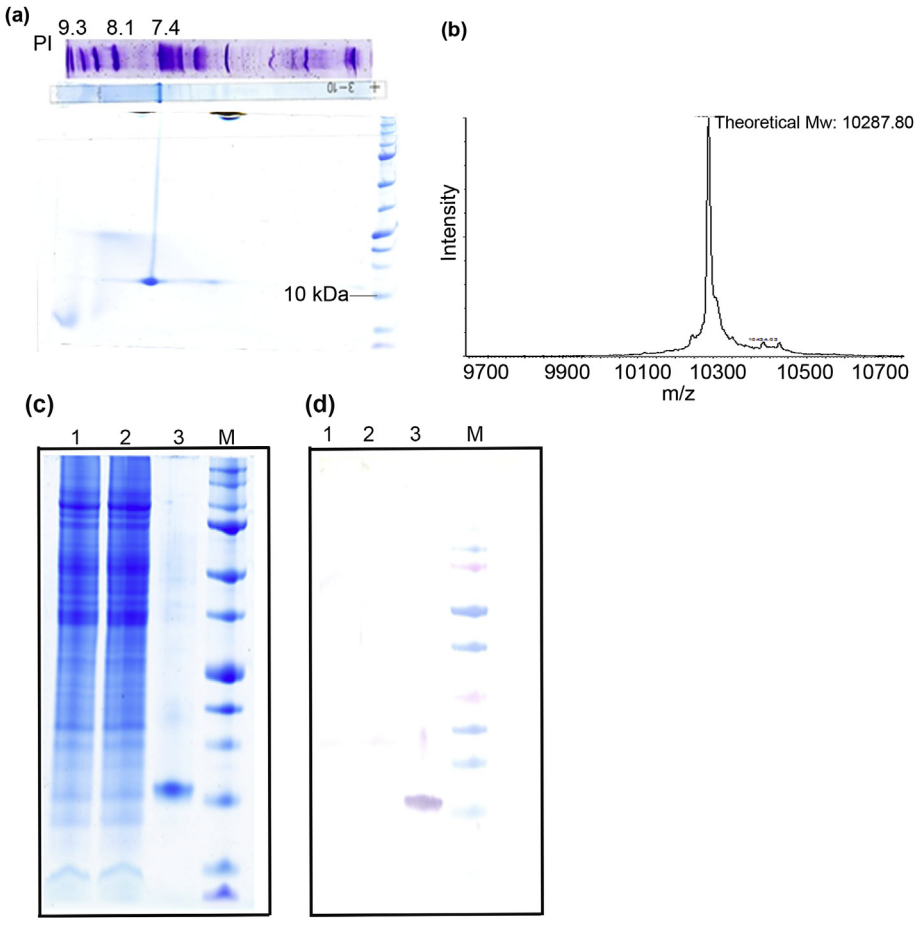



**Fig. 2.** Analysis of purified β-cinnamomin using isoelectric-focusing, MALDI-MS and western blot. (a) Isoelectric-focusing strip of purified basic fraction showed one band at approximately pI 7.61 and the corresponding 2D Tris-Tricine electrophoresis gel loaded with the focusing strip showed one band. (b) MALDI-MS chromatograph of purified β-cinnamomin protein. (c) Tris-Tricine electrophoresis gel of mycelial extracts and M1-liquid extract. The mycelial cell wall extract showed multiple bands and the M1 extract showed one clear elicitin band at 10 kDa. (d) Western blot of corresponding Tris-Tricine gel that showed anti-β-cinnamomin reacted only with the elicitin proteins. Lane 1 = 17.5 μg cell wall protein, Lane 2 = 26.25 μg cell wall protein, Lane 3 = 4.37 μg M1 extract protein and M = Molecular marker.

on the IEF strip and on the 2D-gel (Fig. 2a). The MALDI-MS analysis-using purified protein from several 2D gels produced one peak representing a theoretical molecular weight of 10.287 kDa. This result confirmed that the sample contained only the β-cinnamomin protein (Fig. 2b). This sample along with β-cinnamomin expressed in *E. coli* was used to develop an anti-β-cinnamomin antibody.

The mycelial extract (used as a control) and the M1 extract showed a band corresponding to 10 kDa on gel-electrophoresis (Fig. 2c), however, only the protein with a molecular weight of ~10 kDa was specifically recognised by the β-cinnamomin antiserum. There was no cross reaction with any proteins associated with the cell wall extract (Fig. 2d). Therefore, this experiment showed that the β-cinnamomin antiserum that was purified on a β-cinnamomin affinity column was highly specific for the β-cinnamomin protein (Figure S2).

### 3.3. Identification of *P. cinnamomi* cell wall bound proteins

To examine the association of β-cinnamomin as a covalently bound protein with the *P. cinnamomi* cell wall, isolated cell walls from mycelia were analysed by high-resolution nano LC-Tandem Mass Spectrometry (nano LC-MS/MS). The resulting data were searched for homology using Mascot software against NCBI databases and several known proteins were identified, such as a translation elongation factor 1 alpha, several glycophosphatidylinositol (GPI)-anchored proteins, a glucan 1,3-beta glucosidase, a pyrophosphate-energized vacuolar membrane proton pump and β-cinnamomin (Table 1).

### 3.4. β-cinnamomin production associated with *P. cinnamomi* germ tubes and mycelia

At first, different control experiments were performed by incubating root sections with individual primary (β-cinnamomin-specific or *Phytophthora*-specific) or secondary (Alexa fluor-labelled or Pacific blue-labelled) antisera to check for the presence of non-specific binding, no non-specific binding was found (data not shown). The final experiments were performed using both primary and secondary antisera. *P. cinnamomi* germ tubes showed both blue and red labelling 2 h after cyst germination that indicated the production of β-cinnamomin at this early stage (Fig. 3a). A similar result was also found for *P. cinnamomi* germ tubes 3 h after cyst germination (Fig. 3b) and for those germ tubes that had grown overnight (Fig. 3c).

### 3.5. β-cinnamomin production of *L. angustifolius* roots inoculated with *P. cinnamomi*

β-cinnamomin production was observed from 24 h post inoculation (hpi) throughout the root tissue but was most prominent in the epidermis, cortex and vascular region of *L. angustifolius* roots (Fig. 4a and b and 4e-f). The amount of β-cinnamomin production inside the root tissue increased over time and throughout the root tissue (Fig. 4e–f and Figure S4a-b and e-f). At 24 hpi, the pathogen colonised the endodermis, cortex and vascular region and expanded colonization into these areas over time (Fig. 4c and d, 4 g-h, S4c-d and g-h). No labelling was observed in control tissues that were treated with water alone (Fig. 4i-l). Some of the blue colouration in the central cylinder of the root sections may also have been due to autofluoresence of the highly lignified cell walls of the xylem as was found in control root sections (Fig. 4k-l). The high intensity of red labelling present in the longitudinal sections of inoculated *L. angustifolius* roots indicated a high amount of β-cinnamomin production throughout the different regions of the root (Fig. 5a and b) in conjunction with the presence of the pathogen (Fig. 5c and d).

**Table 1**
Proteins identified as the cell wall-associated proteins from *P. cinnamomi* by high resolution nano LC-MS/MS.

| NCBI Id | Protein description | Peptides | Mascot Score | Species |
|---|---|---|---|---|
| Gi\|6688977 | Translation Elongation Factor 1 Alpha | R.QTVAVGVIK.S | 20 | *P. infestans* |
| | | K.IGGIGTVPVGR.V | 77 | |
| | | M.VATFGPVGLSTEVK.S | 67 | |
| | | R.VETGVIKPGMVATFGPVGLSTEVK.S | 44 | |
| Gi\|262095878 | Predicted GPI-anchored protein | M.KVVNPSGK.I | 40 | *P. infestans T30-4* |
| | | K.VIAIAALPSGGSR.I | 50 | |
| | | R.IAFQSGDNVIVR.E | 85 | |
| Gi\|301104268 | Putative GPI-anchored serine-rich hypothetical protein | M.TAVTSVQAR.V | 32 | *P. sojae* |
| | | M.YVQAEGINVNEQSVK.C | 132 | |
| Gi\|164510747 | Glucan 1,3-Beta-D-Glucosidase | R.AGPDWATADVK.C | 73 | *P. cinnamomi* |
| | | R.FSALVETASTYSK.E | 85 | |
| Gi\|301114941 | Pyrophosphate-Energized Vacuolar Membrane Proton Pump | M.AELPAEVR.D | 42 | *P. infestans T30-4* |
| | | K.SVGVAAMEMVK.E | 23 | |
| | | K.VVENIPEDDPR.N | 16 | |
| | | M.AIISLVFGDFFK.S | 71 | |
| Gi\|301090286 | Triosephosphate Isomerase/Glyceraldehyde-3-Phosphate Dehydrogenase | R.AAIENPKTK.V | 18 | *P. infestans T30-4* |
| | | M.PEQVAIGINGFGR.I | 49 | |
| | | R.GCGQNIIPSSTGAAK.A | 31 | |
| Gi\|301123733 | 5-Methlytetrahydropteroyltriglutamate-Homocysteine Methyltransferase | M.VAVDSATLGFPR.M | 96 | *P. infestans T30-4* |
| Gi\|119287 | β-cinnamomin | -.TACTATQQTAAYK.T | 73 | *P. cinnamomi* |
| Gi\|301107324 | Conserved Hypothetical Protein | R.TTGANGSVITK.T | 42 | *P. infestans T30-4* |
| | | K.IEVQTTTVTK.T | 26 | |

Score indicates the overall quality of the alignment, the higher the score the higher the similarity.

### 3.6. Effect of incubation in β-cinnamomin antiserum of zoospores of P. cinnamomi on lesion formation in L. angustifolius roots

Control roots that were treated with either water alone or the β-the cinnamomin antiserum (1:100) alone did not show any treatment-related effect (Fig. 6a and b). Roots of L. *angustifolius* seedlings that were inoculated with *P. cinnamomi* zoospores produced expanding lesions at 72 hpi (Fig. 6c) whereas when zoospores were pre-incubated with β-cinnamomin antiserum (1:100 dilution) a reduced lesion size was found at 72 hpi (Fig. 6d). However, incubation of roots with zoospores that had been incubated with β-cinnamomin antiserum diluted at either 1:300 or 1:1000 resulted in lesions that were similar in size to those produced by zoospores alone up until 72hpi (Fig. 6e and f respectively). Moreover, zoospores incubated with pre-immune serum (1:100 dilution) (Fig. 6g) produced lesions of similar size to those produced by inoculation with zoospores alone.

The lesion length on infected roots was measured for all treatments from 24 hpi to 120 hpi and in those treatments where zoospores were incubated with β-cinnamomin antiserum (1:100 dilution) significantly smaller lesions were produced from 48 hpi to 120 hpi compared with those produced when roots were infected with zoospores without antiserum. Incubation with the other two dilutions of β-cinnamomin antiserum with zoospores produced similar sized lesions from 24 hpi to 72 hpi but at 120 hpi lesion size was significantly less than those produced when roots were inoculated with zoospores alone. In addition, at each time point zoospores incubated with pre-immune serum produced lesions that were not statistically different in size from those found for zoospore inoculation alone (Fig. 6h). This finding of similar lesion sizes on those roots infected by zoospores that were either not pre-incubated or were pre-incubated with β-cinnamomin demonstrated that the immune serum by itself had no effect either on zoospore infection or on plant root health.

### 3.7. Induction of β-cinnamomin gene expression during early infection stages in L. angustifolius

The primer pairs selected for gene expression produced specific products of the expected size as revealed by a conventional PCR assay (Data not shown). The expression level of the *β-cinnamomin* gene at 3 and 6 hpi was not statistically different to that 0 hpi. However, significantly increased expression was found at 12 hpi (Fig. 7).

## 4. Discussion

The current study investigated the role of β-cinnamomin in virulence through immunodepletion experiments and explored cell wall associated proteins of *P. cinnamomi.* One of the major outcomes from this elicitin work was the separation of elicitin proteins according to their calculated isoelectric point (pI) using an isoelectric focusing system Therefore, this method can be applied to other *Phytophthora* species to explore the nature of elicitins according to the calculated pI. One of the challenges when working with elicitins is their separation from other proteins due to their molecular weight of less than 30 kDa. Widely used SDS-PAGE does not have the resolving power required and, therefore, Tricine-SDS-PAGE, based on a Tricine-Tris buffer system, is the most popular electrophoretic SDS technique for separating smaller proteins, especially those in the range 1–20 kDa. Using this technique, a superior resolution of proteins was achieved in the current study without the necessity of urea and glycine as noted in other studies [31,37]. In the current study, a prominent protein band of 10 kDa in size was derived from a *P. cinnamomi* culture filtrate that indicated the secretion of elicitins into the liquid medium. Further, infiltration of the culture filtrate into tobacco leaves generated necrosis, providing preliminary evidence for the presence of an elicitin. Similar symptoms were also described for tobacco leaves infiltrated with other *Phytophthora* purified elicitins such as cryptogein, parasiticein and capsicein [38].

The elicitin proteins are classified into two groups on the basis of their respective isoelectric point (pI) and they are termed acidic (pI $<$ 5) or basic (pI $>$ 5). To date, more than 30 *Phytophthora* species have been recorded to secrete elicitins which each differ in their pI [30,39]. The *P. cinnamomi* genome has four elicitin genes expressing four proteins that are differentiated based on their pI: β-cinnamomin (pI 7.61), α-cinnamomin (pI 4.45), cinnamomin ha1 (pI 3.19) and cinnamomin ha2 (pI 3.16) [26,39]. Also recently, an RNA-seq study revealed one elicitin-like gene in *P. cinnamomi* [29]. However, the isoelectric focusing investigation of the *P. cinnamomi* culture filtrate in this work revealed three prominent protein bands, indicating the presence of β-cinnamomin, α-cinnamomin and, due to their very close pIs, combined cinnamomin ha1 and cinnamomin ha2. To our knowledge, this is the first report of the successful separation of *P. cinnamomi* elicitin proteins by isoelectric focusing. In addition, purification of β-cinnamomin was achieved and its purity confirmed using isoelectric focusing and

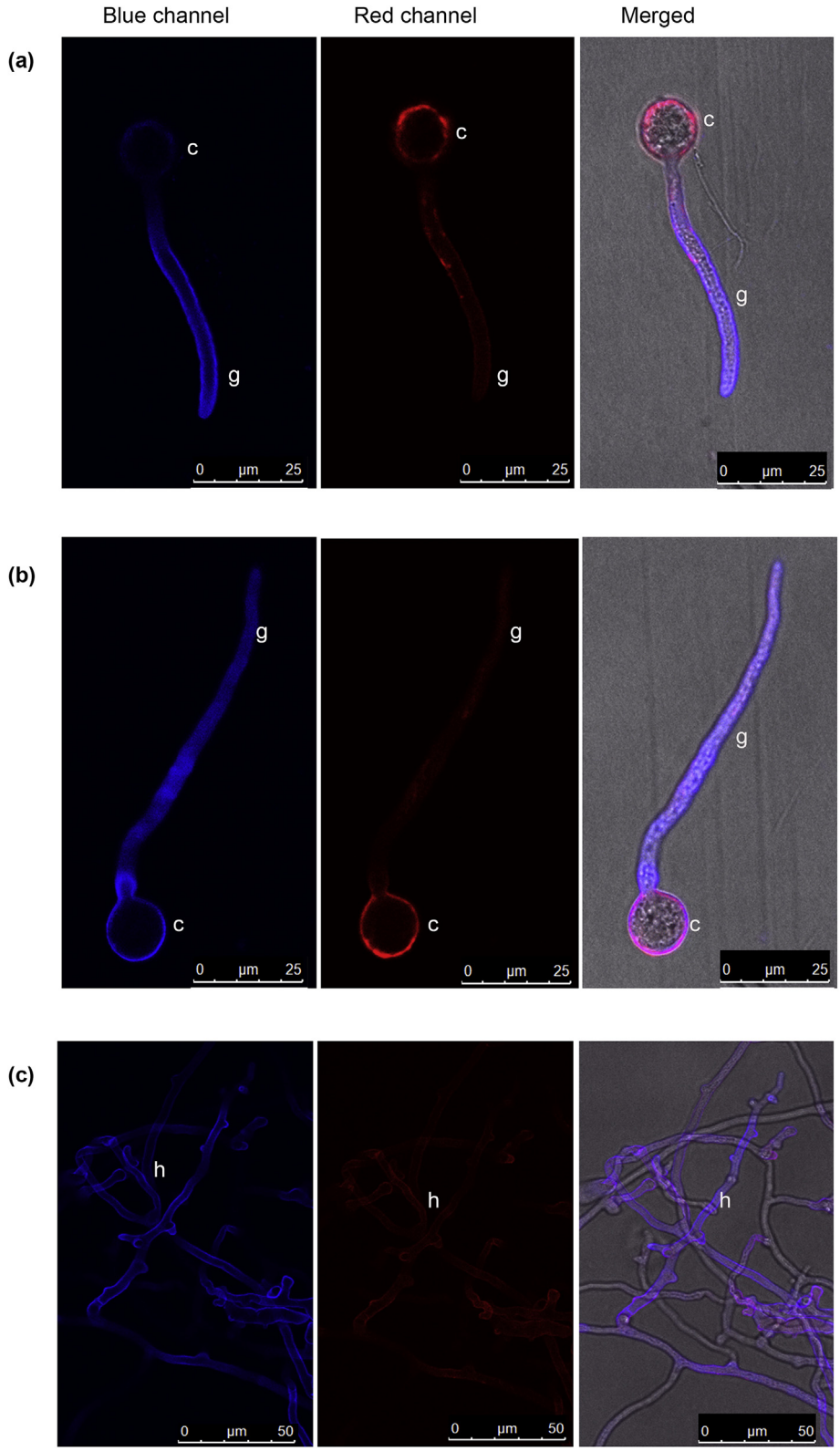



**Fig. 3.** Confocal laser scanning microscope images of β-cinnamomin production by *P. cinnamomi* grown on V8 agar plates. The blue labelling indicates the germ tube or hyphae of the pathogen and the red labelling indicates β-cinnamomin. (a) β-cinnamomin production after two hrs of germ tube growth. Scale = 25 µm. (b) β-cinnamomin production after three hrs of germ tube growth. Scale = 25 µm. (c) β-cinnamomin production in hyphae at 16h after cyst germination c, cyst; g, germ tube; h, hyphae. Scale = 50 µm.

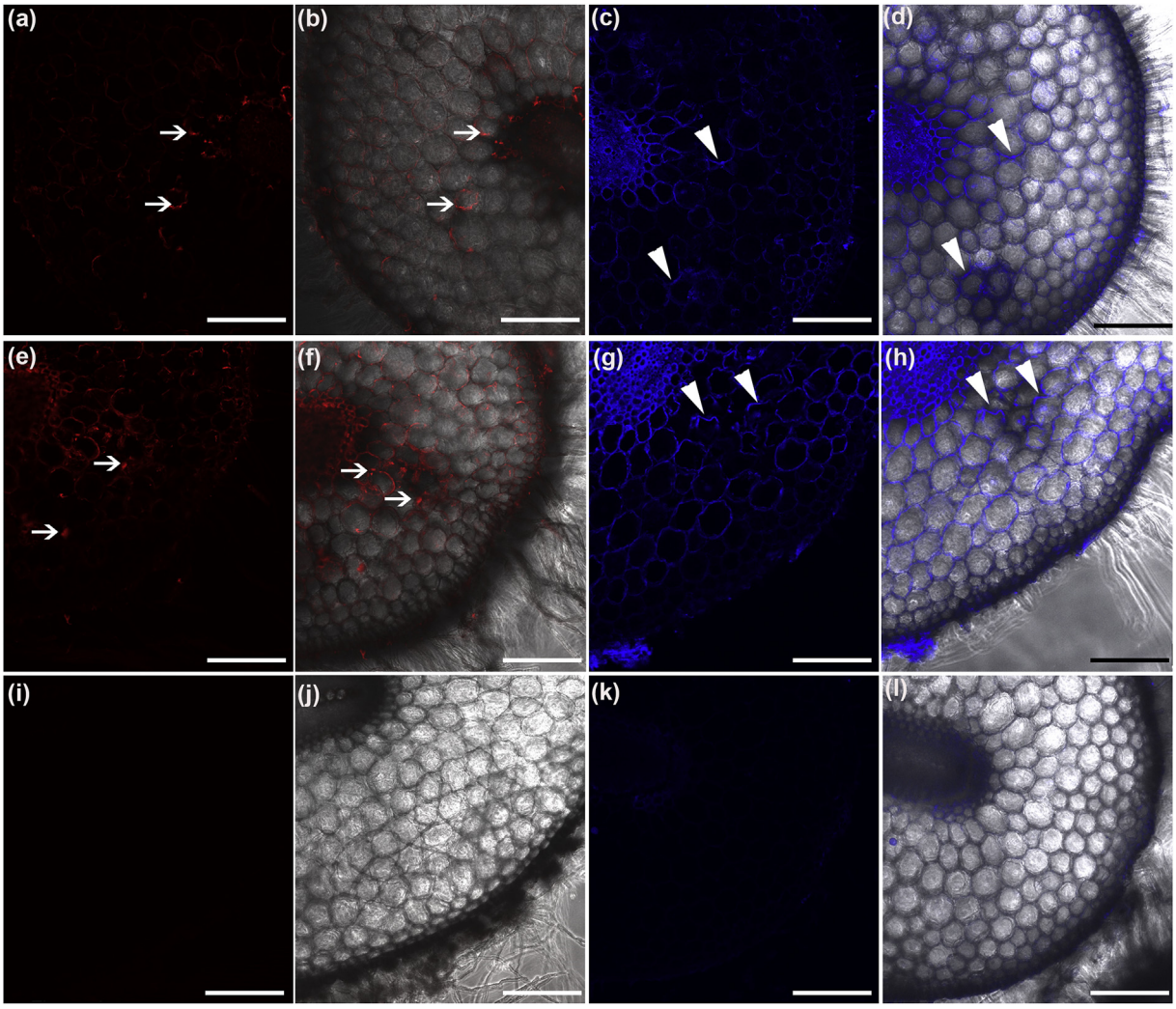



**Fig. 4.** Confocal laser scanning microscope images of *L. angustifolius* roots infected with *P. cinnamomi*. The inoculated roots were sectioned transversely by hand and labelled separately with anti-β-cinnamomin and anti-Phytophthora antibodies. β-cinnamomin shows red-immunolabelling and *P. cinnamomi* shows blue-immunolabelling. (a-b) β-cinnamomin production (arrows) at 24 hpi. (e-f) β-cinnamomin production at 48 hpi. (c-d) and (g-h) *P. cinnamomin* presence throughout the root tissue (arrowheads). (i-l) Mock inoculated control root sections showed no non-specific binding for either of the antibodies. The faint blue colour (k-l) is autofluorescence. Scale bars = 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

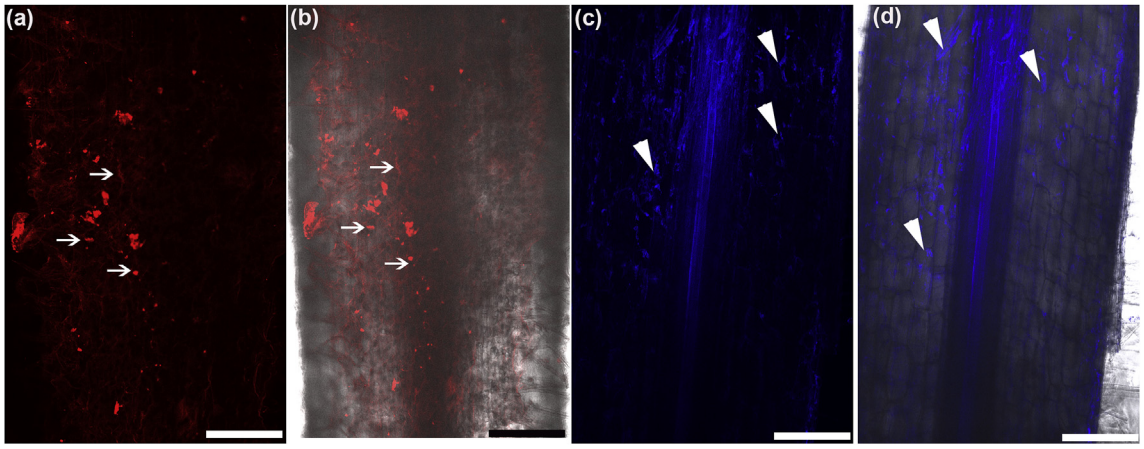
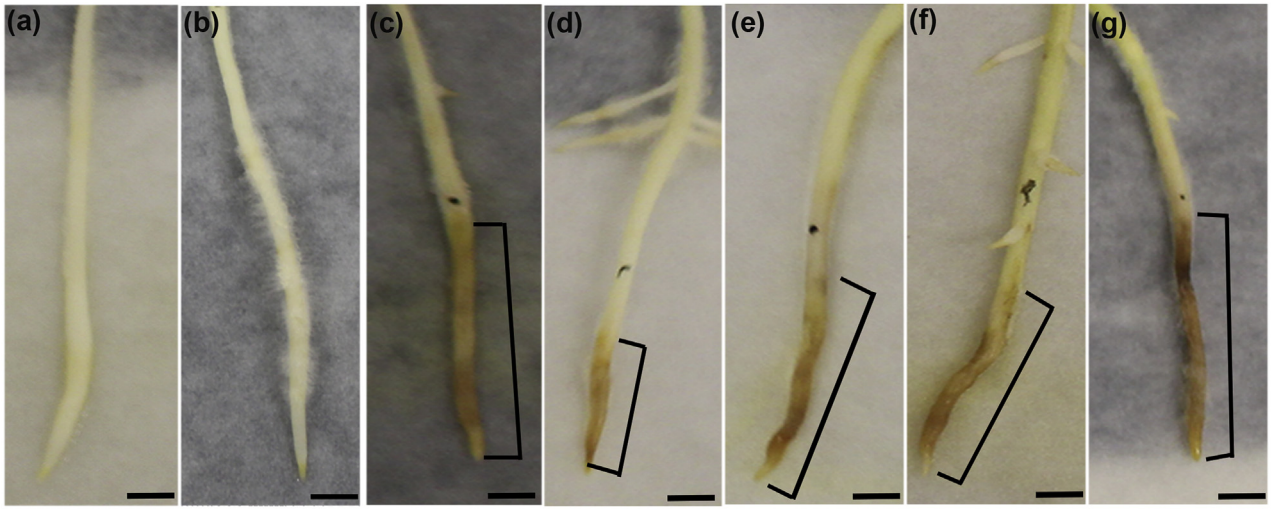



**Fig. 5.** Confocal laser scanning microscopic images of longitudinal sections of *L. angustifolius* roots 72 hpi with *P. cinnamomi*. Root sections were labelled with β-cinnamomin antiserum and Phytophthora-specific antisera. (a-b) Red labelling (arrows) indicates the distribution of β-cinnamomin produced by *P. cinnamomi*. (c-d) Blue labelling (arrowheads) indicates the presence of *P. cinnamomi* hyphae in infected roots. Scale bars = 1mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



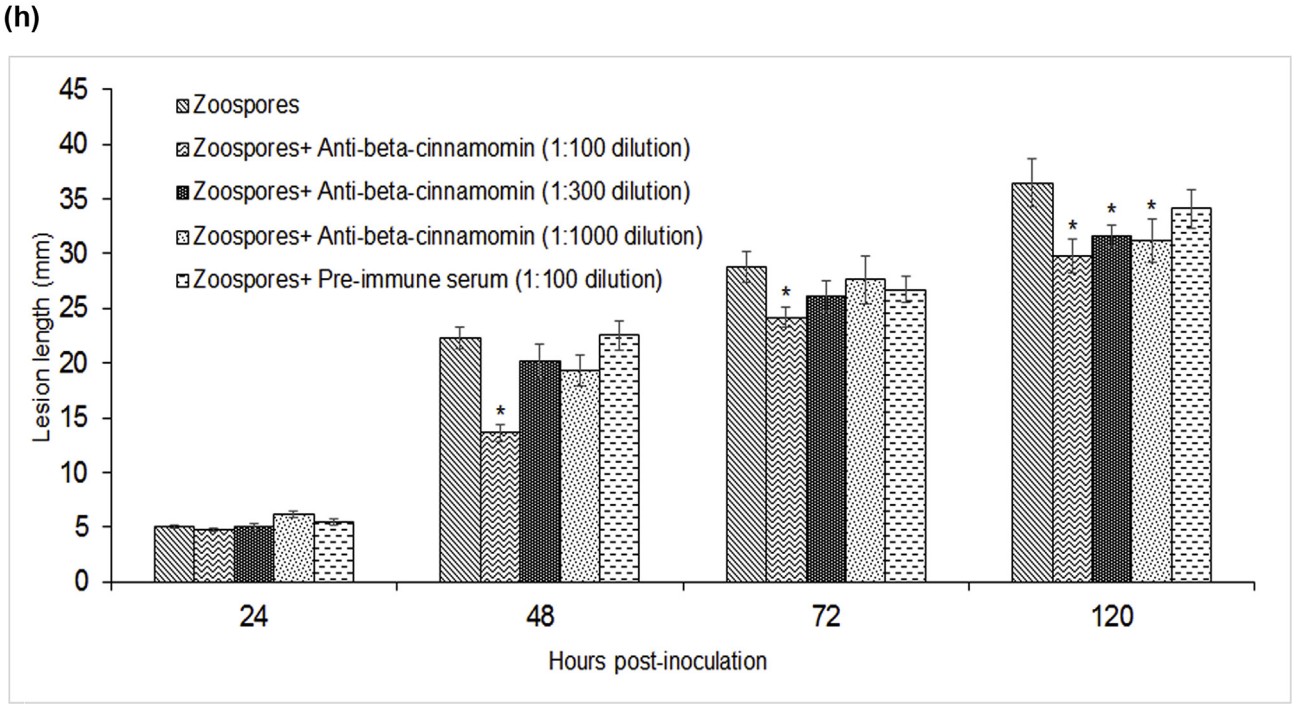




**Fig. 6.** Effect of inhibition of β-cinnamomin on the susceptible interaction between *L. angustifolius* and *P. cinnamomi.* (a) dH2O inoculated control. (b) anti- β-cinnamomin treated control (c) Lesion development in roots inoculated with *P. cinnamomi* zoospores. (d) Lesion development in *L. angustifolius* roots inoculated with zoospores and β-cinnamomin antiserum diluted 1:100, (e) 1:300, (f) 1:1000. (g) Lesion development in roots inoculated with zoospores in pre-immune serum diluted 1:100. Lesions (c-g) are denoted by brackets. Scale bars = 1cm. (h) Lesion length on roots at various times after inoculation either by zoospores pre-incubated with different concentrations of anti-β-cinnamomin antibody or by zoospores alone or mixed with pre-immune serum. Data are the mean of three independent experiments and bars represent one s.e.m. *, denotes significant difference between control (zoospores alone) and other treatments at $P < 0.05$ according to Duncan's multiple range test.

MALDI-MS analysis. Following purification, a new affinity purified β-cinnamomin antiserum was raised and its specificity confirmed for use in a novel immunodepletion assay and *in planta* studies.

In addition to isolation of the elicitins, *P. cinnamomi* cell wall proteins, either covalently or non-covalently bound to cell wall fractions, were also examined. Proteomic studies on oomycetes have previously been performed to compare the proteomes of *P. infestans* and *P. ramorum* [40] and, more specifically, for *P. infestans*, the differential regulation of proteins at different growth stages [41]. The cell wall proteome has also been described previously for *P. ramorum* [32] and *P. infestans* [42] following LC-MS/MS analysis. The present work reports for the first time on the cell wall proteome for *P. cinnamomi* using high resolution nano LC-MS/MS analysis.

Glycosylphosphatidylinositol (GPI) anchored proteins are the most abundant covalently bound proteins in fungal cell walls [43]. However, only a few members of this group of proteins have been identified in *Phytophthora* and only for *P. infestans* and *P. ramorum* mycelial cell wall fractions. This difference highlights that pathogenic oomycetes may share similar ecological niches or growth patterns, but that there are some fundamental differences in biochemistry and physiology of these evolutionarily diverse groups [42]. Two predicted GPI-anchored proteins were identified in the current study from a *P. cinnamomi* mycelial cell wall fraction as covalently bound proteins. The function of these proteins is still not known and was beyond the scope of the current study but future research should target transcript accumulation at different *P. cinnamomi* life cycle stages and exploration of the potential roles of each identified protein.

In the current study, a pyrophosphate-energized vacuolar

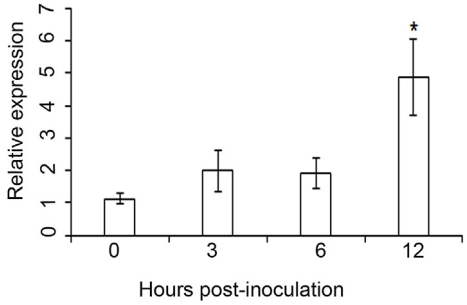



**Fig. 7.** Relative quantification of β-cinnamomin gene expression *in planta* by quantitative real time PCR. RNA was extracted from *L. angustifolius* roots inoculated with *P. cinnamomi* at 0, 3, 6 and 12 hpi. Mean values obtained over two independent experiments each comprised of three replicates and bars represent 1 s.e.m.

membrane proton pump from the *P. cinnamomi* mycelial cell wall was identified. This proton pump has the ability to reduce the extracellular pH and, thus, activate enzymes such as expansins, endoglucanases and polygalacturonases which are involved in cell wall expansion [44,45]. It is therefore likely, that the identified proton pump is involved in cell wall expansion in *P. cinnamomi*. Glucan 1,3-β -D-glucosidases have different roles in plants compared with oomycetes. In plants, 1,3-β -D-glucosidase has been characterized as being mainly involved in plant defence as well as in germination and embryogenesis. In contrast in oomycetes, 1,3-β- D-glucosidase was hypothesized to be involved in the balance between cell wall hydrolysis and synthesis [46,47]. Future work could examine whether the glucan, 1,3-β -D-glucosidase, of *P. cinnamomi* found here, has a similar role in other oomycete pathogens.

Also found in the cell wall fractions of *P. cinnamomi* were Triose phosphate isomerase (TPI) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which catalyse different steps in carbohydrate metabolism. In *P. infestans*, these two enzymes fuse together and form a single transcriptional unit, *tigA,* which is unique in eukaryotes [48]. Moreover, 5-Methlytetrahydropteroyltriglutamate-homocysteine methyltransferase is an adaptive response protein of *P. capsici* when treated with several fungicides. This enzyme is involved in methionine biosynthesis, cysteine biosynthesis and as a eukaryotic translation initiation factor, all of which play important roles in protein synthesis [49–51]. However, in this study, we identified TPI/GAPDH and 5-Methlytetrahydropteroyltriglutamate-homocysteine methyltransferase proteins from the mycelial cell wall of *P. cinnamomi*. More importantly, in the context of this study, β-cinnamomin was found as a covalently bound cell wall protein in the data set. This result indicates that β-cinnamomin is not only secreted into the liquid medium but is also covalently bound to the *P. cinnamomi* mycelial cell wall.

Elicitins have long been considered resistance inducing PAMP molecules or avirulence factors in many *Phytophthora* species. PAMPs are recognised by cell surface localized pattern recognition receptors (PRRs) which can trigger an immune response [10,52]. These proteins induce HR-based local and systemic resistance reactions in plants of the Solanaceae and Cruciferae families. Tobacco plants treated with elicitins showed more resistance to subsequent infection by *P. nicotianae* and *Raphanus sativus* was more resistant to *Xanthomonas campestris* pv. *armoraciae* [53–55]. There are many isolates of *P. nicotianae* and other *Phytophthora* species that secrete elicitins and which are non-pathogenic to tobacco [19,53]. The INF1 elicitin secreted by *P. infestans* was an inducer of resistance in several *Nicotiana* species and a silenced mutant, *inf1,* generated disease lesions on *N. benthamiana* [20]. Collectively, these experiments indicated that *Phytophthora* pathogens produce elicitins to act as avirulence factors in their interaction with plants. However, these findings were only related to the *Phytophthora*-tobacco interactions.

Pathogens secrete proteins not only to induce plant resistance, but they are known to have intrinsic biological function in pathogen virulence during the infection process. Recently, via immunodepletion experiments, α-plurivorin has been described as a virulence factor in *P. plurivora* [16]. The application of α-plurivorin antiserum during inoculation resulted in a complete inhibition of penetration by *P. plurivora* into *F. sylvatica* root tissue. Evidence was provided to show that the acidic elicitin of *P. plurivora* suppressed several defense related genes in *F. sylvatica* roots and therefore it was described as an inducer of effector triggered susceptibility (ETS). In the current study, a similar approach was used to identify the action of the basic elicitin of *P. cinnamomi* in the highly susceptible interaction with *L. angustifolius*.

Zoospores of *P. cinnamomi* encysted on *L. angustifolius* roots 1 h after inoculation and then extended a germ tube. After 3 h, they had penetrated the epidermal cell [56]. β-cinnamomin labeling with an affinity purified antibody of germinating cyst and hyphae both *in-vitro* and *in-vivo* showed the presence of β-cinnamomin. The immune labeling study coupled with nano LC-MS/MS analysis provides strong evidence for the presence of both wall bound and secreted elicitins during the pre- and post-penetration stages of infection. Moreover, β-cinnamomin was produced throughout the area of root tissue invaded by the pathogen from 24 hpi and thus β-cinnamomin released by the pathogen during infection is involved in the early stages of root infection. Our findings concur with those of [16] where α-plurivorin, the only elicitin of *P. plurivora,* was labelled with a specific antiserum and highlighted in fine roots of *F. sylvatica* infected by *P. plurivora*.

Further, to investigate the role of β-cinnamomin in the *P. cinnamomi* infection process an immunosuppression experiment was performed using a β-cinnamomin antiserum. Plants exposed to zoospores that had been treated with antibody showed reduced root lesions compared to plants exposed to zoospores not treated with antibody. All roots inoculated with zoospores that had been incubated with pre-immune serum did not have a significantly reduced lesion length compared to those roots that have been exposed to zoospores in water alone. These experiments showed that treatment of zoospores with nonspecific immunoglobulin proteins did not interfere with the ability of the pathogen to encyst and penetrate an epidermal cell. Moreover, observations of hyphal growth on standard medium showed that the β-cinnamomin antiserum did not have any measurable effect on pathogen growth in culture (Figure S5a-d). These experiments confirm a partial loss of virulence of *P. cinnamomi* in the presence of β-cinnamomin antiserum which suggests specific suppression of the activity of β-cinnamomin by the antibody.

A previous study [27] showed that *β-cinnamomin*-silenced *P. cinnamomi* strains were less aggressive than wild type strains in colonising *Q. suber* root tissue and caused milder disease symptoms. This study did not exclude the possibility of collateral disruption in the pathogen genome by the transformation process that may have interfered with the silenced mutant's aggressiveness. Therefore, the results obtained in the current study provide additional and convincing evidence for a role of β-cinnamomin in the infection process. This result was also supported by an early study of the elicitin-coding sequences, which are present in two *P. nicotianae* isolates (P3461 and P1995) that did not produce elicitins. Both strains showed impaired ability to cause infection in the tobacco and tomato hosts [54]. This inability to cause infection indicates the importance of elicitins in the infection process. In contrast, an *inf1* silenced mutant of *P. infestans* was virulent on potato [20] showing the function of elicitins in different *Phytophthora* species may vary.

Elicitins are highly conserved proteins, secreted by almost all *Phytophthora* species tested [57]. Therefore, it is important to investigate their role as virulence factors in each *Phytophthora*-susceptible plant interaction. Importantly, the results of our immunosuppression studies have demonstrated the critical role in virulence that β-cinnamomin has even though the *P. cinnamomi* genome encodes a number of effectors and other pathogenicity factors.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2019.04.003.

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