Identification of pathogenicity genes in *Phytophthora cinnamomi*

By

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Declaration

I, Anandi Reitmann, hereby declare that this dissertation, submitted to the University of Pretoria for the degree MSc Genetics, contains my own work, and that the content contained within this thesis has not been submitted to any other university or institution.

Anandi Reitmann
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PREFACE

Phytophthora cinnamomi Rands is a pathogenic oomycete that causes devastation in many economically important crop- and forest industries. It is the causal agent of both root rot and stem cankers depending on the specific host interaction. As a soil born pathogen it is impossible to eradicate from invested areas and the application of phosphate is the only control strategy that proves helpful against *Phytophthora* disease. Farmers are experiencing increased pressure to decrease the application of pesticides and to move to organic farming practises. In the light of this, molecular knowledge of plant pathogen interactions will become increasingly valuable in the development of more durable control strategies. To enable successful manipulation of a plant-pathogen interaction, a deep understanding of both genes involved in pathogen infection and the host defence system is required.

Chapter 1 of this thesis is entitled 'Biology of the oomycete pathogen *Phytophthora cinnamomi* Rands'. This chapter provides background on the life cycle of *P. cinnamomi* and describes the host range, disease development as well as current control strategies used to combat this pathogen. A typical interaction between a biotrophic pathogen and its host is described to illustrate the evolutionary arms race between pathogen infection molecules and host defence receptors. Lastly, specific groups of genes that play critical roles during the infection process are discussed in more detail.

Chapter 2 reports on the identification of pathogenicity related genes from the first RNA-seq library of *P. cinnamomi*. An RNA-seq library was produced from cysts and germinating cysts of a high sporulating isolate of *P. cinnamomi* using the Illumina sequencing platform. Paired end reads were *de novo* assembled and unigenes annotated to the protein sequences in the NCBI non-redundant protein database, the Swiss protein database and the protein models in the genomes of *Phytophthora infestans* and *P. cinnamomi*. The dataset was searched for proteins previously implicated in pathogenicity in the literature and for the presence of conserved motifs defining the secretome and RXLR-motif containing proteins in the dataset. Each pathogenicity gene group and their role during infection are discussed in the light of current literature.

Chapter 3 evaluated the expression patterns of seven pathogenicity genes across the *in vitro* developmental phases of *P. cinnamomi*. The expression pattern of each gene across the mycelial, sporulating mycelial, zoospores and cyst and germinating cyst stages is discussed, with potential functional implications it has with regard to its role during infection.

Chapter 4 comments on the success of this project and includes recommendations and aspects that may be interesting for future investigation.

LIST OF ABBREVIATIONS AND SYMBOLS:

ATP	Adenine triphosphate
α	Alpha
aa	Amino acid
RXLR	Amino acid sequence Arginine-any amino acid-Leucine-Arginine
LFLAK	Amino acid sequence Leucine-Phenylalanine-Leucine-Alanine-Lysine
N-terminal	Amino terminal
ANOVA	Analysis of variance
Ta	Annealing temperature
ABC transporter	ATP-binding cassette transporter
avr	Avirulence
bp	Base pairs
BGI	Beijing Genomics Institute
B	Beta
$CaCO_3$	Calcium carbonate
CB	Carbohydrate binding
C-terminal	Carboxyl terminal
CWDE	Cell wall degrading enzyme
CBEL	Cellulose binding elicitor lectin
cm	Centimeter
COG	Clusters of orthologous groups
CDS	Coding DNA sequence
R^2	Coefficient of determination
cDNA	Complementary DNA
C	Contig
CMA	Cornmeal agar
CRN	Crinkling and necrosis-inducing
Ct	Cycle threshold
°C	Degree Celcius
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ETS	Effector triggered susceptibility
ETI	Effector-triggered immunity
ELI	Elicitin
ELL	Elicitin-like
et al.	Et alia meaning 'and others'
etc.	Et cetera meaning 'and so forth'
e.g.	Example
E-value	Expect value
EST	Expressed sequence tags
CBM1	Family 1 carbohydrate binding molecules
FU	Fluorescent units
GO	Gene ontology
M-Value	Gene stability value

GMO	Genetically modified organisms
G	Gigabyte
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
g	Gram
G-proteins	Guanosine nucleotide-binding proteins
CTAB	Hexadecyltrimethylammonium bromide
HMM	Hidden Markov Models
hpi	Hours post inoculation
HR	Hypersensitive response
ID	Identity
ITS	Internal transcribed spacer
JGI	Joint Genome Institute
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPV3	Large peripheral vesicle 3 gene
<	Less than
L	Litre
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
M	Methione
miRNA	Micro RNA
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
mg	Milligram
ml	Millilitre
mm	Millimeter
mM	Millimolar
min	Minute
MAP kinase	mitogen-activated protein kinase
mRFP	Monomeric red fluorescent protein
>	More than
ng	Nanogram
NCBI	National Centre for Biotechnology Information
Nr	NCBI non-redundant protein database
NPP1	Necrosis-inducing *Phytophthora* protein 1
NIP	Necrosis-inducing protein
nt	Nucleotide
ORF	Open reading frame
OD	Optical density or absorbance
PTI	PAMP-triggered immunity
PAMP	Pathogen-associated molecular patterns
%	Percentage
GC content	Percentage guanine and cytocine nucleotides in a sequence
Pc	*Phytophthora cinnamomi*
Pi	*Phytophthora infestans*

PsAvh	*Phytophthora sojae* avirulence homologue
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
dT-primers	Primer sequence consisting of deoxythymine nucleotides
RT-qPCR	Quantitative reverse transcriptase PCR
ROS	Reactive oxygen species
RFU	Relative fluorescence units
R-protein	Resistance protein
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RIN	RNA integrity number
RNA-seq	RNA sequencing
sec	Second
SP	Signal peptide
SNE	Suppressor of necrosis
SNEL	Suppressor of necrosis 1-like
Swiss	Swiss protein database
THRIP	Technology and Human Rescources for Industry Programme
i.e.	That is
3D	Three dimensional
EF-1α	Translation elongation factor 1 alpha
Ubc	Ubiquitin- conjugated enzyme
N	Unassigned nucleotide
U	Unigene
V8	Vegetable juice

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CHAPTER 1

Biology of the oomycete pathogen *Phytophthora cinnamomi* Rands

INTRODUCTION

The class oomycota contains many devastating pathogens affecting crops, forest trees, native vegetation and ornamental plant species, as well as various animal species. The most renown is *Phytophthora infestans* (Mont.) de Bary which was responsible for the great potato famine in Scotland in 1846. *Phytophthora ramorum* Werres, De Cock & Man in`t Veld caused the sudden oak death epidemic in California and Europe (Rizzo et al. 2005) and *Saprolegnia parasitica* Coker is the main pathogen on fresh water fish (salmon and trout) (van West 2006; Phillips et al. 2008). *Phytophthora cinnamomi* Rands is part of this group of destructive pathogens and is able to infect more than 3 000 plant species (Hardham 2005).

Phytophthora cinnamomi was first described by RD Rands in 1922 when it was isolated from cinnamon trees in Sumatra (Rands 1922). Since then it attracted much attention due to the devastating effects is has on natural ecosystems, biodiversity, forestry and agriculture by causing root rot disease and stem cankers. *Phytophthora cinnamomi* is thought to originate from Papua New Guinea (Zentmyer 1988; Hardham 2005), but now has a worldwide distribution affecting the Southern part of Australia, Europe, South Africa, Mexico, Hawaii and the USA (Cahill et al. 2008).

The natural host range of *P. cinnamomi* includes many economically important crops and forest trees such as avocado, pineapple, peach, chestnut, macadamia, oak, pine and eucalyptus (Zentmyer 1980). The economic impact due to *Phytophthora* disease not only includes losses in crop yield and decrease in product quality and value, but large amounts of money are spent annually on control measures (Hardham 2005). Except for the use of phosphite injections (Darvas et al. 1984; Shearer and Fairman 2007; Akinsanmi and Drenth 2013), few other control strategies prove effective against this soil-borne pathogen. A better understanding of the molecular basis that is responsible for a susceptible or resistant host interaction is required to develop more targeted control strategies.

Genome and transcriptome analysis of various pathogenic oomycete species lead to the identification of several gene groups that function in pathogenesis. Functional characterization of pathogenicity proteins is shedding light on the mechanisms employed by the pathogen to manipulate host systems during infection. This review provides background on *P. cinnamomi* biology and disease development and describes pathogenicity gene groups that make *Phytophthora* species successful pathogens.

BIOLOGY OF *PHYTOPHTHORA CINNAMOMI*

Phylogeny and identification

Oomycetes were originally classified as fungi by taxonomists, based mainly on morphological characteristics (sporangium type, antheridium type etc.) and culture characteristics (optimal growth temperature, culture morphology, host range etc.) (Kroon et al. 2012). DNA sequence analysis of the small subunit rRNA showed that oomycetes form a single monophyletic group, more closely related to chrysophytes and diatoms than to fungi (Förster et al. 1990). Today oomycetes are known to differ from fungi in various physical and biochemical ways (Latijnhouwers et al. 2003; Hardham 2005). This includes differences in cell wall composition (cellulose instead of chitin), the presence of non-septate hyphae, the production of wall-less, motile zoospores, the composition of the flagellar apparatus and the structure of the mitochondrial cristae. Further, oomycetes lack the ability to synthesize sterols, only completes meioses directly prior to gametogenesis and differ from fungi in the lysine biosynthesis pathway.

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The *Phytophthora* genus is classified under the kingdom Chromista (phylum: oomycota) within the family Peronosporaceae (Hardham 2005). *Phytophthora* is divided into eight clades based on DNA sequence analysis of seven gene regions (Blair et al. 2008) (Figure 1). *Phytophthora cinnamomi* forms part of clade seven (containing 13 species) together with *Phytophthora sojae* Kaufm. & Gerd. All members of clade seven produce non-papillate sporangia and are mostly root pathogens (Kroon et al. 2012). *Phytophthora infestans* belongs to clade one.

Besides identification of *P. cinnamomi* through DNA sequence analysis of the *ITS* gene region, Kong et al. (2003) developed a PCR based method to rapidly detect and identify *P. cinnamomi*. This approach employs primers directed to the large peripheral vesicle 3 (*LPV3*) gene that encodes a putative storage protein in zoospores. The primer pair amplified the *LPV3* gene region (450 bp in size) with insignificant cross-amplification in 102 isolates from 30 other *Phytophthora* species, 17 isolates from nine *Pythium* spp. and 43 isolates from other water moulds, bacteria and true fungi. In cases where cross-amplification was observed, the primers either produced a different size PCR product or the species had a host range unlikely to overlap with that of *P. cinnamomi*.

Culture morphology

Phytophthora cinnamomi can be cultured on a vast range of nutritional media in the laboratory. Media routinely used to culture *P. cinnamomi* include vegetable juice agar, pea agar, potato dextrose agar and cornmeal agar (Chen and Zentmyer 1970; Zentmyer et al. 1976). On ½ potato dextrose agar, *P. cinnamomi* grows in a rosette pattern (Figure 2A), white to slight yellowish-brown in colour, while it may have an indefinite, radial or coralloid pattern on other nutritional media (Gerrettson-Cornell 1989). Isolates of different mating types show differences in growth rate and cardinal temperatures, with A1 mating type having a higher optimum temperature than mating type A2, though A2 isolates grow faster than A1 isolates on minimal media (Shepherd et al. 1974; Zentmyer et al. 1976). In general, however, cardinal temperatures of *P. cinnamomi* are a minimum of 4-16 °C, optimal 20-32.5 °C and a maximum of 30-36 °C (Zentmyer et al. 1976; Gerrettson-Cornell 1989).

Mycelia have aseptate hypae (Latijnhouwers et al. 2003; Hardham 2005), with frequent irregular shaped hyphal swellings, giving it a coral appearance (Figure 2B). Chlamydospores are present in clumps or as single spores and when mycelia is flooded, almost spherical sporangia (on average 48-75 x 31-46 µm) are produced (Gerrettson-Cornell 1989).

Life cycle of *P. cinnamomi*

Phytophthora cinnamomi is a heterothallic, diploid organism that produces zoospores either through sexual reproduction (through oogonium and antheridium) or through asexual reproduction (by clonal propagation) (Hardham 2005). It is interesting that *P. cinnamomi* mostly reproduces asexually even in the presence of both A1 and A2 mating types (Hardham 2005).

Phytophthora cinnamomi persists in soil or infected plant material for extended periods of time (Zentmyer and Mircetich 1966) until conditions are favourable for growth. In moist and cold conditions chlamydospores, hyphae (asexual) or oogonia (sexual structures) develop sporangia that, through cytoplasmic cleavage, produces 20 to 30 uninucleate, wall-less, biflagellate zoospores (Hardham 2005) (Figure 3). Motile zoospores are most often the agent of infection as they can locate living host tissue by following chemotactic and electrotactic signals (Zentmyer 1961). Recognition of host cell wall carbohydrate molecules initiates the transformation from a zoospore to a walled cyst (Irving and Grant 1984; Hardham and Suzaki 1986). Cysts stably adhere to the plant cell surface, germinate and penetrate host tissue through appresorium formation. In a susceptible host, sporangia are

produced from somatic hyphae on the root surface after two to three days (Hardham 2005). The asexual reproduction cycle repeats several times to rapidly increase the inoculum. Alternatively, sporangia formation is omitted during asexual reproduction and chlamydospores directly germinate to produce hyphae (Hardham 2005).

Host tissue colonization occurs through the spread of somatic hyphae that grow along the plant epidermal anticlinal cell walls with frequent intracellular penetration (Hardham 2001). A biotrophic structure (haustorium) develops, bringing the pathogen plasma membrane in close contact with the plant plasma membrane. This allows the exchange of molecules and nutrients between plant and pathogen (Figure 4).

Trophic lifestyle

Pathogens are divided into three trophic classes based on their manner of acquiring nutrients (troph = nutrition). Biotrophic pathogens infect and feed off living plant material, while necrotrophic pathogens have a saprophytic lifestyle (living off dead material). Pathogens that have sequential biotrophic- and a necrotrophic phases are classified as hemi-biotrophic organisms (Oliver and Ipcho 2004). As *P. cinnamomi* infects symptomless host tissue in front of the lesion margin, it is classified as a hemi-biotroph (Davison et al. 1994; Hüberli et al. 2000; Cahill et al. 2008). Infection of symptomless hosts, however suggest that it does not act as a necrotroph as no necrotic tissue is evident during colonization (Crone et al. 2012). Contrary to this, Shearer and Crane (2012) argues that *P. cinnamomi* have alternating biotrophic and necrotrophic phases (dependent on environmental conditions) and is not accurately described by either of the existing trophic classes. For the purposes of this review, *P. cinnamomi* is regarded as a hemi-biotroph.

PHYTOPHTHORA CINNAMOMI DISEASE DEVELOPMENT AND CONTROL

Host range and distribution of *P. cinnamomi*

Some pathogenic oomycetes are host-specific, marked by signs of co-evolution between host and pathogen, while others are able to infect thousands of species. *Phytophthora cinnamomi* is postulated to have a host range of more than 3 000 plant species (Hardham 2005). This includes various economic important species affecting forest trees and various

crop species worldwide (Table 1). *Phytophthora cinnamomi* infestation not only impacts on plant species of economic value, but also causes disease of indigenous flora in South Africa and Australia (Von Broembsen 1985; Crone et al. 2012). In South Africa, *P. cinnamomi* was reported on 130 different host species including grapevine, pine, eucalyptus, avocado, ornamental plants, fynbos and cultivated protea (the national flower of South Africa) (von Broembsen 1984). *Phytophthora cinnamomi* is regarded to have a nearly worldwide distribution (OEPP/EPPO 2004) reported in the southern part of the Australia, Europe, South Africa, Mexico, Hawaii, North-America, South-East Asia and New-Zealand (Cahill et al. 2008). The large host range of *P. cinnamomi* poses a serious threat to biodiversity as it has been reported in at least 15 of the 25 global biodiversity hotspots worldwide (Myers et al. 2000).

Population structure of *P. cinnamomi* within South Africa

The population structure of a pathogen within a confined area directly impacts on its potential to cause disease. A large, genetically diverse population is more versatile and in the presence of sexual reproduction, allows for high gene flow and high mutation rates, resulting in new variants that may overcome host immunity (McDonald and Linde 2002). The most recent population study of *P. cinnamomi* in South Africa, using isozymes, suggested a clonal population structure (Linde et al. 1997). This classifies *P. cinnamomi* as a low risk pathogen within South Africa according to the criteria stipulated in McDonald and Linde (2002). As this study was completed in 1997 it may not represent the current population diversity of *P. cinnamomi* within South Africa as new variants may have been introduced over the past 30 years.

Disease symptoms

Phytophthora cinnamomi is a versatile pathogen that is able to infect various plant organs resulting in root rot, stem cankers and dieback of young shoots on various hosts (OEPP/EPPO 2004; Hardham 2005) (Figure 5). As a root pathogen, *P. cinnamomi* infection causes progressive brown lesions starting at the tips of feeder roots of susceptible hosts. Excessive root loss leads to leaf yellowing, wilting and eventually tree death (OEPP/EPPO 2004). Stem cankers and root rot symptoms are visible on infected peach trees, marked by wood discolouration, stem lesions and tree gumming that subsequently results in wilting and tree death (Mircetich and Keil 1970).

Above ground disease symptoms only become apparent, once the infection progresses past the stage where control strategies will be effective. Host species that rapidly decline upon *P. cinnamomi* infection are used as indicator species to mark the presence of *P. cinnamomi* in infested areas, before above ground symptoms are visible in host species with slower disease progression. *Stylidium diuroides* (a native herbaceous perennial in Australia) serves as a good indicator for *P. cinnamomi*, as sudden wilting of this perennial, while surrounded by healthy plants, is observed at the end of winter in infested areas (Crone et al. 2012).

Phytophthora cinnamomi was isolated from annual and herbaceous perennial Australian plant species and native plants in South Africa without visible disease symptoms (Von Broembsen 1985; Crone et al. 2012). It is interesting that haustoria were observed during this symptomless colonization (Crone et al. 2012). Symptomless hosts can serve as a reservoir of *Phytophthora* inoculum enabling *Phytophthora* to infinitely persist in invested areas (Crone et al. 2012).

Spread of *P. cinnamomi*

Phytophthora cinnamomi chlamydospores are preserved in soil during dry periods between rainfall events (Zentmyer and Mircetich 1966; Jung et al. 2013). For extended dry periods, it persists in the form of selfed oospores, hyphal aggregations and encased hyphae in host and non-host tissue (Jung et al. 2013). As a soil-borne pathogen it is mainly spread through soil movement (e.g. stuck to the tires of vehicles), rain splash (Podger et al. 1965) and recirculation of invested irrigation water (Hong and Moorman 2005). *Phytophthora cinnamomi* zoospores are transported by flowing surface water up to 75 cm deep into soil just above lateritic soil types (Shea et al. 1983) and by root to root transmission of susceptible plant species (Hill et al. 1994). Feral pigs vector *P. cinnamomi* by ingesting infected plant material (a portion of *P. cinnamomi* remains viable in the digestive tract) or by transporting infested soil collected by their rooting activities (Krull et al. 2013; Li et al. 2013).

Control of *P. cinnamomi*

To date there is no means to eradicate this soil-borne pathogen from invested areas. Many pathogens are controlled by frequent crop rotation on infested land (Curl 1963), but the large host range associated with *P. cinnamomi* and its ability to persist in soil for years (Zentmyer and Mircetich 1966) does not make this a viable option (Mbaka 2011). Fires may decrease *P. cinnamomi* inoculum by decreasing available susceptible host species, but does not seem

to have an effect on pathogen survival (Podger 1990; Moore et al. 2007). Despite this, there are some biological and chemical control measures available to manage *P. cinnamomi*.

Biological control

Biological control refers to the deliberate, controlled establishment or enhancement of organism populations within diseased and invested areas that have an antagonistic effect on a target pathogen, without having adverse effects on the specified ecosystem. In the case of a soil-borne pathogen, such as *P. cinnamomi*, farmers commonly make use of mulches (a combination of compost and soil) (Richter et al. 2011) that enhance natural endophyte populations within soil. Endophytes are endosymbionts that live inside plant tissue without having an apparent adverse effect on the host. Endophytes act as biological control agents by priming the host immune system, leading to a more rapid defence reaction in the case of pathogen attack (Van Wees et al. 2008). The secretion of extracellular hydrolytic enzymes (such as cellulase) into the rhizosphere by endophytes have an antagonistic effect on pathogens by targeting, for example, the *Phytophthora* cell wall (Downer et al. 2002). Various microorganisms have been evaluated for their ability to act as bio-control agents against *P. cinnamomi*. *Trichoderma* showed potential as a bio-control agent as it inhibited the growth of various *Phytophthora* species (*P. cinnamomi, Phytophthora cactorum (*Lebert and Cohn) J. Schröt.*, Phytophthora palmivora* (E.J. Butler) E.J. Butler and *Phytophthora capsici* Leonian) in culture and decreased root rot symptoms resulting from *Phytophthora* infection (Smith et al. 1990; McLeod et al. 1995; Sid Ahmed et al. 1999; Mpika et al. 2009). Other potential bio-control agents enhanced host growth, while suppressing the growth of *P. cinnamomi*. This included *Penicillium funiculosum, Gliocladium* spp. and *Chaetomium globosu* (Drenth and Guest 2004). Despite the identification of parasitic endophytes on *Phytophthora* species, the use of bio-control for a soil pathogen is complicated by the difficulty to establish and maintain endophyte populations.

Chemical control

Many crop and forest pathogens are controlled by the application of fungicides. Differences in the biological nature of oomycetes and fungi, make *Phytophthora* species insensitive to many conventional fungicides (Latijnhouwers et al. 2003). Chitinase synthase inhibitors (e.g. fungicides Nikkomycin and Polyoxin D) are effective against fungi as chitin is the main component of fungal cell walls. The cell walls of oomycetes are primarily constituted of cellulose and are not affected by these fungicides. The chemical metalaxyl targets RNA polymerases (Latijnhouwers et al. 2003) and have been shown to be effective against some

Phytophthora species on crops (Erwin and Ribeiro 1996). A study by Dustan et al. (2010), however, found these fungicides ineffective against *P. cinnamomi* in a field trial.

Phosphite injections seem to be the only effective chemical control strategy against *P. cinnamomi* to date, proving to be effective in *Persea americana* (Darvas et al. 1984), *Eucalyptus marginata*, *Banksia* species (Shearer and Fairman 2007) and *Macadamia* species (Eshraghi et al. 2011; Akinsanmi and Drenth 2013). Phosphite reduces zoospore production (Wilkinson et al. 2001) and leads to deformed hyphae by inhibiting cell wall synthesis and influencing the cytoskeletal sytem of *P. cinnamomi* (King et al. 2010). Phosphite simultaneously primes the host (as seen in *Arabidopsis thaliana*), leading to a quicker, more intense defence response (Eshraghi et al. 2011; Massoud et al. 2012). As the threshold phosphite concentration needed to contain *P. cinnamomi,* while avoiding adverse effects on host species, varies between plant species (Tynan et al. 2001), the application of phosphite to areas containing various natural plant species is not feasible (Dunstan et al. 2010). Research into alternative control agents continues. Farmers, however, experience increased pressure from the global market to move away from chemical pest control and employ organic farming practices.

Other control strategies

An integrated approach proved successful in preventing and even eradicating *P. cinnamomi* from three tested plots, containing native plant species in Australia (Dunstan et al. 2010). This approach involved susceptible host species destruction, application of fungicides and fumigants and the construction of physical root barriers (Dunstan et al. 2010). Other control strategies focus on spread prevention rather than pathogen eradication. Heat treatment of recirculating irrigation water (Hao et al. 2012), vehicle washing and road side sanitation decreases the probability of spreading *P. cinnamomi* to uninvested areas (Goheen et al. 2012). Some industries, such as avocado production, strongly rely on tolerant rootstocks, allowing sufficient fruit production in the presence of *P. cinnamomi* (Smith et al. 2010). In the future, the commercial crop and forest industries will likely move to clonal, genetically modified organisms (GMO) that offer faster development of controlled, more durable resistance to selected pathogens.

OOMYCETE GENOMIC- AND TRANSCRIPTOMIC RESOURCES

In theory, to conserve energy genes are only transcribed into mRNA when their functional products are required by the organism. Therefore, identification of transcripts present in a specific tissue, developmental stage or in response to a specific treatment provides insight into the molecular functioning of the organism under these controlled conditions. In a similar way the transcriptome of specialized developmental structures (with specific biological functions) are expected to be enriched in transcripts enabling that function. Genes associated with pathogenicity, for example, are enriched in pathogen infection structures (germinating cysts) (Ye et al. 2011).

For the past 25 years sequence technology has been applied to transcriptome analysis of various organisms (Gill and Sanseau 2000). Labour intensive procedures (involving cloning and sequencing of cDNA transcripts) allowed the investigation of a few thousand expressed sequence tags (ESTs) in the past (Qutob et al. 2000; Škalamera et al. 2004; Panabières et al. 2005; Krajaejun et al. 2011). Microarray analysis (used since the late 1990s) was the first tool enabling the study of gene expression on a large scale (Leroy and Raoult 2010). One of the disadvantages of this technology is that gene specific probe design is dependent on the availability of genomic sequence data (Wang et al. 2009), which is still lacking for many non-model organisms. Today, RNA-seq analysis is steadily replacing microarray technology in many application (Strickler et al. 2012). As opposed to a hybridization based approach (e.g. microarray), RNA-seq provides researchers with the actual sequence of transcripts, not limiting analysis to known genomic sequence, and allowing detection of novel transcripts, alternative spliced variants and single nucleotide polymorphisms (Wang et al. 2009; Strickler et al. 2012). RNA-seq is far superior to microarray technology with respect to its ability to quantify transcript abundance, allowing the identification of almost all transcripts present in a biological sample (Strickler et al. 2012). The increasing availability of genomic data, for both model and non-model organisms, aids in the correct assembly and annotation of transcripts.

Genomic and transcriptomic data have been generated for various oomycete species (Table 2). Previously, pathogenicity related transcripts were identified by transcriptional analysis of the *in vitro* pre-infection structure (germinating cysts) (Shan et al. 2004a; Grenville-Briggs et al. 2005; Guo et al. 2006; Chen et al. 2011). This approach eliminated the problem of low recovery of pathogen derived transcripts previously reported within cDNA libraries of infected plant material (Beyer et al. 2001; Chen et al. 2011). The increased depth provided by RNA-seq technology, as well as the availability of the genomes of various oomycete- and host species, now enable *in planta* transcriptional analysis of both pathogen and host as sequences can be separated using bioinformatic tools (Ye et al. 2011; Gyetvai et al. 2012).

PLANT - BIOTROPH INTERACTION: PTI AND ETI

Pathogens with an initial biotrophic phase, such as *P. cinnamomi*, need to successfully infect living, reactive hosts. This requires the pathogen to effectively surpass several lines of host defence. The plant defence system includes physical barriers, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). The current accepted model for plant-pathogen interaction is the four phased zig-zag hypothesis proposed by Jones and Dangl (2006) that illustrates the continuous interaction between pathogen molecules and the host immune system (Figure 6).

Pathogen- or microbe associated molecular patterns (PAMP or MAMPs) are pathogen derived molecules that are recognised by host pathogen recognition receptors (PRRs) during a plant-pathogen (or microbe) interaction (Tsuda and Katagiri 2010). PAMPs are typically proteins exposed on the surface of pathogens and are usually conserved between microbes (Boller and Felix 2009) (e.g. flagellin proteins in bacterial flagella (Gómez-Gómez and Boller 2002)). PAMP recognition leads to the activation of a basal defence response, termed PTI (Jones and Dangl 2006; Tsuda and Katagiri 2010). The basal defence response aims to restrict pathogen invasion by strengthening physical barriers (tylose and lignin deposition) (Rookes et al. 2008) and creating a non-favourable environment for the pathogen. This involves the activation of many defence related genes via the mitogen-activated protein (MAP) kinase cascade, the secretion of toxic reactive oxygen species (ROS) and activation of hormonal defence pathways (e.g. salicylic acid, jasmonic acid and ethylene) (Tsuda and Katagiri 2010). Programmed localized cell death is often observed in the area of infection aiming to confine biotrophic pathogens that require living tissue to colonize (Jones and Dangl 2006). This is termed the hypersensitive response (HR).

A successful pathogen protects itself from host defence molecules and actively attacks host defence responses by suppressing PTI (Jones and Dangl 2006). This is mediated by secreted cytoplasmic effector molecules and lead to effector-triggered susceptibility (ETS) (Jones and Dangl 2006; Tsuda and Katagiri 2010). Effector molecules are target specific and contribute to host susceptibility by manipulating host targets within (or outside) plant cells to increase pathogen virulence (Jones and Dangl 2006). Effector molecules are recognized by the corresponding host resistance protein (R protein) (Van Der Biezen and Jones 1998) and activates a second line of defence, called ETI. Effector-triggered immunity can also be activated indirectly by resistance proteins that detect changes in host defence-associated proteins due to the action of pathogen effectors. This indirect recognition was

coined the 'guard hypothesis" (Van Der Biezen and Jones 1998; Dangl and Jones 2001). Effector-triggered immunity involves a more intense, more prolonged and robust defence response than that of PTI (Tsuda and Katagiri 2010). Effectors that evade host detection can suppress ETI, resulting again in ETS. This constant battle between plant and pathogen drives the evolution of both pathogen effector proteins and host resistance proteins (Birch et al. 2006).

OOMYCETE PATHOGENICITY FACTORS

The process of plant infection requires pathogens to accomplish various tasks to successfully colonize hosts. A good pathogen can 1) locate a suitable host, 2) effectively penetrate and colonize the host tissue, 3) protect itself by suppressing host defence responses and counteracting the effect of host hydrolases and toxic compounds and 4) manipulate host physiology to its own benefit, while remaining undetected by the host surveillance system. The above processes are mediated by pathogenicity proteins that function at numerous locations and at different times during infection. Here, various pathogenicity gene groups will be discussed in the light of their role in pathogenicity (Figure 7).

Cell wall degrading enzymes

Pathogen entry is restricted by the plant cell wall, with additional callose, tylose- and lignin deposition in the area of pathogen detection in an attempt to restrict pathogen invasion (Cahill and McComb 1992; Benhamou and Nicole 1999; Rookes et al. 2008). The plant cell wall is primarily composed of cellulose, hemi-cellulose and pectin (Carpita and Gibeaut 1993), with the aerial plant organs covered by the plant cuticle. Plant pathogens possess a large repertoire of cell wall degrading enzymes (CWDEs) that break down the physical barriers (Have et al. 2002) and aid in tissue penetration and colonization. Polygalactorunase, which hydrolyses pectin, is one of the first enzymes secreted by plant pathogens during infection (Jones et al. 1972) and exposes other cell wall components to different CWDEs. Other CWDEs include cellulases (hydrolyzing the 1,4-β-D-glycosidic linkages in cellulose), glucanases and glucosidases (targeting glucose polymers, glucans and glycol-conjugates) and cutinase enzymes (that target cutin) (Have et al. 2002). It is interesting that both the cell walls of plants and *Phytophthora* species are rich in cellulose and β-1,3 and β -1,6 glucans (Bartnicki-garcia and Wang 1987). It is therefore difficult to

attribute the presence of some CWDE to the breakdown of the host's physical barriers or to the biogenesis of the pathogen cell wall (Judelson et al. 2008).

Proteins involved in transport, detoxification and protection against ROS

The oxidative burst is a defence strategy employed by the host that creates a toxic environment for the pathogen. This rapid response involves the generation of ROS (especially superoxide, hydrogen peroxide and nitric oxide) that has a direct toxic effect on the pathogen, but also plays a role in defence signalling and activation of defence related gene expression (O'Brien et al. 2012). Pathogens (including oomycetes) possess several enzymes, involved in counteracting the effect of the oxidative burst by catalyzing the oxidation of free radicals to neutral compounds. Superoxide dismutase, for example, functions as an antioxidant by catalyzing the neutralization of superoxide to oxygen and hydrogen peroxide. Harmful xenobiotic substrates are neutralized by conjunction to the reduced form of glutathione, mediated by the enzyme glutathione s-transferase (Nebert and Vasiliou 2004) or by oxidation by cytochrome P450 enzymes (Črešnar and Petrič 2011).

Besides neutralizing harmful compounds, pathogens actively export toxins (produced by the host or externally introduced) as a survival strategy. The main group of proteins that export toxic compounds are ATP binding cassette (ABC) transporters (Dassa and Bouige 2001). ABC transporters are conserved in plants, fungi, bacteria and oomycetes (Bauer et al. 1999; Dassa and Bouige 2001; van den Brûle and Smart 2002; Torto-Alalibo et al. 2007) and function in pathogen protection and fungicide resistance (Del Sorbo et al. 2000).

Proteins involved in signal transduction and communication

Cell surface guanosine nucleotide-binding proteins (G-proteins) function in various cellular processes including growth, nutrient sensing, conidia formation, fertility, invasive growth, appresorium development and pathogenesis (Latijnhouwers et al. 2003; Li et al. 2007). G-proteins are plasma membrane bound receptors that perceive various extracellular signals and transmit them to intracellular signalling systems (Li et al. 2007; Xue et al. 2008). MAP kinases act downstream of G-proteins and play a fundamental role in the transduction of various intra- and extracellular signals. They are conserved in eukaryotes and function in numerous cellular processes including growth, differentiation and plant defence (Xu 2000; Meng and Zhang 2013). MAP kinases have previously been implicated in pathogenesis, hyphal colonization and appresoria formation in fungal pathogens (Xu 2000).

Effector molecules

The role of effector molecules in infection and virulence is well established (Kamoun 2006). Effector molecules were comprehensively defined by Hogenhout et al. (2009) as ‘‘pathogen proteins and small molecules that alter host-cell structure and function. These alterations either facilitate infection (virulence factors and toxins), or trigger defence responses (avirulence factors and elicitors) or both’’. Apoplastic effectors are secreted into intercellular spaces (the extra haustorial matrix), while cytoplasmic effectors are translocated into host cells where they act in the host cytoplasm or nucleus (Kamoun 2006).

Effector genes of *P. infestans* are predicted to reside in gene sparse, repeat rich regions of the genome, in contrast to housekeeping genes that are found in gene dense and repeat poor regions (Haas et al. 2009). In a similar way *P. sojae* effector genes are flanked by retrotransposons much more frequently than the average gene (R.H.Y.J. and Zody 2008). The repetitive nature of these regions makes them dynamic and more prone to events such as non-homologous recombination and tandem gene duplication and is proposed to drive avirulence gene evolution (Haas et al. 2009). The presence of avirulence gene duplication provides the opportunity for development, through single mutations, of new versions of the specific gene, capable of evading the host surveillance system. This is supported by the large amount of amino acid polymorphisms reported in effector genes that result from high rates of non-synonymous nucleotide substitution (Rehmany et al. 2005; Win et al. 2007).

Apoplastic effector molecules

Host degrading enzyme inhibitors

In the same way that *Phytophthora* secretes CWDE directed at the plant cell wall, the host plant releases enzymes that target invading pathogens. The effect of these enzymes is countered by enzyme inhibitors (Misas-Villamil and van der Hoorn 2008) that target specific host proteases and glucanases. Host degrading enzyme inhibitors reported in *Phytophthora* EST data include Kazal-like proteinase inhibitors (Panabières et al. 2005; Raffaele et al. 2010; Reena et al. 2010), Kazal-like serine protease inhibitors (Le Berre et al. 2008) and other protease inhibitors (Torto-Alalibo et al. 2007). EPIC1 and EPIC2 of *P. infestans* target the Rcr3 protease from tomato (Song et al. 2009), while the protease inhibitor EPI10, inhibit the Kazal-like serine protease P69B subtilase of tomato (Tian et al. 2005).

Elicitins

Elicitin proteins are small extracellular proteins that are associated with infection because they induce a HR in certain plant species (Kamoun et al. 1993a; Kamoun et al. 1993b; Bonnet et al. 1996). Their exact role in infection remains unclear, but their ability to bind sterols and fatty acids (Vauthrin et al. 1999; Yousef et al. 2011) suggests that they are lipid-binding proteins. As *Phytophthora* species lack the ability to synthesize sterols (Latijnhouwers et al. 2003), elicitins may aid in acquiring sterols needed for physiological functions (Jiang et al. 2006). Based on the spacing of cysteine residues in the elicitin domain, this family of proteins is divided into 17 clades (Jiang et al. 2006). Elicitins from the same clade show similar expression patterns during infection. *Phytophthora infestans* elicitin INF1 is one of the first and best characterized elicitins and is used to define elicitin-like (ELLs) genes (genes that share the 98 amino acid elicitin domain of the INF1 elicitin). As INF1 is known to induce a HR in tobacco (Kamoun et al. 1998), it is regularly used to evaluate the ability of other proteins to suppress plant immunity (Bos et al. 2006; Gilroy et al. 2011; Yaeno et al. 2011; Cheng et al. 2012).

Pathogen adhesion

In order to establish host colonization, zoospores need to stably adhere to the host surface to germinate. Genes implicated in this process include cellulose binding and elicitor lectin (CBEL) proteins (Gaulin et al. 2002), mucin-like proteins (Torto-Alalibo et al. 2007) and adhesins (Robold and Hardham 2005). CBEL is part of the family 1 carbohydrate binding (CBM1) proteins, with the carbohydrate binding (CB) domain coupled to PAN/Apple modules (Larroque et al. 2012) facilitating binding to carbohydrate substrates and to other proteins (Tordai et al. 1999). The CB domain is recognized by the host surveillance system and leads to the activation of the HR (Séjalon-Delmas et al. 1997; Gaulin et al. 2006). Accordingly, CBEL is classified as a PAMP. Other molecules that aid in pathogen adhesion are adhesins (Robold and Hardham 2005) and mucins, present in an adhesive substance that is secreted by cysts (Gubler et al. 1989; Hardham and Gubler 1990). In this way CBEL, mucin- and adhesin- genes act as virulence factors, as they facilitate infection.

Necrosis-inducing proteins

Oomycetes, fungi and bacteria possess genes that encode necrosis-inducing proteins (NIP) (Fellbrich et al. 2002). NIPs induce host cell death as part of the infection strategy (Bae et al. 2006) in pathogens with a necrotrophic phase. In hemi-biotrophic pathogens, NIPs are thought to facilitate the switch between biotrophy and necrotrophy, as it is expressed during late infection in the *P. sojae*-soya bean interaction (Qutob et al. 2002; Ye et al. 2011). The

expression pattern of NIPs can therefore serve as a marker for the switch between biotrophy and necrotrophy (Kelley et al. 2010).

Cytoplasmic effector molecules

While apoplastic effector molecules act extracellularly, the target molecules of cytoplasmic effectors are inside host cells. Two main groups of cytoplasmic effectors are proposed namely the RXLR effector family and the Crinkling and necrosis-inducing (CRN) protein family.

RXLR effectors

RXLR effector genes have been identified in the genomes of all plant pathogenic oomycete species with a biotrophic phase (Table 3). This protein family is characterized by the presence of a conserved amino acid sequence RXLR (Arg-any amino acid-Leu-Arg) in its N-terminal, preceded by a signal peptide allowing its secretion, and a variable C-terminal domain (Stassen and Van den Ackerveken 2011) (Figure 8). The RXLR motif shows striking similarity to the host-targeting signal present in proteins of the malaria parasite *Plasmodium* (Rehmany et al. 2005) and was originally thought to be crucial for effector translocation into the host cytoplasm (Whisson et al. 2007). The role of the RXLR motif in effector translocation is currently under debate (Ellis and Dodds 2011). Another less conserved amino acid sequence of importance is the EER region (Glu-Glu-Arg) that sometimes follows the RXLR motif by about 30 amino acids towards the C-terminal (Grouffaud et al. 2010).

The variable C-terminal region of RXLR proteins show little sequence conservation between RXLR gene homologues (Win et al. 2007). This is believed to contain the effector domain (Win et al. 2007; Jiang et al. 2008), responsible for the vast range of functions performed by RXLR effectors. *Phytophthora infestans* effector AVRblb2 acts in the haustorial space and increases virulence by preventing the secretion of the host papain-like cysteine protease C14 into the apoplast (Bozkurt et al. 2011). Avr3a from *P. infestans* stabilizes host ubiquitin E3-ligase CMPG1 (Bos et al. 2010), thereby suppressing INF1-induced cell death (Bos et al. 2006).

RXLR proteins are recognized by the host surveillance system either directly (via interaction with plant resistance proteins) or indirectly (by detection of changes in host defence-associated proteins). *Phytophthora sojae* Avr1b is recognized by soya bean resistance protein Rps1b (Dou et al. 2008) and *P. infestans* Avr3a and Avr4 are recognized by potato resistance proteins R3a (Bos et al. 2006) and R4 (Van Poppel et al. 2009) respectively.

Adaptive effector evolution to avoid recognition by host resistance proteins is evident in differential recognition of proteins produced from different RXLR gene alleles. Two alleles of *Avr3a* of *P. infestans* exist, where one variant is recognized by potato resistance protein R3a, while the other is not (Armstrong et al. 2005). *Phytophthora sojae* RXLR effector Avr3a and Avr5 are allelic variants recognized by resistance proteins Rps3a and Rps5 in soya bean (Dong et al. 2011). As RXLR proteins directly interact with host resistance proteins, they can be utilized to identify novel plant resistance genes. Vleeshouwers et al. (2008) rapidly identified and cloned the resistance genes *Rpi-sto1* and *Rpi-pta1* of *Solanum stoloniferum* and *Solanum papita* respectively by using a candidate gene approach starting with computational prediction of *Avr* genes in the *P. infestans* genome (Vleeshouwers et al. 2008).

Comparison of the three dimensional (3D) structures of four oomycete effector proteins revealed the presence of a conserved structural fold formed by α-helical structures with loops of variable regions between helixes (Win et al. 2012). This conservation was significant as similarity was not evident at amino acid sequence level. Boutemy et al. (2011) suggest that the W-Y domains, previously discovered by Jiang et al. (2008) in the in the C-terminal of RXLR proteins, are critical for the formation of the helical structures constituting the conserved fold. Approximately 44 % of predicted RXLR genes in *Phytophthora* contain a W-Y motif in their C-terminals, with up to 11 W-Y domain repeats (Boutemy et al. 2011). The variable loop regions between α-helixes allow evolution and diversification of effector proteins without influencing this conserved 3D structure (Boutemy et al. 2011).

Crinkling and necrosis-inducing proteins

Crinkling and necrosis-inducing (CRN) proteins have been reported in many oomycete species including *P. infestans* (Haas et al. 2009), *Pythium ultimum* Trow (Lévesque et al. 2010), *P. sojae* and *P. ramorum* (Tyler et al. 2006). While the presence of RXLR effector genes is restricted to pathogens with a biotrophic phase, the CRN effector gene family is expanded in pathogens with a necrotrophic phase (Stam et al. 2013). CRN effectors harbour an N-terminal signal peptide followed by the characteristic LFLAK motif (Leu-Phe-Leu-Ala-Lys) (Win et al. 2007; Haas et al. 2009). CRN family members of *P. sojae* and *P. capsici* have been implicated in suppression of host defences (Liu et al. 2010; Chen et al. 2013). Analysis of *P. capsici* CRN effectors showed that few induce necrosis in plants (Stam et al. 2013). The localization of CRN effectors in the host nucleus and their regulated expression patterns suggest that they function during infection, even if not directly in pathogenesis (Stam et al. 2013).

CONCLUSION

Phytophthora cinnamomi is a destructive, wide host range, plant pathogen resulting in economic losses in crop and forest industries worldwide. Effective control of *P. cinnamomi* is yet to be achieved and until then *P. cinnamomi* will remain a threat to biodiversity and agriculture. The molecular basis for *P. cinnamomi*'s ability to successfully colonize such a large range of plant species is not known. Studies, employing genomic and transcriptomic tools to investigate disease development have proved valuable in other oomycete pathosystems and will be equally useful to investigate *P. cinnamomi* (with the first draft of the *P. cinnamomi* genome recently released). Various gene groups have been implicated in oomycete pathogenesis with most current pathogenesis related research focussing on effector proteins (especially RXLR cytoplasmic effectors). Ultimately, extensive knowledge of pathogen and host is required to start comprehending disease phenotypes and to exploit this knowledge for the development of better control strategies.

TABLES

Table 1. Economically important crops and forest trees affected by *Phytophthora cinnamomi*.

Host	Disease	Disease name	Reference
Forrest trees			
Pinus sylvestris	Root rot	-	(Chavarriaga et al. 2007)
Pinus echinata	Root rot	Little leaf disease	(Tainter 1997)
Pinus radiata	Wilting, root rot	-	(Butcher et al. 1984; von Broembsen 1984)
Pinus occidentalis	Root rot	Little leaf disease	(Jung and Dobler 2002)
Pinus (Other)		-	(von Broembsen 1984)
Eucalyptus marginata	Root rot	Phytophthora dieback	(Podger et al. 1965; Shea et al. 1983)
Eucalyptus grandis	Root rot	-	(von Broembsen 1984)
Eucalyptus (Other)		-	(von Broembsen 1984)
Quercus suber (Cork Oak)	Root rot	Sudden Oak death	(de Sampaio e Paiva Camilo-Alves et al. 2013)
Quercus rotundifolia (Holm Oak)	Root rot	Sudden Oak death	(de Sampaio e Paiva Camilo-Alves et al. 2013)
Crops			
Vaccinium corymbosum (Blueberry)	Root rot, crown rot	Phytophthora root rot	(Larach et al. 2009)
Castanea sativa (Chestnut)	Stem canker	Ink disease	(Vannini and Vettraino 2001; Akilli et al. 2012)
Juglans regia (Walnut)	Root rot, crown rot	-	(Mircetich and Matheron 1983; Kurbetli 2013)
Prunus persica (Peach)	Root rot, stem canker	-	(Mircetich and Keil 1970)
Ananas comosus (Pine apple)	Heart rot	Heart rot	(Rohrbach 1985)
Persea americana (Avocado)	Root rot	Phytophthora root rot	(Wager 1942; Aveling and Rijkenberg 1986; Sepúlveda-Chavera et al. 2013)
Macadamia	Root rot, stem canker	Phytophthora trunk canker	(Mbaka 2011; Serfontein and Fourie 2011)

Table 2. Sequence data available for oomycete pathogens with a focus on infection related studies.

Species	Clade*	Data type	Reference
Phytophthora cinnamomi	7	genome	Available, not published
Phytophthora infestans	1	EST, genome	(Kamoun et al. 1999; Grenville-Briggs et al. 2005; Randall et al. 2005; Guo et al. 2006; Judelson et al. 2008; Haas et al. 2009; Judelson et al. 2009; Henriquez and Daayf 2010; Gyetvai et al. 2012)
Phytophthora sojae	7	EST, RNA-seq, genome	(Qutob et al. 2000; Tyler et al. 2006; Chen et al. 2007; Torto-Alalibo et al. 2007; Ye et al. 2011)
Phytophthora ramorum	8	genome	(Tyler et al. 2006)
Phytophthora parasitica (=Phytophthora nicotianae)	1	EST	(Shan et al. 2004b; Škalamera et al. 2004; Panabières et al. 2005; Natividade Targon et al. 2007; Rosa et al. 2007; Le Berre et al. 2008)
Phytophthora capsici	2	EST, RNA-seq, genome	(Reena et al. 2010; Lamour et al. 2012; Chen et al. 2013)
Phytophthora cactorum	1	EST	(Chen et al. 2011)
Phytophthora colocasiae	-	EST	(Sharma et al. 2009)
Pseudoperonospora cubensis	NA	RNA-seq	(Savory et al. 2012)
Hyaloperonospora arabidopsidis	NA	EST, genome	(Baxter et al. 2010; Cabral et al. 2011)
Pythium ultimum	NA	EST, genome	(Cheung et al. 2008; Lévesque et al. 2010)
Pythium insidiosum	NA	EST	(Krajaejun et al. 2011)
Saprolegnia parasitica	NA	EST, genome	(Torto-Alalibo et al. 2005; Jiang et al. 2013)

* Clades currently distinguished within the genus *Phytophthora* (Blair et al. 2008).

EST – Expressed sequence tags

Table 3. Number of RXLR genes predicted in the published genomes of pathogenic oomycetes

Organism	Number of predicted RXLR genes	Reference	Main Hosts	Trophic life style
Hyaloperonaspora arabidopsidis	134	(Baxter et al. 2010)	*Arabidopsis thaliana*	Biotroph
Phytophthora infestans	563	(Haas et al. 2009)	Potato	Hemi-biotroph
Phytophthora sojae	396	(Jiang et al. 2008)	Soya bean	Hemi-biotroph
Phytophthra ramorum	374	(Jiang et al. 2008)	Oak, multiple	Hemi-biotroph
Phytophthora capsici	357	(Lamour et al. 2012)	Pepper and cucurbits	Hemi-biotroph
Saprolegnia parasitica	0	(Jiang et al. 2013)	Amphibians, crustaceans, fish and insects	NA
Pithium ultimum	0	(Lévesque et al. 2010)	Multiple	Necrotroph

FIGURES

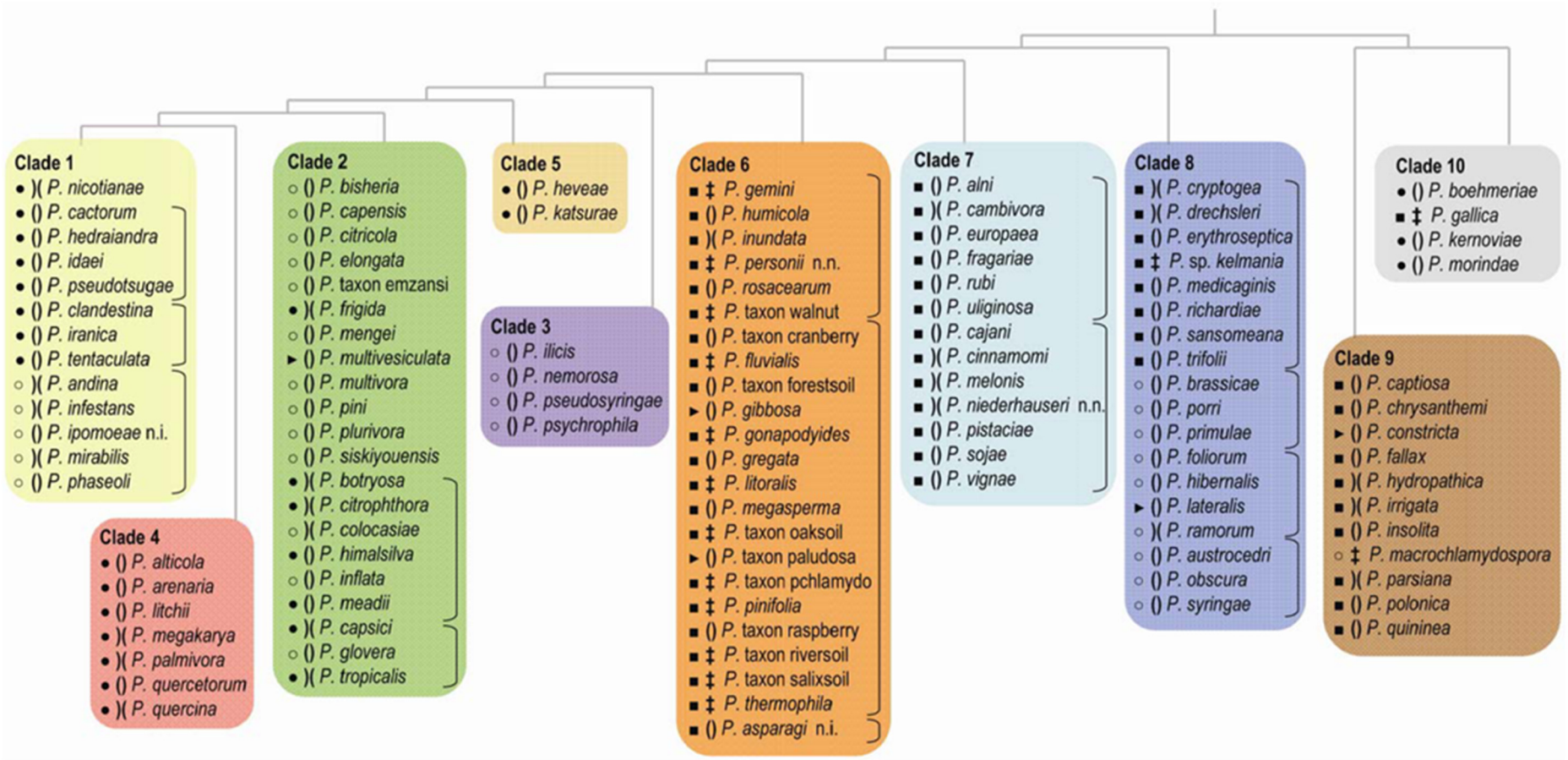

Figure 1. Illustration of the clades currently distinguished within the genus *Phytophthora*. The relationship is depicted as reported in Blair et al. (2008). Sporangia: papillate (●), semi-papillate (○), nonpapillate (■), non- to semipapillate (►), a mixture of nonpapillate and semipapillate (►). Heterothallic species are marked as)(, homothallic species as (), and sterile species (i.e., oogonia unknown or rarely produced) as ‡. n.i. = nomen invalidum; n.n. = nomen nudum. Image obtained from Kroon et al. (2012).

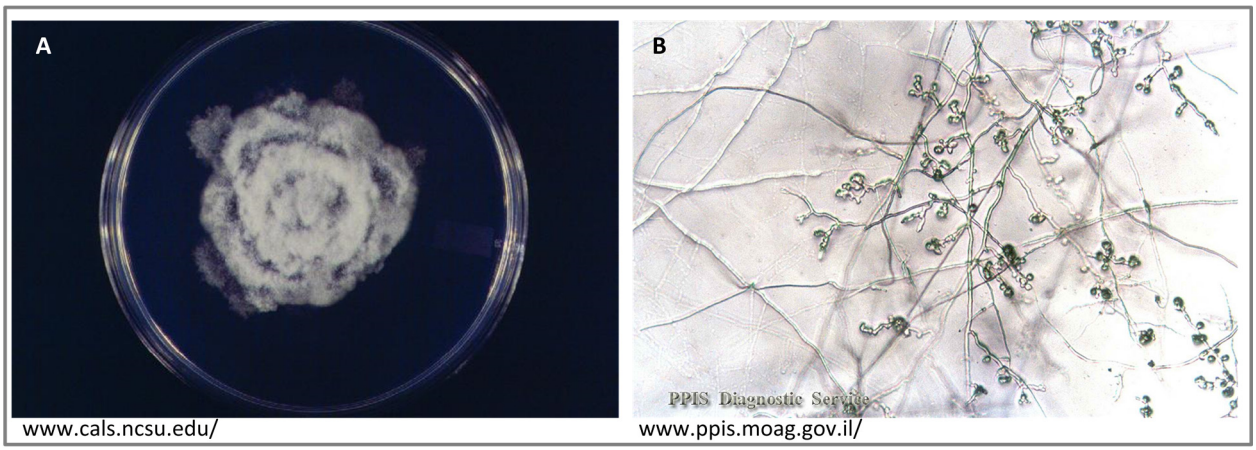

Figure 2. *Phytophthora cinnamomi* culture morphology. A) The typical rosette pattern of *P. cinnamomi* on potato dextrose agar (PDA) B) *Phytophthora cinnamomi* mycelia with the presence of hyphal swellings.

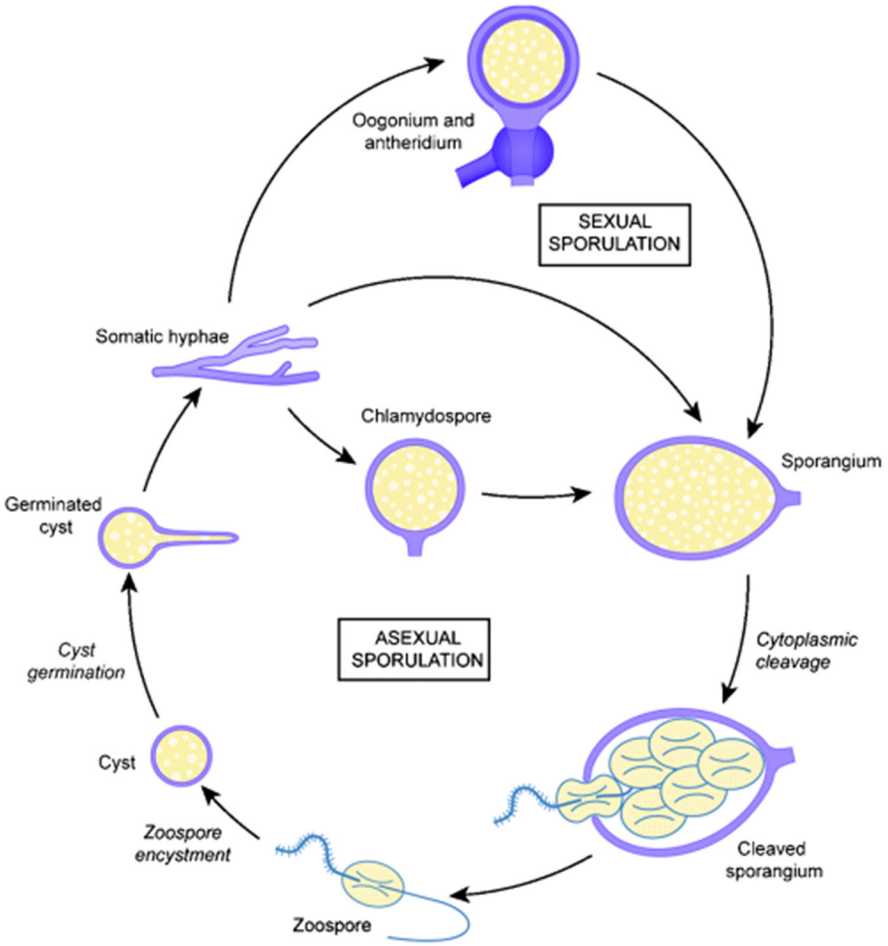

Figure 3. The life cycle of *Phytophthora cinnamomi*. Sexual or asexual reproduction leads to the formation of sporangia from which uninucleate, biflagellate zoospores are release. Zoospores encyst once in contact with the plant surface and germinate in order to locate a suitable point of entry into the plant tissue. Host colonization occurs by the spread of somatic hyphae and increases the inoculum by frequent asexual reproduction. Image obtained from Hardham (2005).

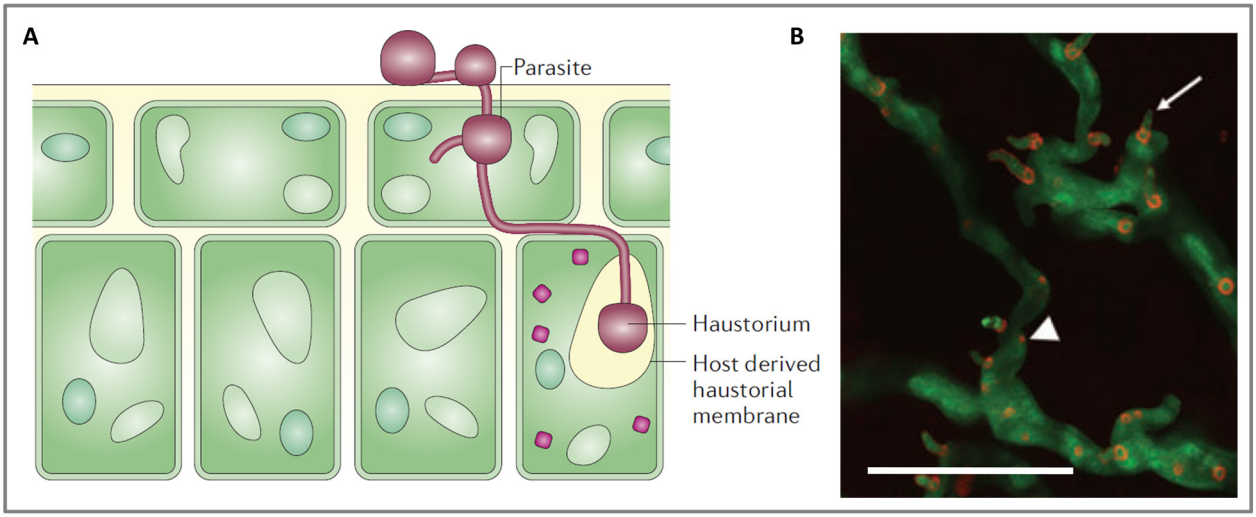

Figure 4. Haustorium structure present in plant-biotrophic interactions. Haustoria are biotrophic feeding structures that forms by intracellular penetration of hyphae. A) Host colonization occurs through the spread of intercellular and intracellular hyphae (purple). Nutrients and effector molecules (purple squares) are secreted into the extra-haustorial space (between the haustorial membrane and the plant derived extra-haustorial membrane) and translocates into the host cell. B) Confocal microscopic image of transformed *Phytophthora infestans*, expressing green fluorescent protein (GFP) to mark the cytoplasm of hypae (green filter) during colonization of *Nicotiana benthamiana* leafs. Pihmp1 (P*. infestans* haustorial membrane protein 1) fused to monomeric red fluorescent protein (mRFP) is visible (red filter) in the haustorial plasma membrane. The arrow indicates a haustorium, while the arrow head shows an attempted point of entry, where a haustorium failed to develop. Images were obtained from Haldar et al. (2006) (A) and Avrova et al. (2008) (B). Scale bar 50 µm.

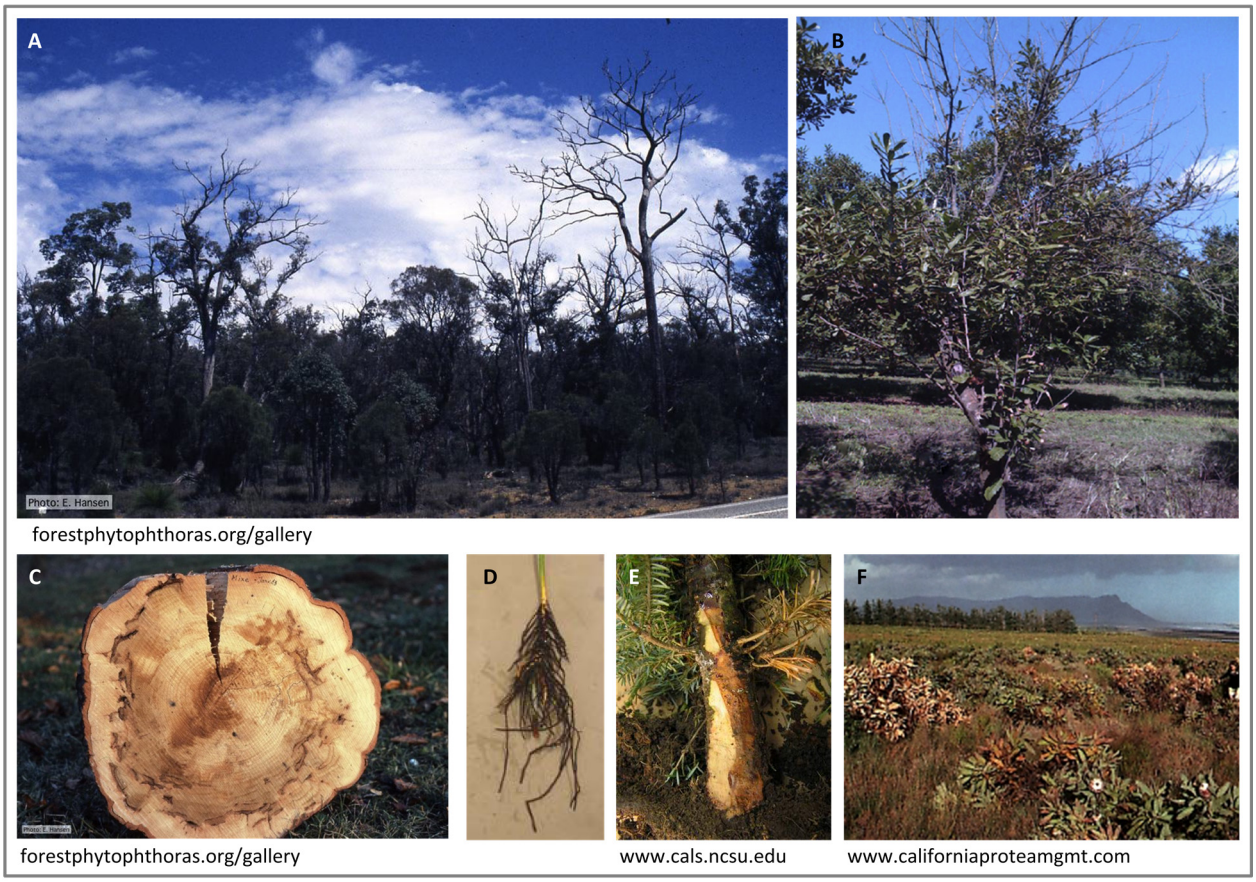

Figure 5. *Phytophthora cinnamomi* disease symptoms on various host species. A) *Eucalyptus marginata* dieback in the Jarrah forest in Australia B) A diseased macadamia tree with pale to yellow leafs and signs of stress C) Lesion in the trunk of an oak tree in France D) Phytophthora root rot on a susceptible *Persea americana* rootstock R0.12 E) Wood discolouration of the stem of Fraser fir (*Abies fraseri*) F) Dieback of Protea (*Proteaceae*) in California, US. Images were obtained from Akinsanmi and Drenth (2013) (B) and Engelbrecht et al. (2013) (D).

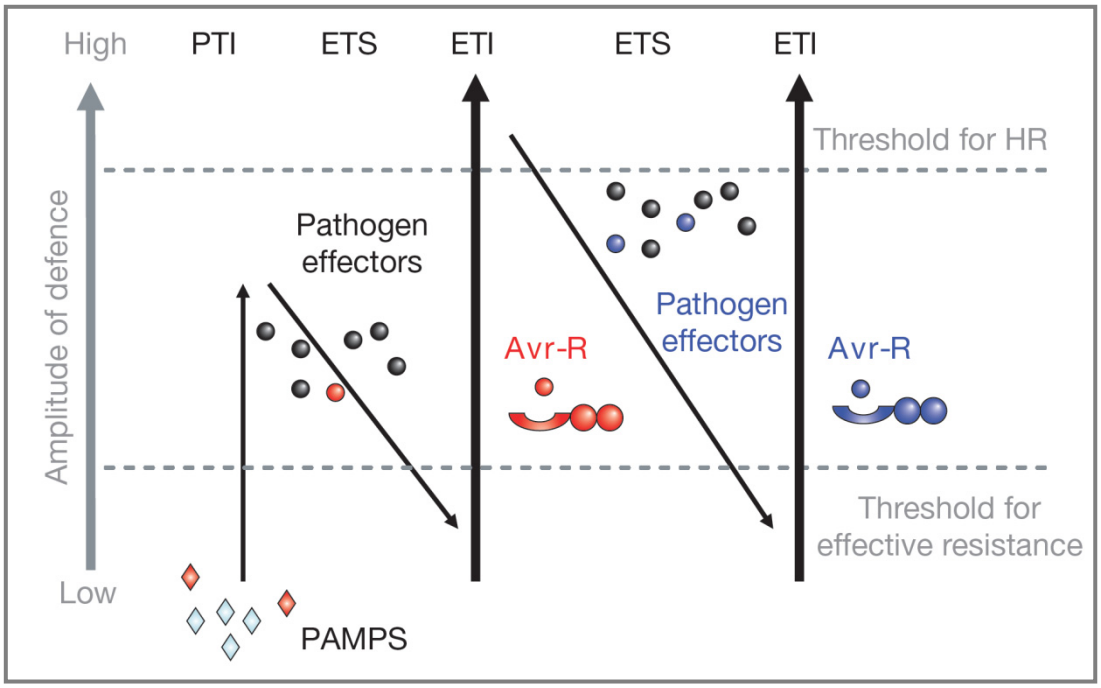

Figure 6. A plant-biotroph interaction illustrating the four phased zig-zag model for the interaction between the plant immune system and pathogen virulence/avirulence factors. This model was proposed by Jones and Dangl (2006). Recognition of pathogen associated molecular patterns (PAMPs) by pathogen recognition receptors (PRR) leads to the activation of a basal defence response termed PAMP-triggered immunity (PTI). Pathogens posses effector molecules that are able to interfere with the plant immune system and suppress PTI, resulting in effector-triggered susceptibility (ETS). Effector proteins are recognized by plant resistance proteins (R), resulting in a second defence response, effector-triggered immunity (ETI). Development of novel effector proteins overcomes ETI, again resulting in ETS. This continuous plant-pathogen interaction drives the development of novel effector variants able to evade host recognition and suppress ETI, in turn driving the evolution of plant resistance genes. Image obtained from Jones and Dangl (2006).

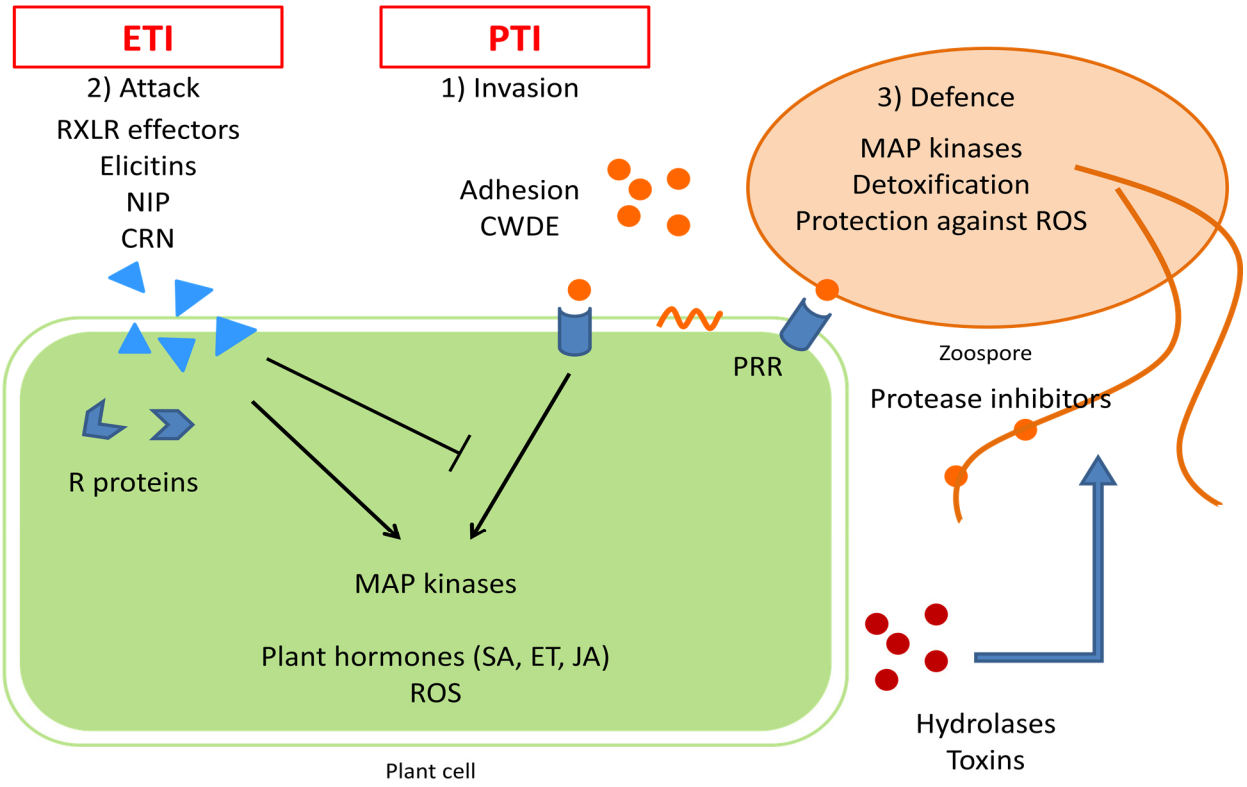

Figure 7. Catogories of pathogenicity genes involved in the molecular events of a plant-pathogen interaction. 1) Invasion: Proteins that aid in pathogen adhesion and cell wall degrading enzymes (CWDE) are critical to ensure host tissue penetration and colonization. 2) Attack: Effector molecules manipulate host physiology and suppress plant defence responses. Some effectors elicit host defence responses if recognized by the host surveillance system. 3) Defence: Mitogen-activated protein (MAP) kinases aid in signal transduction and communication to enable a rapid response to the host defence strategies. Proteins involved in metabolite transport, detoxification, protection against reactive oxygen species (ROS) and protease inhibitors help to protect the pathogen from host attack. PAMP-triggered immunity (PTI) is a basal defence response resulting from host recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs), while effector-triggered immunity (ETI) is activated when effector proteins are detected by host resistance proteins (R proteins). NIP – necrosis-inducing protein, CRN – Crinkling- and necrosis-inducing protein.

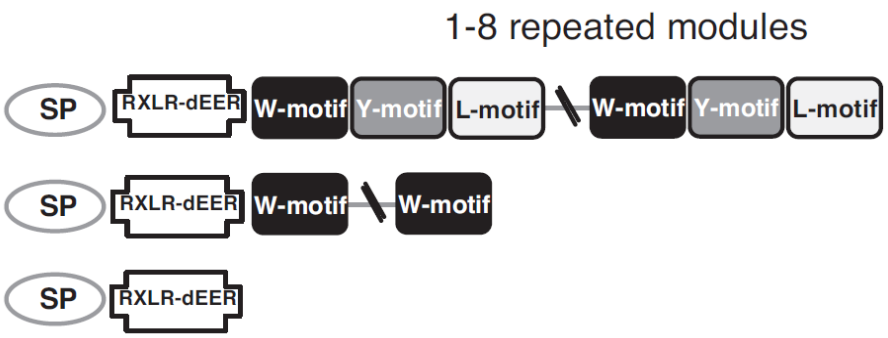

Figure 8. Structure of cytoplasmic RXLR effector family proteins present in biotrophic oomycete pathogens. RXLR effector proteins are named after the four amino acid motif (Arg-any amino acid-Leu-Arg) that follows a signal peptide (SP) in the N-terminal of the protein. The C-terminal, containing the less conserved effector domain, may contain none to numerous repetitions of W-, Y- and /or L-motifs (Jiang et al. 2008) underpinning a conserved structural fold in the mature protein (Boutemy et al. 2011). Image obtained from Jiang et al. (2008).

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CHAPTER 2

RNA-seq analysis of cysts and germinating cysts of *Phytophthora cinnamomi*

ABSTRACT

Phytophthora cinnamomi is an economically important pathogen that is able to successfully infect more than 3 000 plant species. As a hemi-biotrophic pathogen, it requires an arsenal of proteins that enables it to surpass the host defence system and facilitate infection of living hosts. We aimed to identify the repertoire of pathogenicity related genes to this oomycete. An RNA-seq library of the cyst and germinating cyst developmental stage of *P. cinnamomi* was produced using the Illumina sequencing platform. Over 70 000 unigenes were identified from 225 049 contigs that were assembled from 13 million paired end reads. Contaminant sequences were eliminated using bioinformatic tools, resulting in 37 534 unigenes showing homology to *Phytophthora* gene models which were used in further analysis. More than 2 000 unigenes had a putative role in pathogenesis. Cell wall degrading enzymes and genes involved in the protection against oxidative stress were most abundant among them. This included polygalacturonase, glucanase, glucosidase and reductase enzymes. Many transcripts that aid in detoxification and metabolite transport (cytochrome P450 and ABC transporters) were present. A preliminary search revealed 46 putative RXLR effector genes, together with elicitin-like and necrosis-inducing protein transcripts. This work is the first step towards understanding the molecular basis that is responsible for *P. cinnamomi*'s success as a wide host-range pathogen.

INTRODUCTION

Phytohthora cinnamomi Rands is a hemi-biotrophic oomycete that belongs to the kingdom of the Chromista (Hardham 2005). This pathogen causes rot of feeder roots of woody plants, eventually leading to chlorosis on leafs, wilting and dieback of trees (OEPP/EPPO 2004). In some species, stem cankers and dieback of young shoots are also observed (Hardham 2005). As a broad host range pathogen, with a nearly worldwide distribution (OEPP/EPPO 2004), it is a threat to natural ecosystems, biodiversity, forestry and agriculture. The economic impact due to *P. cinnamomi* infestation is evident in the food industry (avocado, pineapple, peach, chestnut and macadamia) and the forest industry, affecting eucalyptus, pine and oak trees (Zentmyer 1980; Zentmyer 1985). Losses due to *Phytophthora* infestation not only include decreases in crop yield and product value, but also large amounts of money spent annually on control measures.

As a soil-borne pathogen there are no means to eradicate *P. cinnamomi* from invested areas as it can survive in moist soil or dead plant material as chlamydospores for up to six years (Zentmyer and Mircetich 1966). The main effective control strategy to date is the use of phosphite injections (chemical), which has proved helpful in combating *P. cinnamomi* in *Persea americana* (Darvas et al. 1984), *Eucalyptus marginata* and *Banksia* species (Shearer and Fairman 2007). Mulches (a combination of compost and soil) are also widely used to control *Phytophthora* diseases (Richter et al. 2011), as it enhances natural microbial activity and increases exogenous cellulose enzymes that target *Phytophthora* cell walls (Downer et al. 2002). Other control measures focus on spread prevention, including road side sanitation, vehicle washing (Goheen et al. 2012) and treatment of circulating irrigation water (Hao et al. 2012). In some industries, such as avocado production, farmers strongly rely on tolerant rootstocks to produce sufficient fruit in the presence of *P. cinnamomi* (Smith et al. 2010).

Phytophthora cinnamomi is heterothallic but preferably reproduces asexually, even with the presence of both the A1 and A2 mating types (Hardham 2005). The main agent of infection in both cases is uninucleate, biflagellate, motile zoospores that are released from sporangia (Hardham 2005). They are spread to suitable hosts through rain splash and soil movement (soil attached to vehicle wheels etc.) (Podger et al. 1965) and locate living plant material by responding to chemo- and electrotactic signals (Zentmyer 1961). Contact with plant cell wall carbohydrate molecules induces zoospore encystment (Irving and Grant 1984; Hardham and Suzaki 1986), followed by tissue penetration that occurs through specialized structures

called appressoria. Within two to three days, sporangia appear on the plant surface (Hardham 2005) and the asexual reproduction cycle may repeat several times, rapidly increasing the inoculum at a single site.

Disease results from a compatible plant-pathogen interaction, where the pathogen successfully evades host detection and invades and colonizes host tissue. In an incompatible plant-pathogen interaction a strong phenotypic response is observed at the site of infection, involving localized cell death (hypersensitive response) (Jones and Dangl 2006) and callose deposition (Benhamou and Nicole 1999) which effectively restricts pathogen growth. This is the result of recognition of pathogen-associated molecular patterns (PAMP) by pathogen recognition receptors (PRR) (so called PAMP-triggered immunity) and detection of avirulence factors by host resistance proteins (effector-triggered immunity) (Jones and Dangl 2006; Tsuda and Katagiri 2010). To understand this system, insight into both plant defence pathways as well as knowledge of pathogenicity genes (especially the pathogen effector repertoire) is critical.

Pathogenicity genes are defined as pathogen derived proteins or molecules that enable infection of a suitable host. They may act at different times (early or late infection) and at various locations (apoplast, cytoplasm, nucleus). RXLR effector Avrblb2 from *Phytophthora infestans* (Mont.) de Bary acts at the haustorial interface, preventing the secretion of host papain-like cysteine protease C14 into the apoplast, thereby enhancing potato susceptibility to *P. infestans* (Bozkurt et al. 2011). Necrosis- and ethylene inducing peptide (NEP1) (characterized from *Fusarium oxysporum)* on the other hand, is targeted to the host cell wall and cytosol, assisting plant cell death as part of disease development in necrotrophic pathogens (Bae et al. 2006). Expression analysis of NEP1-like proteins in *Phytophthora sojae* Kaufm. & Gerd. indicated two main induction points namely germinating cysts and during late infection of soya bean (Ye et al. 2011). Identification and functional characterization of pathogenicity genes gives insight into the mechanisms employed by pathogens to establish infection.

Little is known about the molecular events that govern a successful *P. cinnamomi* infection. Transciptomic analysis proved a useful tool in identifying infection related transcripts in other oomycete species (Panabières et al. 2005; Randall et al. 2005; Chen et al. 2007). This approach involved sequencing of cDNA clones and expressed sequence tags (ESTs) from various developmental structures, yielding a few thousand ESTs for analysis (Qutob et al. 2000; Škalamera et al. 2004; Panabières et al. 2005; Krajaejun et al. 2011). The expansion of available genomic data and the application of next generation sequencing technology to

transcriptome analysis now enable the identification of almost all transcripts present in a sample, including low abundant transcripts that remained elusive in previous approaches (Strickler et al. 2012). Not only does this approach allow identification of novel transcripts, alternative spliced variants and single nucleotide polymorphisms, it also allows transcript quantification (Wang et al. 2009; Strickler et al. 2012).

RNA-seq has emerged as the preferred technology for gene expression analysis as it by far exceeds the sensitivity and depth provided by microarray analysis (Wang et al. 2009). Ye et al. (2011) successfully profiled the expression of 30 pathogenicity gene families from *P. sojae*, during *in vito* growth as well as *in planta* infection. Currently the genomes of seven oomycete species have been published (Tyler et al. 2006; Haas et al. 2009; Baxter et al. 2010; Lévesque et al. 2010; Lamour KH et al. 2012; Jiang et al. 2013) and can be used to aid sequence assembly and transcript annotation. RNA-seq technology can also be applied to organisms lacking a reference genome through *de novo* assembly (Strickler et al. 2012; Ward et al. 2012).

In this chapter, we generated the first RNA-seq library of the cyst and germinating cyst stages of *P. cinnamomi* from a high sporulating isolate. Paired end reads were *de novo* assembled and annotated based on homology to *P. infestans* protein models. More than 2 000 pathogenicity transcripts were identified that will serve as candidates for further investigation.

MATERIALS AND METHODS

Identification and culturing of *P. cinnamomi* isolates

All *P. cinnamomi* isolates used in this study were obtained from the *P. cinnamomi* collection maintained by the Fruit Tree Biotechnology Programme at the University of Pretoria, South Africa. This included isolates originating from avocado trees in the Tzaneen area in South Africa (Table 1). Cultures were purified from long term storage stock by isolating a single hyphal tip from water agar onto ½ potato dextrose agar (PDA) (19.5 g/L PDA (Merck, Darmstadt, Germany), 7.5 g/L agar and distilled water). Isolates were maintained on 5 % V8 (vegetable juice) agar (cleared V8 juice (Campbell Soup Company, USA), 0.1 g/L $CaCO_3$, 20 mg/L *B*-sitosterol, 17 g/L agar and distilled water).

Isolate identities were confirmed by a species specific PCR, using primers directed against the *LPV3* gene (Kong et al. 2003). DNA was extracted from *P. cinnamomi* mycelia using the PrepMan™ Ultra Reagent according to the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The PCR was performed on the 2720 Thermal Cycler (Applied Biosystems) under the following cycling conditions: 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min, finishing with an 8 min incubation at 72 °C. Each PCR reaction of 25 µl contained 2.5 µl 10X PCR reaction buffer (Roche Diagnostics, Mannheim, Germany), 200 mM dNTPs (Bioline, London, UK), 3 mM $MgCl_2$, 0.2 µM of each *LPV3* primer, 2 units Fast Start Taq polymerase (Roche Diagnostics), Sabax water and 25 ng purified DNA. PCR products were analysed with gel electrophoresis (2 % agarose gel, 80 V) yielding a product of 450 bp in size.

Screening of the sporulation ability of *P. cinnamomi* isolates

Phytophthora cinnamomi is known to produce variable amounts of zoospores. This study required a high sporulating isolate to enable the collection of a large amount of zoospores. To identify a high sporulating isolate, 12 *P. cinnamomi* isolates were screened for their ability to produce sporangia. Agar blocks (5 % V8 agar), 2 x 2 mm in size, containing a 3-day-old *P. cinnamomi* culture, were placed in 90 mm petri dishes filled with 20 ml distilled water. The water was replaced after 4 hours incubation at 23 °C under florescent light and left overnight. Sporangia production was scored by counting the amount of sporangia produced per agar plug under a confocal microscope. A value of 0 to 3 was assigned per block, where a score of 0 represented no sporangia, 1 represented 1-7 sporangia, 2 represented 7-15 sporangia and a score of 3 was assigned to blocks containing 16 sporangia or more. Sporangia that had already released zoospores were also considered when assigning scores. This process was repeated three times, analyzing three agar plugs per isolate.

Evaluation of the growth of isolate CNVDB1 and CNVDB29 on different media at different temperatures

The growth rate and morphology of a high sporulating isolate (CNVDB29) and a low sporulating isolate (CNVDB1) was compared on three types of culture media across six temperatures. A mycelium agar plug (6 mm in diameter) was placed in the centre of each 90 mm petri dish (containing 20 ml of agar) with the mycelia facing downwards. This was done for each isolate on ½ PDA, cornmeal agar (CMA) (17 g/L CMA and distilled water) and 5 % V8 agar. The cultures were incubated at 10, 15, 20, 25, 30 and 35 °C. Each unique condition

(isolate, media and temperature) contained five biological replicates. The diameter of each culture was measured on the horizontal and vertical axis, after four days of growth.

Statistical analysis

Data generated from growth analysis of isolate CNVDB1 and CNVDB29 was statistically analyzed using analysis of variance (ANOVA) followed by a Least Square Means Differences Student's t-test using the JMP '9 software ($p>0.05$) (SAS Institute, Cary, North Carolina).

Enrichment for germinating cysts

Zoospores of isolate CNVDB29 were produced using an adjusted protocol of Chen and Zentmeyer (1970). Sporangia were produced by incubating multiple small 10 % V8 agar blocks (approximately 2 x 2 mm in size) containing a 3-day-old *P. cinnamomi* culture, in 5 % V8 broth (cleared V8 juice, 0.1 g/L $CaCO_3$, 20 mg/L *B*-sitosterol, distilled water). V8 agar plates contained 17 g/L agar in addition to the reagents present in V8 broth. After two days incubation under fluorescent light at 23 °C, the mycelia plugs were washed with three changes of distilled water and incubated under the same conditions in filtered river water until sporangia developed (1½ days). The river water was filtered twice through chromatography paper (MN260 Macherey-Nagel, Düren, Germany). Zoospore release was induced by washing the sporulating mycelia with two changes of distilled water at 4 °C, followed by incubation at 4 °C for 12 min. Zoospore release started approximately 1 hour later, while being incubated at 23 °C. The spores were harvested through a nylon sieve and counted with the Superior haemocytometer (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany). On average zoospore concentrations of $4x10^4$ spores/ml were obtained. To enrich for germinating cysts, zoospores were vigorously shaken for 2 min to induce encystment and incubated in the dark at 19 °C for approximately 90 min. Cysts and germinating cysts were harvested via centrifugation (10000 rpm at 4 °C for 5 min) in the Centrifuge 5810 R (Eppendorf, Hamburg, Germany). Pellets were immediately frozen in liquid nitrogen and stored at -70 °C. Cysts and germinating cysts were photographed with the AxioCam MRc (Zeiss, Göttingen, Germany) coupled to the Axioskop microscope (Zeiss).

RNA extraction and quality assessment

Tri-Reagent (Ambion, Life technologies, Carlsbad, CA, USA) was used, according to the manufacturer's instructions, to obtain total RNA from cysts and germinating cysts. All RNA

samples were treated with DNase I enzyme (Fermentas, Life Sciences, Hanover, USA) and purified using the Qiagen RNeasy cleanup kit according to the manufacturer's instructions (Qiagen, Valencia, California, USA). RNA concentrations and OD 260/280 and 260/230 ratios were determined with the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA). The final RNA quality, of the sample to be sequenced, was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Sequencing and cDNA library assembly

A cDNA library of cysts and germinating cysts of *P. cinnamomi* was prepared by the Beijing Genomics Institute (BGI) through fragmentation of mRNA enriched RNA (oligo dT-primers) and random hexamer primed cDNA synthesis. The paired end cDNA library was sequenced on the HiSeq2000 Illumina platform. BGI assembled reads into contigs using Trinity (Grabherr et al. 2011). Gaps between contigs from the same transcript were filled with the remaining reads and residual gaps then filled with N's to obtain unigenes. This was achieved by aligning unigene sequences with @blastdb via blastx (E-value >1x10^{5}). Accordingly, unigenes are sequences that contain minimal unassigned nucleotides and could not be extended on either side. The orientation and coding regions of unigenes were determined according to the best blast hit in the National Centre for Biotechnology Information (NCBI) non-redundant protein database, the Swiss protein database, Clusters of orthologous groups (COG) database and KEGG classes (in that order). ESTScan (Iseli et al. 1999) (http://estscan.sourceforge.net/) was used to predict the coding region and direction of unigenes in cases where the unigenes did not significantly align to any protein sequences in the mentioned databases. The depth of each unigene was calculated by dividing the base number of mapped reads by the unigene sequence length. Unigene coverage was determined by the ratio of the base number on unigene covered by reads and the unigene sequence length.

Functional annotation

Unigenes were annotated using the blastx algorithm (E-value >1x10^{5}) against the NCBI non-redundant protein database, the Swiss protein database, Clusters of orthologous groups database as well as the protein models predicted in the genomes of *P. infestans* (*Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/ downloaded February, 2013) and *P. cinnamomi* v1.0 (http://genome.jgi.doe.gov/Phyci1/Phyci1.home.html, downloaded March, 2013).

Signal peptides were predicted in unigene amino acid sequences starting with methionine, using neural networks and Hidden Markov Models (HMM) (Nielsen and Krogh 1998) in SignalP v3.0 (Nielsen and Krogh 1998; Bendtsen et al. 2004; Emanuelsson et al. 2007) employing a Sprob cut-off: 0.7 (Kebdani et al. 2010). Sequences containing predicted transmembrane helices downstream of the signal peptide as predicted by TMHMM v2.0 default parameters (Krogh et al. 2001) were discarded.

The method described in Whisson et al. (2007) was used to identify putative RXLR sequences using Hidden Markov Models (HMM). The longest coding DNA sequence (CDS) was identified in each contig, allowing open ends, but not open starts to a CDS. Reg-ex was used to analyse the candidate sequences for the physical presence of an RXLR sequence (Arg-any amino acid-Leu-Arg). Putative RXLR proteins were compared to predicted RXLR effector genes from *P. infestans* (http://www.broadinstitute.org/ downloaded February, 2013), *P. sojae* (Wang et al. 2011) and *Hyaloperonospora arabidopsidis* (Cabral et al. 2011) via blastx analysis using cut-off values as in Tyler et al. (2006) and Jiang et al. (2008) (E- value >1x10^{8}). Protein sequences were aligned with Mafft Version 7 (Katoh and Standley 2013), trimmed in MEGA 4 (Tamura et al. 2007) and visual outputs created with GeneDoc Version 2.7.000 (Nicholas et al. 1997).

RESULTS

Screening of the sporulation ability of *P. cinnamomi* isolates

Zoospore production in *P. cinnamomi* is known to be inconsistent. Numerous isolates (as listed in Table 1) were screened for their ability to produce high amounts of sporangia (resulting in higher amounts of zoospores) constantly. A species specific PCR, targeting the *LPV3* gene of *P. cinnamomi* (Kong et al. 2003), confirmed the identity of all twelve isolates that were assessed for sporulation ability (Figure 1). Isolate CNVDB29 produced the most sporangia (>16 sporangia per agar block) consistently across three independent experiments (Figure 2). Isolate CNVDB1 scored the lowest in the sporangia production screening, with a mean score of 0.25 and a standard deviation of 0.433. Isolate CNVDB29 was therefore selected to generate material for RNA-seq analysis.

Characterization of isolate CNVDB1 and CNVDB29 in terms of growth

The optimal temperature (as a measure of most growth after four days) was determined for isolate CNVDB1 and CNVDB29 at temperatures 10 to 35 °C. Isolate CNVDB1 was regularly used in laboratory experiments, requiring prolonged maintenance in culture. In contrast to this isolate CNVDB29 was freshly isolated from the field, six months prior to this study. Both isolate CNVDB1 and CNVDB29 grew the slowest at 10 °C and 35 °C on all culture media (Table 2). Isolate CNVDB1 grew the most at 25 or 30 °C after four days on all three nutritional media. The optimal temperature for isolate CNVDB29 was similar to that of isolate CNVDB1 on ½ PDA and CMA, but was lower (20 or 25 °C) on V8 agar. The optimal growth temperature differs between isolates when compared on the same nutritional media.

The effect of media constitution on the growth of both isolates was evaluated at 25 °C. Isolate CNVDB1 grew significantly slower on CMA than on ½ PDA at 25 °C, but did not differ significantly between culture media ½ PDA and V8 agar (Table 2). The growth rates of isolate CNVDB29 differed significantly between each type of nutritional media. The growth rate of isolate CNVDB1 was compared to isolate CNVDB29 on ½ PDA, CMA and V8 agar at 25 °C. Isolate CNVDB29 grew significantly slower than isolate CNVDB1 on ½ PDA at 25 °C, while CNVDB29 grew more rapidly than CNVDB1 on CMA agar. The growth rate did not differ significantly between the two isolates on V8 agar.

Morphological differences were observed between isolates CNVDB1 and CNVDB29 when compared on the same culture media. This difference in appearance between isolates was most prominent on ½ PDA and V8 agar (Figure 3). Isolate CNVDB1 presented with the expected rosette pattern, characteristic of *P. cinnamomi* on ½ PDA (Gerrettson-Cornell 1989), while isolate CNVDB29, grew in a white, dense, radial shape. Isolates CNVDB1 and CNVDB29 looked morphologically more similar on CMA, both growing in rosette patterns, but isolate CNVDB29 appeared whiter and more vigorous than isolate CNVDB1. On V8 agar, isolate CNVDB29 again seemed to grow more vigorous than isolate CNVDB1, forming bumps on the surface of the agar, while isolate CNVDB1 grew uniform in the bottom zone and on top of the agar. The hyphae of isolate CNVDB1 seemed slightly thinner and appeared more transparent than those of isolate CNVDB29 on V8 agar.

RNA extractions and quality assessment

High quality total RNA was extracted from cysts and germinating cysts of isolate CNVDB29 of *P. cinnamomi* (Figure 4). To meet the requirement of 40 µg of total RNA required by BGI to allow for cDNA library construction, RNA from multiple experiments were pooled (Table 3).

The final concentrations of the subsets of pure RNA, as determined with the Nanodrop, ranged from 260 to 1500 ng/µl. The wavelength emission ratio's (both OD 260/280 and OD 260/230) of all subsamples were higher than 1.95. The final RNA library did not contain equal amounts of RNA from each biological replicate. An RNA integrity number (RIN) of 8.6 (>8) was assigned to the final RNA library that was used for cDNA library construction and sequencing (Table 4).

Sequencing and assembly of the cyst and germinating cyst cDNA library of *P. cinnamomi*

A cDNA library of the cyst and germinating cyst phase of *P. cinnamomi,* isolate CNVDB29, was sequenced on the Illumina HiSeq 2000 platform, generating 13 032 666 paired end reads (1 G data). From the 1 billion nucleotides sequenced, 0.01 % could not be assigned unambiguously (N percentage) with a Q20 value of 95.3 % (Table 5). This indicates that 95.3 % of the nucleotides sequenced have 99 % accuracy, with an error rate of 1 in a 100. Overlapping reads were *de novo* assembled into 225 049 contigs (Table 6). The majority of the contigs (67.21 %) were 100-200 nt in length (mean contig length = 213 nt, N50 = 257 nt) (Table 6 and Figure 5), which is consistent with the mean read length currently obtained with this sequencing technology (2 x 100 nt for a paired end library). Almost 10 % of the contigs were 400 nt or longer in length. In total 72 220 unigenes were identified in the dataset by combining contigs originating from the same transcript. Over 80 % of the unigenes were 100-500 nt in length (mean length = 386 nt, N50 = 391 nt) (Figure 6). Unigene coverage ranged from 4.86 % to 99.97 %, with 66 % of the unigenes having coverage of 90 % or more. Unigene depth ranged from 0.0549 to 6066.

Cyst and germinating cyst cDNA library annotation

Functional annotation based on protein sequence homology

Unigenes were annotated based on homology (E-value >$1x10^5$) to protein sequences in publicly available databases (NCBI non-redundant protein database [Nr], Swiss protein database [Swiss]) and to protein models predicted in the genomes of *P. infestans* and *P. cinnamomi* (Figure 7). It is standard to use the Nr and Swiss databases to annotate sequence data of organisms lacking genomic data (as was the case when this project was initiated). The genome of *P. infestans* was utilized, as it represents a species closely related to *P. cinnamomi* and has been revised frequently since its release in 2009 (Haas et al.

2009). Of the 72 220 unigenes, 16.47 % could not be assigned a putative function as it did not share significant sequence homology with any proteins in the four databases (Table 7). The remaining unigenes showed homology to proteins in at least one database (68.5 % with Nr and Swiss proteins, 47.47 % with *P. infestans* proteins and 50.54 % with *P. cinnamomi* proteins) with some unigenes showing homology to unigenes from various databases (Figure 8). Approximately 22 745 unigenes showed homology to proteins in all three datasets (Nr and Swiss, *Pi* and *Pc* annotated datasets).

The unigenes showing homology to proteins in the non redundant protein database included only one unigene that showed homology to a gene originating from a *Phytophthora* species and one from *Pythium* (data not shown). Further investigation into the species highly represented in the Nr annotated unigene dataset revealed that approximately 42 % of the genera belonged to the domain bacteria and 8.7 % to the domain fungi (Figure 9). These unigenes (that do not show homology to *Phytophthora* proteins) were regarded as contaminant sequences (likely originating from other organisms present in the river water used to induce sporulation) and were not included in further analysis.

The remainder of the study focused on unigene sequences that showed homology to either *P. infestans* or *P. cinnamomi* protein sequences (37 534 unigenes) (Figure 8). A total of 8.7 % of these unigenes (3 254 unigenes) only showed homology to *P. cinnamomi* protein models and were not present in the *Pi* annotated dataset. The *Pc* annotated dataset provides confidence that transcripts originate from *P. cinnamomi*. The functional annotations indicated in the *Pi* annotated unigene dataset (representing 53 % of the unigenes), however was used to infer unigene function. This is because the *P. infestans* genome annotations are likely more reliable (revised multiple times) than those of the *P. cinnamomi* genome (which is only in its first version, has not been published and is based on homology to other *Phytophthora* proteins).

Each unigene does not represent a single gene of *P. cinnamomi*. Unigenes are only fragments of transcripts, as total RNA is fragmentized prior to cDNA synthesis to enable sequencing. Accordingly, many unigenes may originate from the same mRNA transcript, with multiple copies of identical mRNA transcripts in a sample (depending on gene expression levels). To estimate the amount of unique genes that were expressed in the cyst and germinating cysts sample, only a single representative of each unique protein identity (from the *Pi* annotated unigene dataset) was retained. A unique gene, in this study was defined as a unique protein identity, where numerous unigenes could correspond to a single protein ID based on protein homology. A total of 10 209 unigenes with unique annotations

(unique *Pi* protein identity numbers) remained. This included 80.42 % (7 707 unigenes) of the 9 583 core *Phytophthora* orthologous genes, predicted by Haas et al. (2009). Within the *Pi* annotated unigene dataset, only 6.3 % represented ribosomal subunit RNA and 36 % were hypothetical proteins.

Clusters of orthologous groups (COG) functional classification

The clusters of orthologous groups of proteins (COG) database provided functional annotations for 48.37 % of the 37 534 unigenes in the *Pi* and *Pc* annotated unigene datasets. Enrichment analysis divided the unigenes into 26 functional categories (Figure 10). The function of approximately 4.25 % of unigenes with homology to proteins in the COG database (771 unigenes) was unknown. Highly represented classes (excluding general function prediction only) included those involved in translation (21.73 %), post translational modification (10.24 %) and amino acid transport and metabolism (9.66 %). A total of 30.78 % of transcripts were dedicated to transport and metabolizing compounds (including amino acids, carbohydrates, lipids, nucleotides, coenzymes and inorganic ions). Unigenes involved in energy production and conversion were also abundant (7.4 %). Unigenes that play a role in replication and cell wall biogenesis (total of 1 871 unigenes) support the process that is associated with cysts germination. Functional categories that could potentially contribute to pathogenesis (1 457 unigenes) include signal transduction mechanisms (4.25 %), secretion (1.99 %) and defence mechanisms (1.25 %). The defence mechanism category mainly comprised of proteins involved in transport (215 unigenes). A single vancomycin resistance protein formed part of this category supporting *Phytophthora* resistance to vancomycin reported previously (Eckert and Tsao 1962).

Putative secreted proteins

Secreted proteins can be identified by the presence of a signal peptide in the N-terminal of a protein (SignalP v3.0 Sprob >0.7). The start codon (methione) could be identified in the predicted open reading frame of 2 245 of the 37 534 unigenes in the *Pi* and *Pc* annotated unigene datasets (Figure 7). Unigenes lacking a start codon were excluded from this analysis as we could not be confident that the unigene sequence represented the first 40 amino acids of proteins. A total of 113 unigenes contained a predicted signal peptide. Three of the unigenes containing predicted signal peptides were eliminated due to the presence of predicted transmembrane helices (TMH), suggesting that they will not be secreted but retained in cell membranes. Ninety eight unigenes had a function assigned based on homology to the *P. infestans* protein models and included nine conserved

hypothetical proteins and 16 unigenes that were identified as pathogenicity genes later in this study (as listed in Table 9). Some unigenes were only present in the *Pc* annotated unigene dataset (11 unigenes) and may be *P. cinnamomi* specific (includes Unigene12323, Unigene15167, Unigene19168, Unigene24069, Unigene32988, Unigene37984, Unigene40431, Unigene5155, Unigene54017, Unigene55798 and Unigene58247). Another 25 unigenes with a predicted signal peptide (lacking TMH) did not show homology to proteins in any of the databases (part of the 16.47 % unannotated unigenes in Table 7).

Potential RXLR effector genes

Computational methods were employed to identify putative RXLR-motif (Arg-any amino acid-Leu-Arg) containing transcripts from the longest CDS (containing a start codon) identified in each contig using HMM (Figure 7) (Whisson et al. 2007). The CDS used in this analysis depended on computational predictions considering nucleotide sequence, while the CDS used to define unigenes depended on sequence homology to existing predicted CDS in protein databases (identified by blastx analysis). As RXLR effector genes are expected to be under high selective pressure (frequently evolving), considering all potential CDS (regardless of homology to known proteins) is important. Therefore the longest CDS in each contig was considered for identification of putative RXLR proteins and not the identified unigene sequences. A total of 46 putative RXLR effector transcripts were identified, with 21 sequences harbouring a predicted signal peptide (Table 8). Five of the 46 sequences, although predicted by the HMM, did not contain an actual RXLR sequence. Four of the predicted RXLR transcripts did not show significant homology to proteins predicted in the *P. cinnamomi* genome (blastx E-value >1x10^5).

Pathogenicity related genes identified from the cyst and germinating cyst cDNA library of *P. cinnamomi*

Specific pathogenicity candidate genes

The *Pi* annotated unigene dataset was manually screened for specific pathogenicity candidate genes as reported in the literature. To do this, the putative pathogenicity gene categories used in Torto-Alalibo et al. (2007) was adjusted (Table 9). The number of unigenes with annotations that fall within each category is indicated in column 2. As various unigenes (e.g. unigene 5 and 17) in some cases correspond to a single *Pi* protein identity number (e.g. PITG2648), we eliminated functional redundancy by removing unigenes with

identical annotations. Column 3 indicates the putative number of unique transcripts (without redundancy) that fall within a pathogenicity gene category.

The selection of pathogenicity unigenes depended on matching specific search terms (e.g. glucanase), to gene names in the *Pi* annotated unigene dataset. To ensure that all potential pathogenicity candidate unigenes were included, alternative names for some proteins were considered. In all instances proteins described as '–like' were included (e.g. Adhesin-like protein) together with the actual gene name (e.g. adhesin). Flocculation protein was incorporated under the category mucins and enolase under the category lyases. Cathepsin Z was included in the category cysteine proteases. The group representing protease inhibitors also contained ecotin and proteins with a Kazal-domain.

The most represented gene catagories were hydrolases, followed by genes involved in the protection against oxidative stress and detoxification (Table 9). Effector genes were also highly abundant, representing 85 unigenes. It is interesting that no cellulase enzymes were present in the *Pi* annotated unigene dataset. Closer investigation indicated that unigenes showing homology to cellulase enzymes in the Nr and Swiss databases were annotated as hydrolase-, glucanase- or glucosidase enzymes (that also have the ability to digest cellulose) in the *Pi* annotated dataset. Unigenes annotated as glutathione synthase, ubiquitin proteases and cell surface glycoproteins were absent. A total of 2 392 pathogenicity related unigenes were identified from the *P. infestans* annotated unigene dataset.

RXLR effector gene homology

Analysis using the blastx algorithm (E-value >1x10^{8}) revealed four sequences with significant protein sequence homology to known RXLR proteins of *P. sojae* and *P. infestans* (Table 10). There was no significant homology to any of the 17 predicted RXLR proteins of *Hyaloperonospora arabidopsidis* (Gäum.) (Cabral et al. 2011). Closer analysis of the aligned protein sequences (Figure 11) and the alignment statistics (amino acid similarity >70 %) provides a better indication of protein similarity. Transcript C14063 and C15073 represented the full length of the corresponding best protein hit (with amino acid similarity < 70 %). The sequence C31843 covered 50 % of PsSNEL2 (83 % similarity) and C83210 covered 71 % of PsAvh448 (60 % similarity) and thus do not represent the full length transcript. C31843 (only 95 amino acids in length) shared 100 % identical amino acids with Phyci1-16230 that is 158 amino acids in length (predicted protein from the *P. cinnamomi* genome) and may represent the full length transcript of C31843. Phyci1-16230 and SNEL2 shared 66 % identical- and 71 % similar amino acids (with 16 % gaps).

The percentages gaps present in the alignments are in some cases accounted for by stretches of amino acids present in one sequence but absent in the other. For example, in the alignment of C31843 to SNEL2 from *P. sojae* (an ortholog of PsSNEL1), a stretch of 20 consecutive amino acids, present in SNEL2 and absent in C31843, accounts for the 22 % non-identical amino acids (Figure11 C). In a similar way, C83210 had two stretches of 8 and 10 amino acids respectively, that is present in the *P. cinnamomi* contig, but absent from PsAvh448 (the best blast hit in *P. sojae*) (Figure 11 D). This could result from insertions, deletions or alternative splicing events within the different species.

The protein models predicted in the *P. cinnamomi* genome were not always the same as the open reading frame (ORF) identified from the transcriptomic data. For example, Phyci1-172110 (predicted from the *P. cinnamomi* genome), does not contain the first 58 amino acids present in the reading frame of C15073 and accordingly the RXRL-motif is not present in Phyci1-172110 either (Figure 11 B).

DISCUSSION

In this study more than 2 000 pathogenicity genes were identified from the *in vitro* pre-infection structures of the hemi-biotrophic pathogen, *P. cinnamomi*. The cyst and germinating cyst dataset was filtered to eliminate contaminant sequences by selecting genes with homology to *Phytophthora* species (*P. infestans* and *P. cinnamomi*). The resulting 37 534 unigenes were used in further analysis (from which 8.7 % were only present in *P. cinnamomi* and not in *P. infestans*). Pathogenicity unigenes identified in *P. cinnamomi* included unigenes with homology to gene families implicated in pathogenesis in the literature (>2 000 unigenes), 110 putative secreted unigenes (containing a signal peptide and lacking a TMH) and 46 unigenes containing an RXLR-motif. Pathogenicity gene families included, among others, 20 unigenes with homology to protease inhibitors, nine necrosis inducing *Phytophthora* proteins, four small cysteine rich proteins and various cell wall degrading enzymes.

This is the first large scale transcription analysis of cysts and germinating cysts of *P. cinnamomi*. Reads were assembled, using an assemble-then-align approach as illustrated for Illumina sequence data from the *Phytophthora* - *Rubus idaeus* (red raspberry) interaction (Ward et al. 2012). This approach surpasses the problem of mapping reads that contain

exon-exon boundaries back to a genome. Repeating the assembly using the genome of *P. cinnamomi* (recently made available, but not published) as reference sequence, may improve the quality of the assembly and accuracy of predicted protein models from this dataset. Accordingly, both *de novo* assembly and assembly to a reference genome provide valuable information.

The increased sensitivity and high through-put provided by RNA-seq technology (Strickler et al. 2012), enabled the identification of 34 280 unigenes (showing homology to *P. infestans* protein models) representing 10 209 unique transcripts from 72 220 unigenes. This provides a more comprehensive overview of the cyst and germinating cyst developmental stage than the few thousand expressed sequence tags (maximum 2 500 ESTs) obtained in similar single stage transcriptomic studies, in the past (Qutob et al. 2000; Škalamera et al. 2004; Krajaejun et al. 2011). This approach allowed the identification of low abundant transcripts, which included some pathogenicity genes reported to be expressed at low levels during cyst germination in other studies (e.g. RXLR effector genes and polygalactorunases (Ye et al. 2011)).

Previous infection studies performed *in planta* reported very low- to no retrieval of pathogen derived transcripts after sequencing, due to low pathogen mass relative to plant tissue in the sample (Beyer et al. 2001; Chen et al. 2011). Studying the pre-infection structure (germinating cysts) to gain insight into infection related genes has proved helpful in surpassing this problem (Shan et al. 2004; Grenville-Briggs et al. 2005; Guo et al. 2006; Chen et al. 2011). Germinating cysts represent a critical structure in oomycete infection and are accompanied by large scale transcriptional reprogramming, including upregulation of infection related genes (Chen et al. 2011; Ye et al. 2011; Chen et al. 2013). Accordingly, the transcriptome of the cyst and germinating cyst stage of *P. cinnamomi* was investigated with the aim of identifying pathogenicity related genes.

The isolate (CNVDB29) used in this study was selected because it produced the most zoospores consistently and grew vigorously in culture. The growth rates of this high sporulating isolate (CNVDB29) were compared to a low sporulating isolate (CNVDB1) on three different media across various temperatures. The cardinal temperatures for both isolate CNVDB1 and CNVDB29 were consistent with the temperatures reported in the literature (minimum 4-16 °C, optimal 20-32.5 °C, maximum 30-36 °C) (Zentmyer et al. 1976; Gerrettson-Cornell 1989), although the optimal temperature of isolate CNVDB29 was slightly lower (25 °C) on V8 agar than isolate CNVDB1. The two isolates responded differently to various nutritional contents and also appeared morphologically different especially on ½

PDA. This is consistent with Zentmyer et al. (1976) that reported varying growth rates (ranging from 41 – 85 mm after four days incubation on at 25 °C on PDA) between isolates on the same media. The authors also noted that isolates grew at different rates in response to different nutrition content. Isolate CNVDB1 produced less zoospores and grew less vigorously on V8 agar than isolate CNVDB29. Prolonged maintenance of an isolate on media in the laboratory with repeated transfers can decrease vigour, virulence and especially ability to sporulate in fungi (Nakasone et al. 2004). The same could be true for oomycetes as may be the case for isolate CNVDB1 which was regularly used in laboratory experiments.

Isolate CNVDB29 was selected to produce the first cyst and germinating cyst cDNA library of *P. cinnamomi* with the aim of identifying pathogenicity related transcripts. Functional enrichment analysis of the cyst and germinating cyst dataset of *P. cinnamomi* indicated that genes involved in metabolism (30.78 %), translation, posttranslational modification and protein turnover (31.97 %) and energy production and consumption (7.4 %) were most abundantly in this developmental stage. A study by Chen et al. (2011), found similar dominant functional classes during functional enrichment analysis of a pooled infection related subtractive hybridization library consisting of genes enriched in *Phytophthora cactorum (*Lebert and Cohn) J. Schröt. germinating cysts and strawberry infection. Transcripts associated with the transcription process were highly represented in the *P. cactorum* study (9.5 %), reflecting the similar 6.2 % of transcription related genes present in the *P. cinnamomi* cyst and germinating cyst library. In a similar way *P. sojae* metabolism, protein synthesis, transcription process and energy production related transcripts were most abundant in soya bean-infected EST libraries (Qutob et al. 2000; Chen et al. 2007).

The transcriptomic dataset was specifically searched for effector molecules as their function in pathogenicity in oomycetes are well recorded (Kamoun 2006). We identified various apoplastic effectors (acting extracellularly) expressed in the germinating cyst phase of *P. cinnamomi*. Protease inhibitors (representing 20 unigenes) are employed by many pathogens as a counter defence to host enzyme attack (Misas-Villamil and van der Hoorn 2008). Some protease inhibitors, previously reported in *Phytophthora* EST data include Kazal-like proteinase- and protease inhibitors (Panabières et al. 2005; Torto-Alalibo et al. 2007; Raffaele et al. 2010; Reena et al. 2010) and Kazal-like serine protease inhibitors (Le Berre et al. 2008). The Kazal-like serine protease P69B subtilase of tomato, for example, is targeted by protease inhibitor EPI10 from *P. infestans* (Tian et al. 2005). Orthologs of this specific serine protease inhibitor was identified in *Phytophthora ramorum* Werres, De Cock & Man in`t Veld (Meijer et al. 2006) and *Phytophthora palmivora* (E.J. Butler) E.J. Butler (Chinnapun et al. 2009) and is also present in this dataset from *P. cinnamomi*. Some

protease inhibitors are species specific, like EPIC1 and EPIC2 that seem to be restricted to *P. infestans* (Tian et al. 2007). These inhibitors target the Rcr3 protease from tomato (Song et al. 2009). In support of this, both EPIC1 and 2 were absent from the *P. cinnamomi* dataset. However, EPIC 3, 4, 5 and 6, reported in other *Phytophthora* species (Tian et al. 2004; Tian et al. 2007) were also detected in this study.

Other apoplastic effectors identified from *P. cinnamomi* include 20 elicitin transcripts. Elicitins are devided into 17 clades (Jiang et al. 2006) and induce a hypersensitive response (HR) in some plant species, specifically the *Solanaceae* and *Cruciferae* families (Kamoun et al. 1993a; Kamoun et al. 1993b; Bonnet et al. 1996). They are characterized by a signal peptide followed by a 98 amino acid conserved elicitor domain (with six cysteine residues in a specifically spaced pattern) in the N-terminal of the protein (Kamoun 2006). The exact role of elicitins in pathogenicity remains elusive, but their ability to bind sterols (Vauthrin et al. 1999; Yousef et al. 2011) suggests a general role as lipid-binding proteins. One of the best characterized elicitins is INF1 from *P. infestans* that acts as a PAMP and induces HR in tobacco plants (Kamoun et al. 1998). Several other studies exploited the avirulence function of INF1 to screen the ability of other effectors to suppress host defence responses (Bos et al. 2006; Gilroy et al. 2011; Yaeno et al. 2011; Cheng et al. 2012). None of the *P. cinnamomi* unigenes showed homology to INF1.

A total of 47 unigenes that aid in pathogen adhesion were identified. This included seven cellulose binding and elicitor lectin proteins (CBEL), 10 adhesins and 30 mucin transcripts. CBEL proteins, found in the cell walls of oomycete species (Séjalon-Delmas et al. 1997), can bind to cellulose components of the host cell wall (Gaulin et al. 2002). It also acts as a PAMP, as the cellulose binding domain of CBEL is recognized by the host surveillance system and leads to activation of the HR in tobacco plants (Gaulin et al. 2006). *Phytophthora cinnamomi* cyst attachment is further enhanced by adhesive material, containing adhesin proteins (Robold and Hardham 2005), that is secreted by the spore after incystment (Gubler et al. 1989; Hardham and Gubler 1990). The data generated in this study support these previous reports from *P. cinnamomi,* as 40 unigenes corresponding to adhesin and mucin proteins were expressed during *in vitro* cyst germination in this study.

The two main groups of proposed cytoplasmic effectors include the RXLR effector family and the Crinkling and necrosis-inducing (CRN) protein family (Haas et al. 2009). From a single developmental stage we identified 46 putative RLXR effectors. This is the first report of RXLR effectors in *P. cinnamomi* and is likely an underestimation, since it was identified from a transcriptomic library (and not the genome) and could only consider sequences starting

with methione. This class of cytoplasmic effectors are characterized by the sequence Arg-any amino acid-Leu-Arg (RXLR) within the first 70 amino acids of the mature protein. The RXLR-motif was previously thought to be responsible for effector translocation into host cells, but the role of this motif is currently under debate (Ellis and Dodds 2011). RXLR effectors are directly associated with avirulence as their recognition by host resistance proteins activates effector-triggered immunity (ETI). Examples include *P. infestans* Avr3a and Avr4 recognition by potato resistance proteins R3a (Bos et al. 2006) and R4 (Van Poppel et al. 2009) respectively and *P. sojae* Avr1b recognition by soya bean resistance protein Rps1b (Dou et al. 2008). Positive selection, to avoid resistance gene recognition, is evident in differential recognition of *Avr* gene alleles as seen in *Avr3a* of the *P. infestans*-potato interaction (Armstrong et al. 2005) and for *Avr3b* in the *P. sojae*-soya bean pathosystem (Dong et al. 2011). RXLR effector genes enhance disease susceptibility by suppressing host defence responses in various ways. *Phytophthora sojae* effector Avh331 influences mitogen-activated protein kinase (MAPK) mediated defence pathways in *Arabidopsis* by disrupting MAPK signalling and suppressing downstream resistance gene activation (Cheng et al. 2012). Avr3a from *P. infestans* suppresses INF1-induced cell death (Bos et al. 2006) by stabilizing host ubiquitin E3-ligase CMPG1, required for INF1-induced cell death (Bos et al. 2010).

Except for an N-terminal signal peptide and the RXLR-motif, little sequence conservation remains between members of the RLXR family and RXLR orthologs and paralogs (Jiang et al. 2008; Haas et al. 2009). This lack of similarity accommodates their diverse functions (dependent on the C-terminal amino acid sequence (Win et al. 2007; Jiang et al. 2008)) and most likely result from positive selection to remain undetected (Tyler et al. 2006; Win et al. 2007; Jiang et al. 2008; Qutob et al. 2009). For this reason it is difficult to define RXLR gene orthologs. Jiang et al. (2008) reported as low as 30 % amino acid identify between paralogs in *P. sojae* and *P. ramorum* and on average 70 % amino acid identity between 1 000 randomly selected *P. sojae* RXLR proteins to the most similar *P. ramorum* protein. Based on this, the *P. cinnamomi* contig C31843 is suggested as an ortholog of the suppressor of necrosis 1-like protein 2 (SNEL2) (Wang et al. 2011) from *P. sojae* (78 % identical amino acids, with C31843 representing 50 % of the hit sequence). The full length protein predicted in the *P. cinnamomi* genome that corresponds to C31843 (jgi-Phyci1-16230) shares 66 % amino acid with PsSNEL1 (71 % similarity), which further supports this suggestion. SNEL2 was named a paralog of PsSNEL1, which in turn is an ortholog of suppressor of necrosis 1 protein (SNE1) of *P. infestans* (Wang et al. 2011). SNE1 was shown to be localized in the host nucleus (tomato) and has the ability to suppress cell death induced by effector recognition or pathogen induced necrosis during the necrotrophic phase of *P. infestans*

(Kelley et al. 2010). It will be interesting to see if the protein produced from C31843 is indeed an ortholog from SNE1 and functionally similar. Unigene sequences do not represent full length transcripts, but in this case only the N-terminal region of each transcript. As great sequence divergence is expected in the C-terminal of RXLR proteins, comparison of full length protein sequences is expected to share even less amino acids with other RXLR proteins. Cluster analysis (including construction of phylogenetic gene trees) and evaluation of synteny between genomic regions (where the genes are located) should be considered before confirming any of the RXLR genes identified from *P. cinnamomi* as orthologs.

A total of 30 unigenes with homology to Crinkling- and necrosis-inducing proteins (CRN) were found in *P. cinnamomi*. The presence of the characteristic LFLAK motif (Leu-Phe-Leu-Ala-Lys) (Win et al. 2007; Haas et al. 2009) needs to be determined before the identity of the related unigenes can be confirmed. This can be achieved by employing the HMM developed by Haas et al. (2009) together with the pipeline developed by Stam et al. (2013) to identify CRN proteins in *Phytophthora capsici* Leonian. This cytoplasmic effector group was originally identified as elicitors in tomato (Torto et al. 2003) and CRN members of *P. sojae* and *P. capsici* have since been implicated in suppression of host defences (Liu et al. 2010; Chen et al. 2013). Unlike RXLR effectors that seem to be restricted to biotrophic and hemi-biotrophic pathogens, the CRN effector family is expanded in both hemi-biotrophic and necrotrophic oomycete pathogens (Stam et al. 2013). Characterization of *P. capsici* CRN effectors indicated that most CRN domains do not elicit cell death in plants, but their regulated expression patterns and localization to the host nucleus suggest that they are functionally important during infection even if not directly in pathogenesis (Stam et al. 2013).

Except for effector molecules, unigenes representing other infection related gene families were also investigated. Necrosis- and ethylene- inducing proteins (NIPs) induce cell death in the host as part of the infection strategy (Bae et al. 2006) and is present in oomycetes, fungi and bacteria (Fellbrich et al. 2002). The *P. cinnamomi* dataset contained nine unigenes corresponding to the necrosis-inducing *Phytophthora* protein 1 (NPP1) that was first identified in *Phytophthora parasitica* Dastur (Fellbrich et al. 2002). Pathogens are divided into three categories based on their lifestyle. A biotrophic pathogen feeds of living plant tissue, a necrotroph kills its host and lives off necrotic material, while a hemi-biotrophic pathogen first infects a living host and then induces necrosis as it enters the necrotrophic phase (Oliver and Ipcho 2004). NLPs are suggested to facilitate the switch from biotrophy to necrotrophy in hemi-biotrophic pathogens, as it is expressed during late infection of the *P. sojae*-soya bean interaction (Qutob et al. 2002; Ye et al. 2011). This expression pattern of NLPs can be exploited to serve as a marker for the switch between biotrophy and

necrotrophy (Kelley et al. 2010). *Phytophthora cinnamomi* was first defined as a necrotroph, but has also been described as a hemi-biotropic pathogen as it can colonise symptomless host tissue (Davison et al. 1994; Hüberli et al. 2000; Cahill et al. 2008). Shearer and Crane (2012) argued that *P. cinnamomi* does not fit into either of the defined trophic categories, but has alternating biotrophic and necrotrophic phases depending on environmental conditions. *In planta* expression profiling of the NIPs identified in the *P. cinnamomi* dataset may shed light on the switch between trophic phases in *P. cinnamomi*, together with investigation of notable changes in pathogen metabolism that is associated with each phase.

Plant pathogens secrete enzymes directed to break down components of the plant cell wall (Have et al. 2002). The cell wall serves as a physical barrier for pathogen entry, tissue penetration and colonization and is primarily made up of cellulose, hemi-cellulose and pectin (Carpita and Gibeaut 1993). We identified over 900 hydrolytic enzymes transcribed in the *P. cinnamomi* cyst and germinating cyst phase. Enzymes that break down the primary cell wall include polygalactorunases, cellulases, glucanases and gucosidases. No cellulase enzymes were identified in the *P. cinnamomi* library. Unigenes that showed homology to cellulase enzymes in the Nr and Swiss databases were however annotated as hydrolase-, glucanase- or glucosidase enzymes (that also has the ability to digest cellulose) in the *Pi* annotated dataset. Secretion of enzymes that break down cellulose by oomycete species is interesting, as it is the main component of their own cell wall (Hardham et al. 1994). Cutin, that forms part of the plant cuticle, is degraded by cutinase enzymes (representing three unigenes in the *P. cinnamomi* dataset). Further, three chitinases were present in the dataset. Since chitin is not present in plants, these enzymes may be directed at competing or antagonistic fungi which have cell walls that mainly consist of chitin (Bowman and Free 2006).

Discovery of novel or species specific transcripts is not possible with a homology based annotation approach. More than 2 000 unigenes (present in the *Pc* annotated unigene dataset) did not show homology to proteins in public available databases or the *P. infestans* genome (Figure 9). These unigenes may represent transcripts that are unique to *P. cinnamomi*. *Phytophthora cinnamomi* specific pathogenicity genes, especially effector genes, are interesting candidates for further investigation as they may be responsible for its wide host range. Identification of conserved motifs within full length transcripts may prove a more useful approach in characterizing *P. cinnamomi* specific transcripts than a homology based approach. A further 9 805 unigenes (16.47 % of predicted unigenes) did not show homology to any proteins in the four databases considered (including the *P. cinnamomi* genome) (Table 7). This may be explained by incorrect assembly and prediction of CDS of

the *P. cinnamomi* transcriptomic or genomic data, although this cannot account for the complete set of unannotated unigenes. Large amounts of transcripts identified by RNA-seq analysis in other studies, with available genomes, identified novel exons that mapped to unannotated regions of the genome (Cloonan et al. 2008; Morin et al. 2008; Mortazavi A et al. 2008). Accordingly, the large amount of unannotated unigenes in the *P. cinnamomi* library may represent CDS that have not been identified previously or even other types of RNA (e.g. miRNA).

Signal peptides were predicted in 110 unigenes (lacking TMH) that showed homology to proteins models from *P. infestans* and *P. cinnamomi*. Unigene sequences do not represent full length transcripts and hence some of the unigenes with predicted signal peptides may contain TMH in the region that was not sequenced (retaining the protein in membranes). Any secreted protein has the potential to act as a pathogenicity protein, as they are able to leave the pathogen to directly interact with host proteins. A total of 11 unigenes, harbouring signal peptides, may potentially be specific to *P. cinnamomi* and the function of a further 9 signal peptide-containing unigenes is unknown (conserved hypothetical proteins). Further investigation is required to determine if they are pathogenicity factors.

The *P. cinnamomi* transcriptomic library will make a significant contribution to the available molecular tools to study *P. cinnamomi* in the future. The experimental approach used in this study, however, had numerous shortcomings. A number of *P. cinnamomi* isolates produced variable amounts of zoospores inconsistently. This forced us to produce spores in a non-sterile system, leading to the presence of a large amount of non-*Phytophthora* transcripts in the dataset. Contaminant sequences were successfully eliminated from the *P. cinnamomi* dataset through bioinformatic filtering. Due to the nature of the sample constitution in this study specifically (pooling total RNA from multiple experiments), we could not utilize this data to capture gene expression levels, which is one of the main advantages offered by RNA-seq analysis. With the *P. cinnamomi* genome recently made available, differential gene expression analysis *in planta* will enable a better understanding of pathogenesis in the future. RNA-seq technology has transformed the field of transcriptome analysis, but there is still room for improvement. The Illumina sequencing platform still only produces short reads, introducing bias as the RNA or cDNA needs to be fragmented during library preparation (Wang et al. 2009; Strickler et al. 2012). Short reads also complicate assembly of repeat rich regions, making it difficult to determine if the reads result from abundant transcripts or from cDNA amplification (Wang et al. 2009). The future of RNA-seq will focus on enabling strand specific libraries (which are laborious and inefficient at the moment) and increasing the accuracy and depth of the library by combining long read 454 technology with short read

Illumina libraries (Wang et al. 2009). The *P. cinnamomi* dataset was annotated with a homology based approach which did not allow the identification of novel transcripts. This dataset, however still holds the potential to identify novel transcripts by identifying conserved motifs within unigenes lacking significant homology to known proteins.

CONCLUSION

This is the first study that investigated pathogenicity genes in *P. cinnamomi*. Using RNA-seq and a *de novo* assembly approach, we generated the first transcriptomic library available for *P. cinnamomi*. We successfully identified over 2 000 pathogenicity unigenes from the cyst and germinating cyst phase that included genes similar to those implicated in pathogenicity in other *Phytophthora* species. Among the unigenes identified were a range of effector genes (including RXLR effectors) that serve as good candidate genes for further investigation. The transcriptomic data generated in this study, together with *P. cinnamomi* genomic data, is a valuable tool that can be utilized to investigate *P. cinnamomi* genes *in planta* and to study alternative splicing within transcripts. This information serves as a basis to start unravelling *P. cinnamomi* phytopathology.

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The Beijing Genomics Institute constructed the cDNA library (from total RNA generated by us), completed the standard bioinformatic analysis including library quality assessment, read assembly, CDS prediction and unigene annotation to the NCBI Non-redundant protein database. Prof. Paul Birch and Dr. Remco Stam (The James Hutton Institute, Scotland), predicted putative RXLR proteins from contigs generated by BGI. This work was supported by funding from the National Research Foundation THRIP Grant TP 2011060300010, The Hans Merensky Foundation and Westfalia.

TABLES

Table 1. *Phytophthora cinnamomi* isolates used in this study.

Isolate ID	Host	Root/Soil	Location	Farm
CNVDB1	*Persea americana*	Unknown	Tzaneen, Limpopo, SA	Unknown
CNVDB5	*Persea americana*	Unknown	Unknown, SA	Unknown
CNVDB18	*Persea americana*	Unknown	Tzaneen, Limpopo, SA	Westfalia Block 1
CNVDB19	*Persea americana*	Roots	Tzaneen, Limpopo, SA	Westfalia Block 1
CNVDB27	*Persea americana*	Roots	Tzaneen, Limpopo, SA	Westfalia Test Block
CNVDB29	*Persea americana*	Roots	Tzaneen, Limpopo, SA	Westfalia Test Block
CNVDB32	*Persea americana*	Roots	Tzaneen, Limpopo, SA	Theuns Botha
CNVDB34	*Persea americana*	Soil	Tzaneen, Limpopo, SA	Theuns Botha
CNVDB39	*Persea americana*	Soil	Tzaneen, Limpopo, SA	Theuns Botha
CNVDB40	*Persea americana*	Soil	Tzaneen, Limpopo, SA	Francois Voster
CNVDB41	*Persea americana*	Roots	Tzaneen, Limpopo, SA	Francois Voster
CNVDB43	*Persea americana*	Unknown	Tzaneen, Limpopo, SA	Francois Voster

SA – South Africa

Table 2. Growth (mm) of *Phytophthora cinnamomi* isolate CNVDB1 and CNVDB29 on three different nutrition media after four days incubation at temperatures ranging from 10 °C to 35 °C.

Isolate	Medium	10 °C	15 °C	20 °C	25 °C[#]	30 °C	35 °C
CNVDB1	½ PDA *	14.15	26.1	38.75	55.6 [a]	53.2	6.7
	CMA**	12.15	22.9	37.55	52.6 [b]	59.55	6.15
	V8***	13.05	32.9	46.15	54.45 [ab]	55	6.25
CNVDB29	½ PDA *	13.75	20.95	28.75	37.4 [c]	40.75	6.45
	CMA**	11.2	17.65	36.8	55.45 [a]	61.3	6.5
	V8***	13.75	33.15	55.1	52.95 [b]	44.8	6.65

* ½ Potato dextrose agar ** Cornmeal agar ***Vegetable juice agar

[#] Analysis of variance, followed by a Least Square Means Differences Student's t-test were performed separately on samples incubated at 25 °C. Growth measurements containing the same letters do not differ significantly (P = 0.05).

Table 3. Quality and quantity of subsets of purified total RNA from cysts and germinating cysts of *Phytophthora cinnamomi* isolate CNVDB29, pooled for cDNA library construction.

Biological replicate	Volume (µl)	RNA concentration (ng/µl)	OD 260/280	OD 260/230
1	9	1500.9	2.16	2.31
2 a	9.56	784.4	2.17	2.21
2 b	8	263.0	2.15	1.95
3 a	11.77	1299.9	2.19	2.20
3 b	8	432.7	2.13	2.24
Total	46.33	903.8		

Table 4. RNA quality of the cyst and germinating cyst library of *Phytophthora cinnamomi* isolate CNVDB29 as determined by the Agilent 2100 software.

	Concentration (ng/µl)	Volume (µl)	Total mass (ng)	RIN*	28S:18S	OD 260/280	OD 260/230
RNA Isolate CNVDB29	375.0	73	27.375	8.6	1.4	2.13	1.6

*RNA integrity number

Table 5. The quality of the sequenced cDNA library of *Phytophthora cinnamomi* cysts and germinating cysts, isolate CNVDB29.

Sample	Total Reads	Total Nucleotides (nt)	Q20 percentage	N percentage	GC percentage
Cyst and germinating cyst library	13 032 666	1 172 939 940	95.30 %	0.01 %	46.26 %

Table 6. Quality and contig length distribution of the cyst and germinating cyst cDNA library of *Phytophthora cinnamomi.*

	100-200 nt	200-300 nt	300-400 nt	400-500 nt	>500 nt	N50[1]	Mean	All contigs
Number of contigs	151 248	34 598	17 635	8 137	13 431	257	213	225 049
Percentage of contigs	67.21 %	15.37 %	7.84 %	3.62 %	5.97 %	-	-	-

Table 7. Number of unigenes from the *Phytophthora cinnamomi* cyst and germinating cyst transcriptomic dataset that showed significant homology to proteins in protein databases as indicated (E-value >1x10^{5}).

	Total	Nr*	Swiss**	*P. infestans* protein models§	*P. cinnamomi* protein models#	No hits***
Number of unigenes	72 220	47 330	38 364	34 280	36 500	9 805
Percentage unigenes with significant blastx hit (%)	-	65.54	53.12	47.47	50.54	16.47

* NCBI non-redundant protein database

** Swiss protein database

§ *Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)

http://genome.jgi.doe.gov/Phyci1/Phyci1.home.html

*** no hits in Nr, Swiss, *Pi* protein models and *Pc* protein models

Table 8. Putative RXLR effector transcripts from the cyst and geminating cyst library of *Phytophthora cinnamomi* with the best protein match from the *P. cinnamomi* genome indicated, as determined through blastx analysis (E-value >1x10^{5}). The presence of a signal peptide (SP) and actual RXLR sequence is indicated.

Contig	SP	RXLR motif	E-value	Bit score	Protein ID of best blast hit
Contig1990	Yes	Yes	0	649.82	jgi-Phyci1-116704
Contig3889	No	Yes	0	730.32	jgi-Phyci1-241851
Contig14564	No	No	4.64E-139	489.57	jgi-Phyci1-88880
Contig8139	Yes	Yes	3.06E-135	477.25	jgi-Phyci1-83483
Contig1480	No	Yes	1.43E-127	451.82	jgi-Phyci1-253206
Contig22794	Yes	No	6.83E-124	440.65	jgi-Phyci1-219078
Contig38826	No	Yes	1.97E-96	347.82	jgi-Phyci1-113905
Contig2006	No	Yes	1.37E-92	334.72	jgi-Phyci1-91296
Contig31644	Yes	Yes	4.66E-89	323.17	jgi-Phyci1-359507
Contig12983	No	Yes	3.76E-82	300.44	jgi-Phyci1-184564
Contig14063*	Yes	Yes	2.64E-79	290.04	jgi-Phyci1-30885
Contig9909	No	No	2.22E-68	253.45	jgi-Phyci1-19419
Contig29027	No	Yes	8.97E-68	252.29	jgi-Phyci1-92480
Contig17530	Yes	Yes	9.26E-66	245.36	jgi-Phyci1-292291
Contig2921	Yes	Yes	1.86E-64	240.74	jgi-Phyci1-500310
Contig5149	No	Yes	3.24E-64	240.35	jgi-Phyci1-509188
Contig33937	Yes	Yes	9.67E-61	228.02	jgi-Phyci1-374529
Contig28374	No	Yes	6.68E-59	221.86	jgi-Phyci1-85009
Contig46416	No	Yes	5.49E-56	212.23	jgi-Phyci1-400789
Contig33615	Yes	Yes	1.39E-55	210.3	jgi-Phyci1-80513
Contig15073*	Yes	Yes	1.84E-50	194.13	jgi-Phyci1-172110
Contig170656	No	Yes	2.70E-50	192.59	jgi-Phyci1-91700
Contig14204	Yes	Yes	3.94E-46	180.26	jgi-Phyci1-232994
Contig17531	Yes	Yes	7.27E-46	179.1	jgi-Phyci1-292291
Contig66803	Yes	Yes	3.52E-42	165.62	jgi-Phyci1-557761
Contig19961	No	Yes	6.90E-42	165.24	jgi-Phyci1-20391
Contig43424	No	Yes	7.59E-36	145.98	jgi-Phyci1-85378
Contig33954	No	Yes	1.85E-35	143.28	jgi-Phyci1-214852
Contig83210*	Yes	Yes	1.90E-32	133.26	jgi-Phyci1-246465
Contig81356	Yes	Yes	2.46E-32	132.88	jgi-Phyci1-89800
Contig129865	Yes	No	8.93E-30	124.41	jgi-Phyci1-23225
Contig5551	Yes	Yes	1.50E-29	123.63	jgi-Phyci1-442085
Contig2634	No	Yes	7.69E-29	121.32	jgi-Phyci1-290439
Contig19412	No	Yes	4.26E-26	112.46	jgi-Phyci1-274179
Contig17839	No	Yes	9.96E-26	112.85	jgi-Phyci1-7143
Contig2637	No	Yes	8.18E-23	101.29	jgi-Phyci1-290439
Contig84507	Yes	Yes	3.81E-20	92.43	jgi-Phyci1-381040
Contig118237	No	No	1.15E-13	70.86	jgi-Phyci1-95101
Contig31843*	Yes	Yes	4.82E-12	65.47	jgi-Phyci1-16230

Contig78872	No	Yes	2.70E-10	59.69	jgi-Phyci1-492221
Contig112596	No	Yes	7.83E-10	58.15	jgi-Phyci1-97937
Contig146670	No	Yes	2.25E-09	56.61	jgi-Phyci1-7055
Contig57984 **	Yes	Yes	0.04	33.88	jgi-Phyci1-86540
Contig82707 **	Yes	Yes	0.23	30.03	jgi-Phyci1-106207
Contig17241 **	No	Yes	0.67	28.49	jgi-Phyci1-91991
Contig145768 **	No	Yes	5.76	25.41	jgi-Phyci1-24980

* contigs with significant homology to known RXLR effector proteins

** contigs not showing significant homology to protein models predicted in the genome of *P. cinnamomi*

Table 9. Unigenes with a putative role in *Phytophthora cinnamomi* pathogenicity. Pathogenicity unigenes were identified from the *Phytophthora infestans* protein model annotated unigene dataset.*

Putative pathogenicity unigenes **	Number of unigenes	Number of unique *Pi* ID numbers
Adhesion	**47**	**10**
CBEL protein	7	3
Adhesins	10	3
Mucins	30	4
Hydrolases	**978**	**311**
Hydrolases	236	80
Serine proteases	179	63
Cysteine protease/proteinases	120	26
Serine carboxypeptidases	3	1
Ubiquitin proteases	0	0
Cutinases	3	2
Esterases	179	58
Lyases	136	27
Glucosidases	53	21
Glucanases	55	24
Polygalacturonases	11	8
Cellulases	0	0
Chitinases	3	1
Inhibitors of proteases	**20**	**11**
Protease inhibitors	20	11
Effectors	**85**	**60**
RXLR effector genes	26	20
Elicitins and elicitin-like	20	13
Crinkling and necrosis-inducing proteins	30	22
Necrosis-inducing *Phytophthora* protein 1	9	6
Small cysteine-rich proteins	**4**	**2**
Small cysteine-rich proteins	4	2
Detoxification, drug resistance, and metabolite transport	**594**	**118**
Cytochrome P450	25	13
Putative ABC transporters	569	105
Protection against oxidative stress	**544**	**117**
Glutathione s-transferases	26	10
Peroxidases	75	12
Reductases	394	88
Superoxide dismutases	34	3
Glutaredoxins	15	4
Glutathione synthetases	0	0
Signal transduction and regulation	**117**	**43**
Mitogen-activated protein kinases	74	23
Myb-like proteins	43	20
Cell surface glycoproteins	0	0

Virulence genes	**3**	**3**
Virulence	3	3
TOTAL	**2392**	**675**

* Pathogenicity gene categories modified from Torto-Alalibo et al. (2007)

** Functional annotations are based on protein sequence homology and not on the presence of conserved motifs

Table 10. Protein sequence homology between predicted RXLR proteins of *Phytophthora cinnamomi, Phytophthora sojae* and *Phytohthora infestans* as determined through blastx analysis (E-value >1x10^{8}). The statistics for the overlapping alignment region is indicated.[*]

Contig ID	Protein ID	Annotation	E-value	Bit Score	Alignment length (Unigene to blast hit)	Hit protein length	% Identity	% Positive	% Gaps	Species
C14063	304654199[a]	PsAvh42	6.69E-25	102.83	160	153	46	63	11	*P. sojae*
C15073	304654141[a]	PsAvh65	3.07E-11	57.38	155	163	31	48	25	*P. sojae*
C15073	PITG_14203[b]	RXLR	2.56E-19	90.51	155	153	38	54	9	*P. infestans*
C31843	304654104[a]	PsSNEL2	4.15E-11	55.84	95	189	78	83	5	*P. sojae*
C83210	304654139[a]	PsAvh448	1.52E-08	46.98	82	115	43	60	21	*P. sojae*

[*] Only alignments with E-value >1x10^{8} are listed here

[a] Protein ID numbers from NCBI

[b] ID numbers from the *Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)

FIGURES

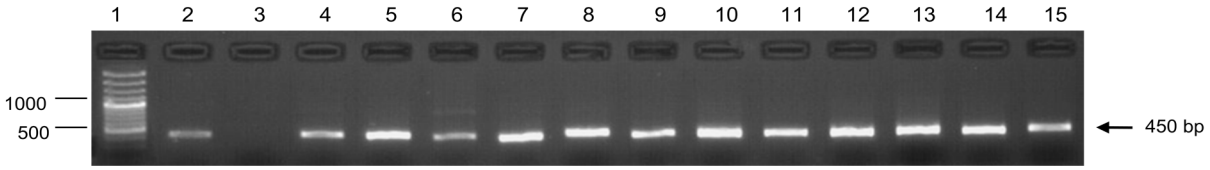


Figure 1. Species-specific PCR, using the *LPV3* primer pair (Kong et al. 2003)**, to confirm the identity of *Phytophthora cinnamomi* isolates.** The successful amplification of a PCR product of 450 bp in size, confirmed the identity of all 12 isolates as *P. cinnamomi.* 1) O'GeneRuler 100 bp DNA ladder 2) CMW33388 as a positive control 3) Negative control 4) CNVDB34 5) CNVDB32 6) CNVDB43 7) CNVDB41 8) CNVDB1 9) CNVDB19 10) CNVDB40 11) CNVDB27 12) CNVDB29 13) CNVDB39 14) CNVDB18 19) CNVDB5.

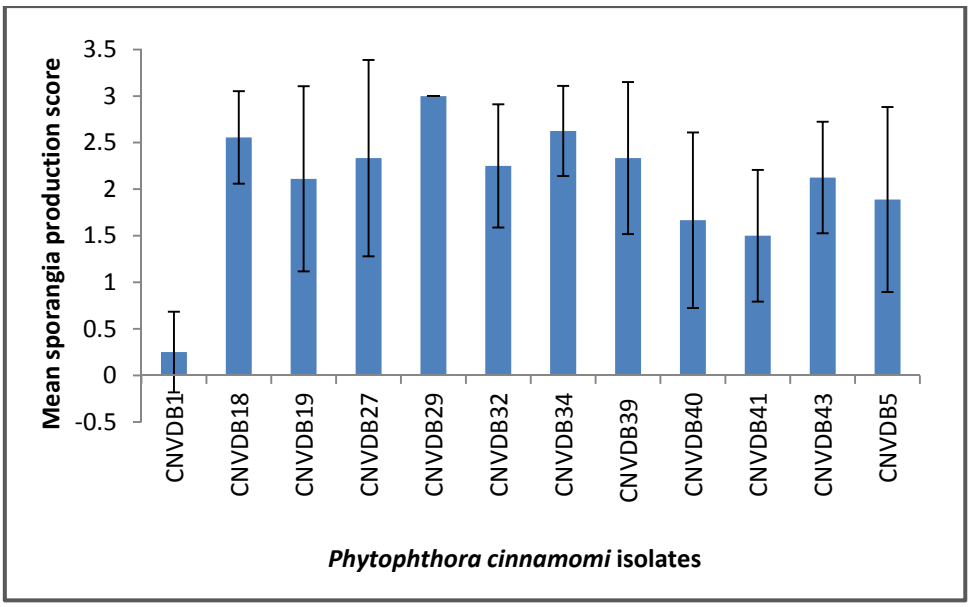


Figure 2. The sporangia production ability of 12 *Phytophthora cinnamomi* isolates. A score of 0 (no sporangia) to 3 (> 15 sporangia) was assigned to each agar block after counting the amount of sporangia produced for each isolate under a confocal microscope. The mean sporangia production score for each isolate was determined from three biological replicates and three technical replicates. The error bars represent standard deviation.

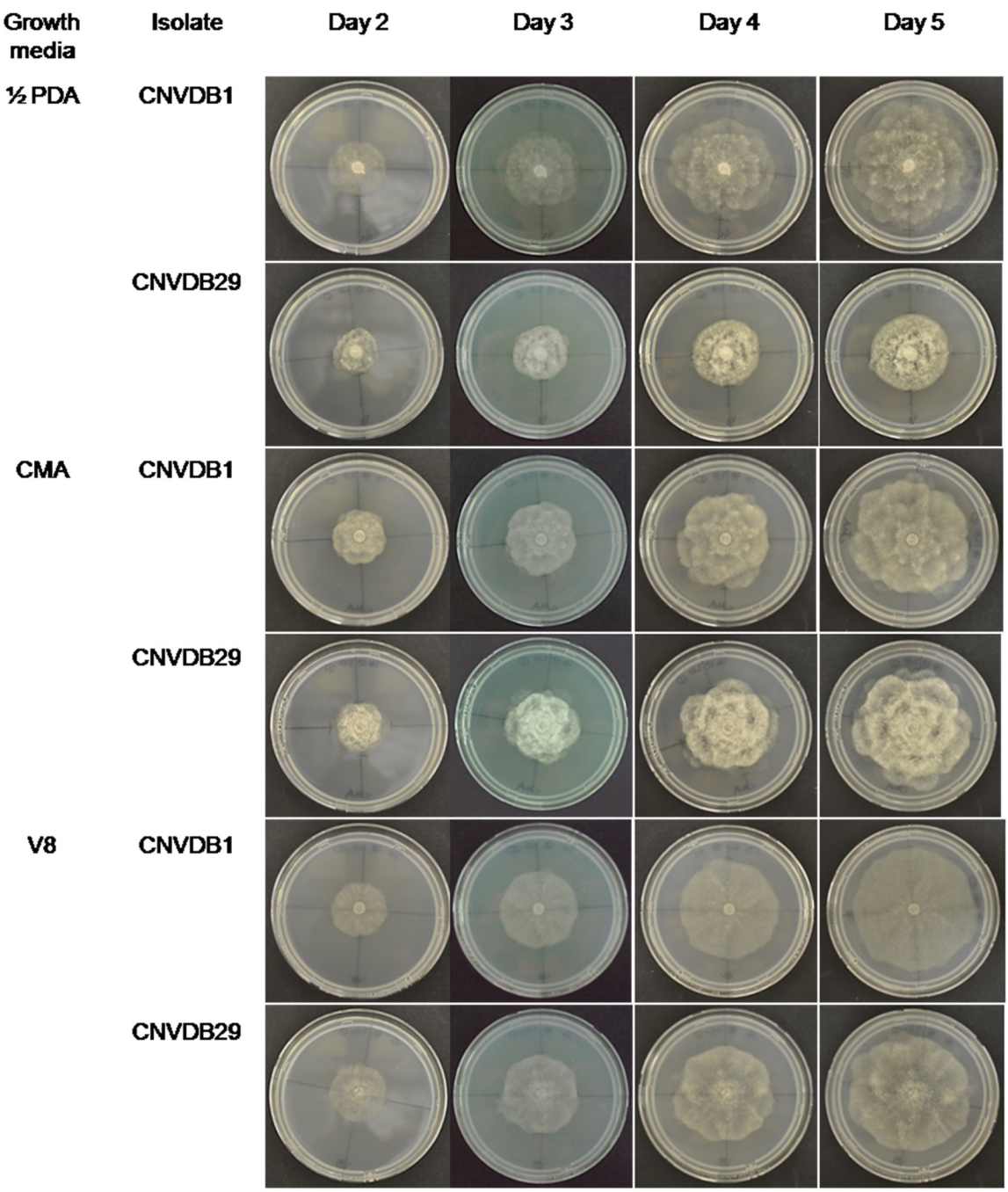


Figure 3. Growth morphology of *Phytophthora cinnamomi* isolates CNVDB1 and CNVDB29 on ½ potato dextrose agar (½ PDA), cornmeal agar (CMA) and vegetable juice (V8) agar at 25 °C over 4 days.

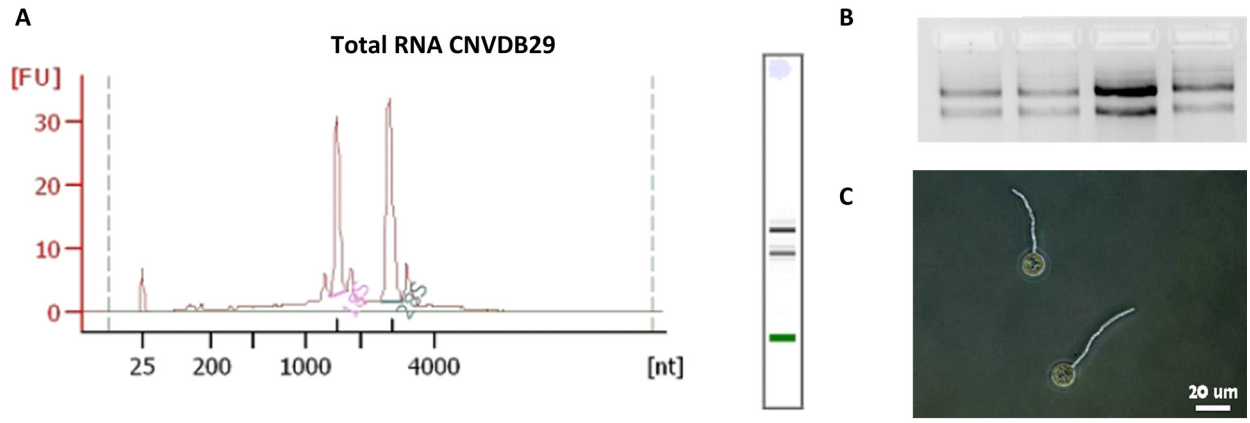


Figure 4. Electropherogram (A) and gel electrophoresis analysis (B) of pure total RNA, used for cDNA library construction, extracted from cysts and germinating cysts (C) of *Phytophthora cinnamomi* isolate CNVDB29. A) An RNA integrity number (RIN) of 8.6 was assigned to the sample using the Agilent 2100 Bioanalyzer software. B) Lane 1-4 represent pure total RNA from four independent experiments that were pooled to constitute the final sample that was used to produce the Illumina cDNA library. C) Stereo microscope image of germinating cysts of *P. cinnamomi* isolate CNVDB29. Scalebar = 20 µm.

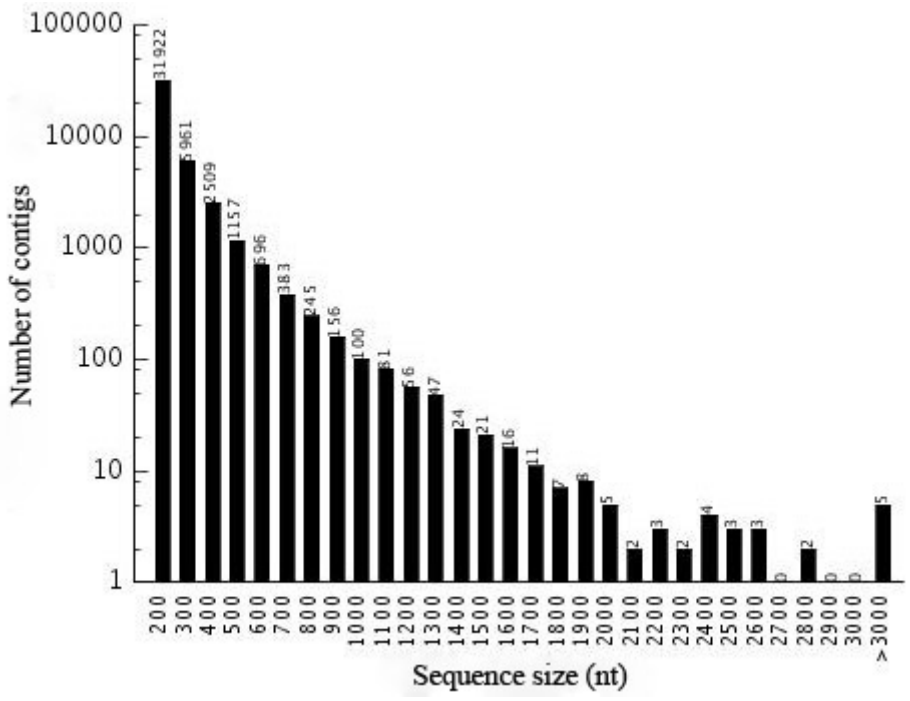


Figure 5. Contig length distribution of the cyst and germinating cysts cDNA library of *Phytophthora cinnamomi* isolate CNVDB29 generated through Illumina sequencing on the HiSeq 2000 instrument at the Beijing Genomics Institute. The image was generated by the BGI.

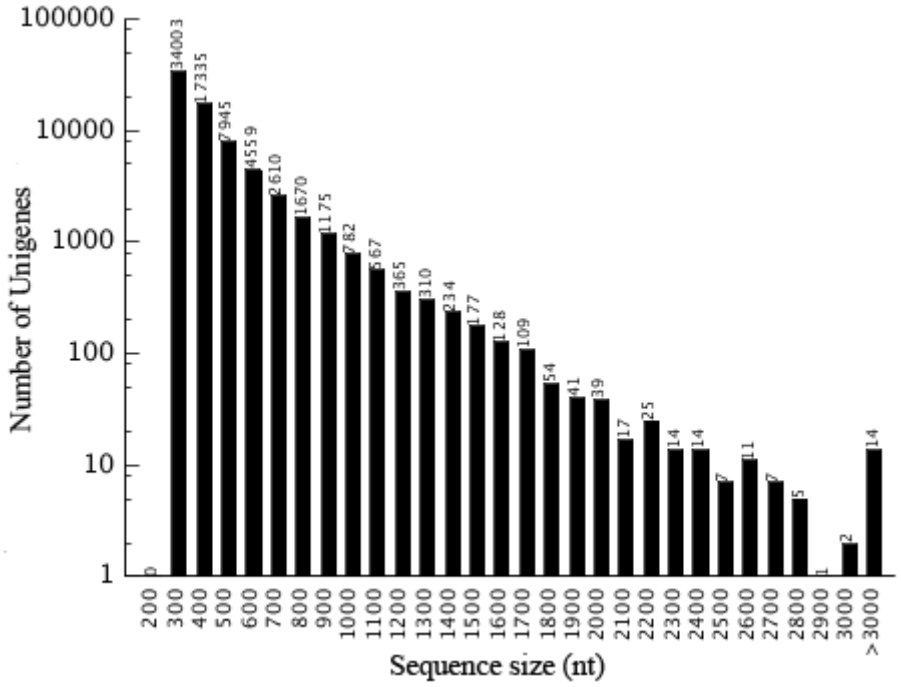


Figure 6. Length distribution of unigenes of the cyst and germinating cyst cDNA library of *Phytophthora cinnamomi* isolate CNVDB29. The image was generated by the BGI.

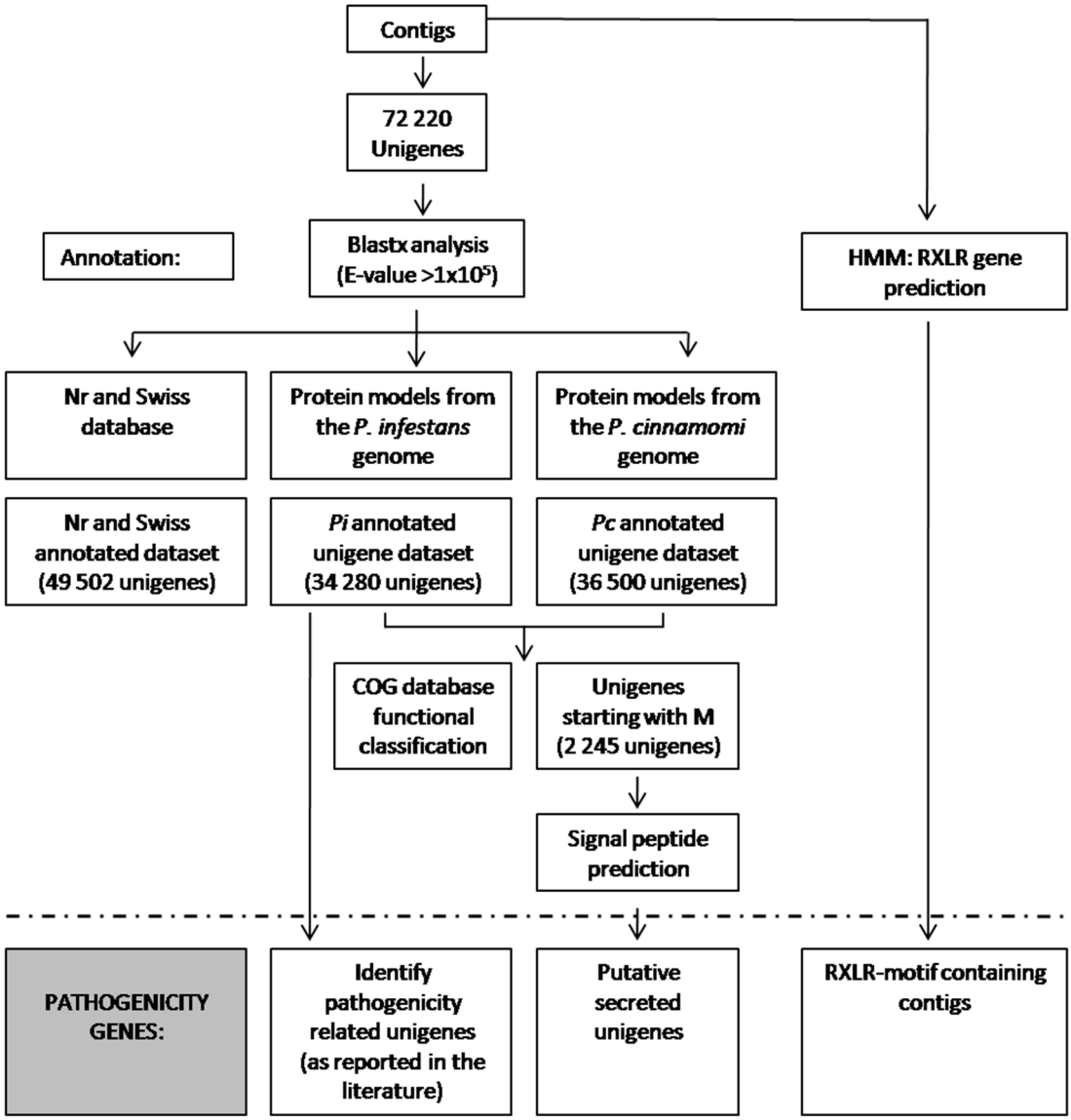


Figure 7. Workflow diagram indicating the strategy used to annotate the cysts and germinating cysts cDNA library of *Phytophthora cinnamomi*. Functions were assigned to unigenes based on protein sequence homology (E-value >1x10^5) to public available protein databases (NCBI non-redundant protein database [Nr], Swiss protein database [Swiss]) and to protein models predicted in the genomes of *Phytophthora infestans* (closely related species) and *P. cinnamomi*. Functional enrichment analysis was performed on the *Pi* and *Pc* annotated unigene dataset via blastx analysis (E-value >1x10^5) to the Cluster of orthologous (COG) proteins database. Putative secreted proteins were predicted (SignalP v3.0) from unigenes in which the start codon (methione) could be identified in the *Pi* and *Pc* annotated unigene dataset to ensure that the unigene sequence represented the N-terminal of the protein. Putative RXLR-motif containing transcripts were identified from the longest open reading frame (containing a start codon) predicted in each contig, using Hidden Markov Models (HMM). The *Pi* annotated unigene dataset was used to identify *Phytophthora* pathogenicity genes as reported in the literature. Putative secreted unigenes and RXLR-motif containing unigenes were also considered pathogenicity related genes.

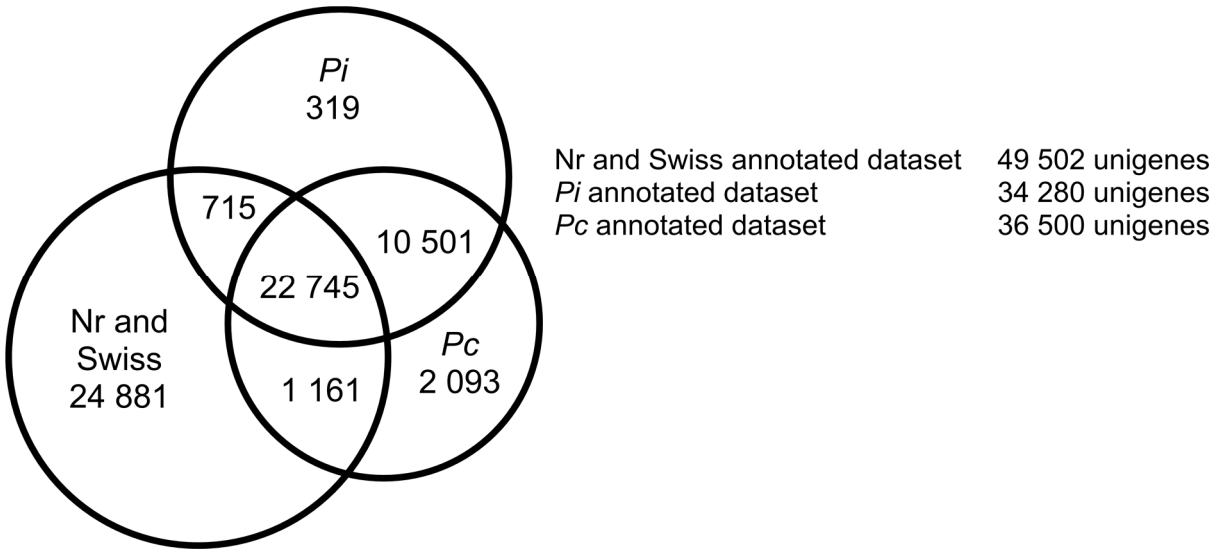


Figure 8. Venn diagram illustrating the overlap of unigenes that showed homology to proteins in more than one protein database during blastx analysis (E-value >1x10^{5}). The numbers inside the circles indicate the number of unigenes that showed homology to protein in the NCBI non-redundant protein database and Swiss- protein database (Nr and Swiss), the protein models predicted from the genomes of *Phytophthora cinnamomi* (*Pc* annotated dataset) and *Phytophthora infestans* (*Pi* annotated dataset). Grand totals are indicated on the right.

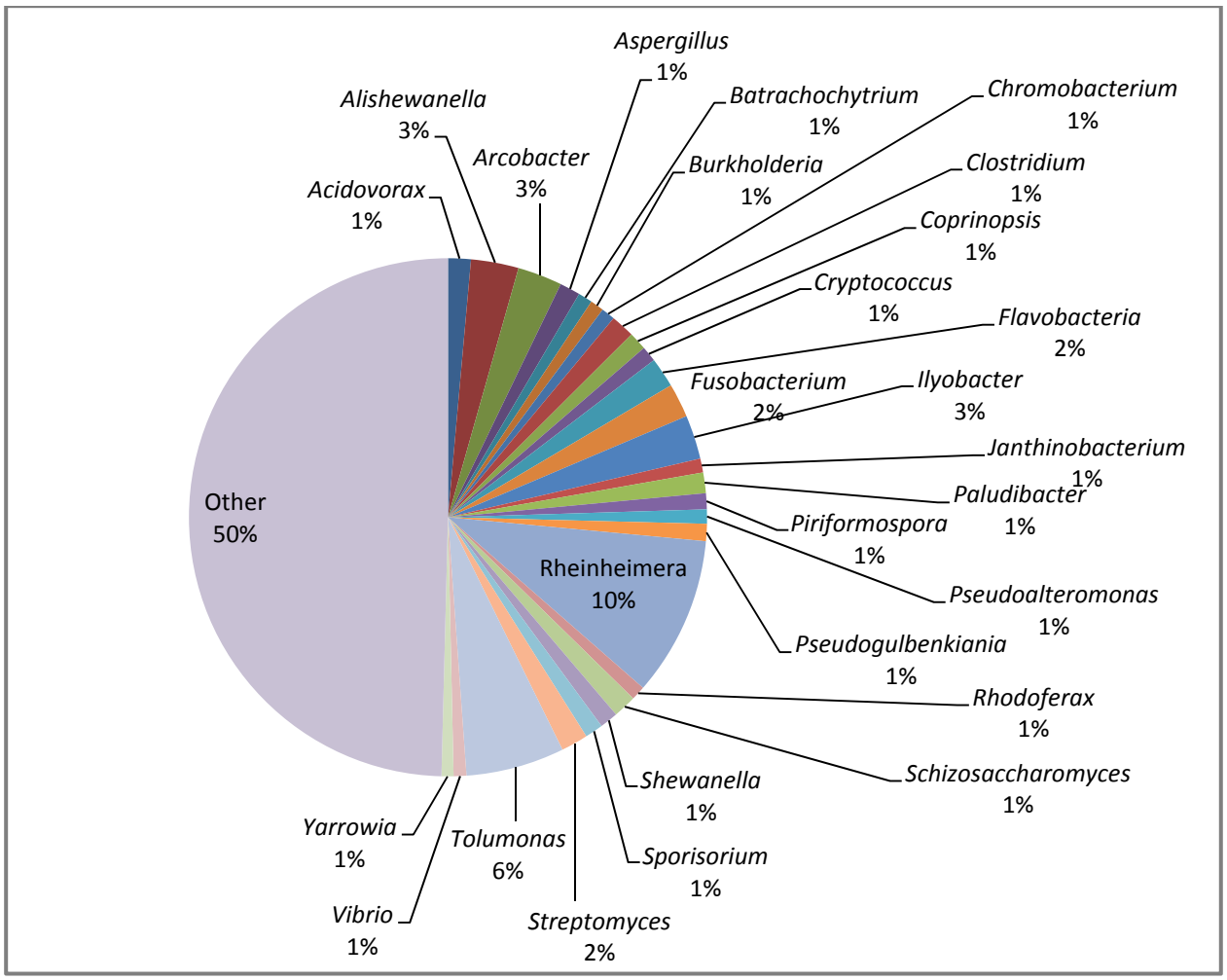


Figure 9. Division of genera representing at least 1 % of the unigenes present in the Nr annotated unigene dataset of *Phytophtora cinnamomi* cysts and germinating cysts. Unigene functional annotations are based on protein sequence homology as determined with blastx analysis (E-value >1x10^{5}) to the NCBI non-redundant protein database. Approximately 42 % of the unigenes showed homology to proteins originating from the domain bacteria and 8.7 % from the domain fungi. Genera that presented less than 1% of the dataset were not included in the analysis and are designated as 'other genera' in the figure (50 %).

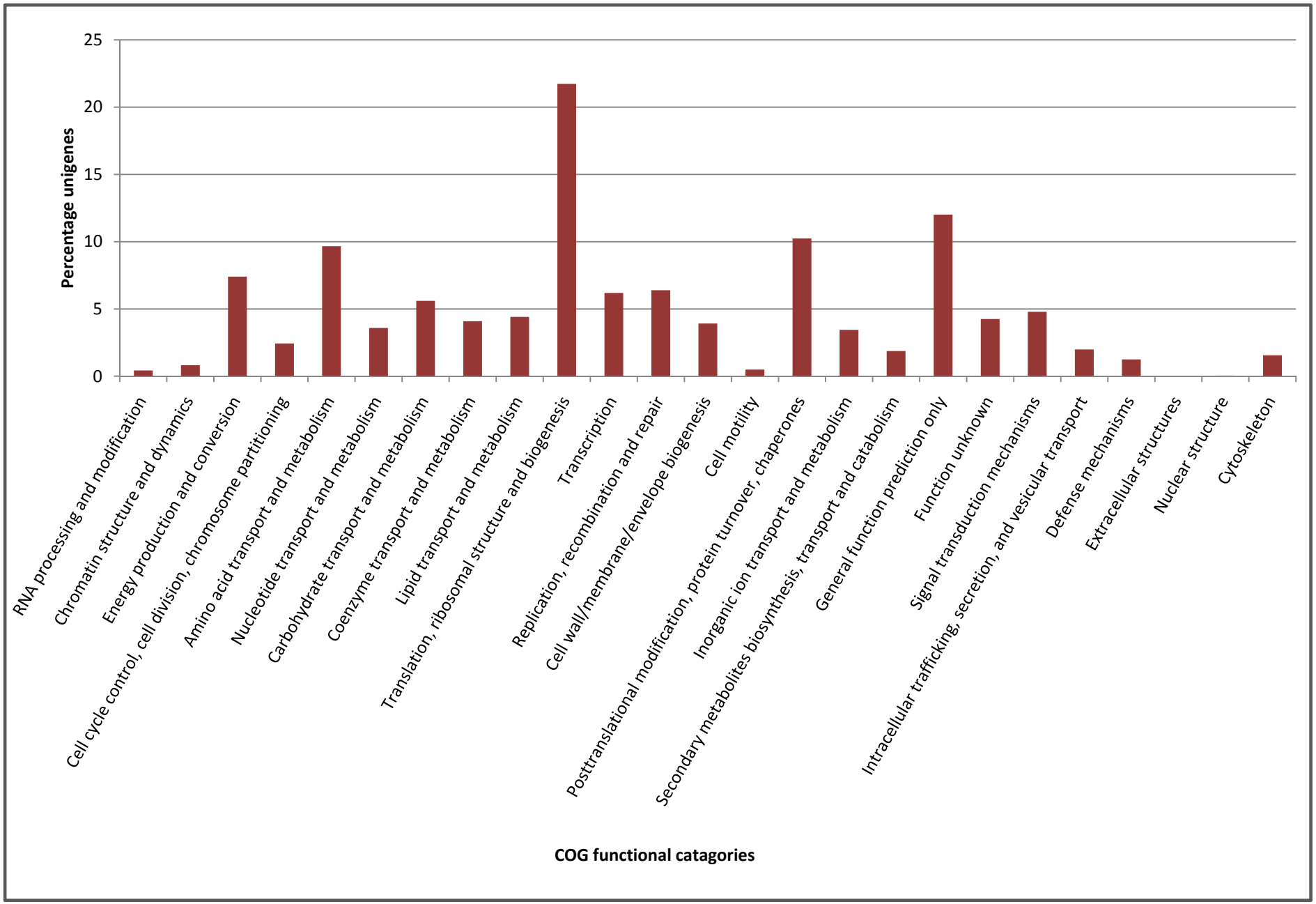
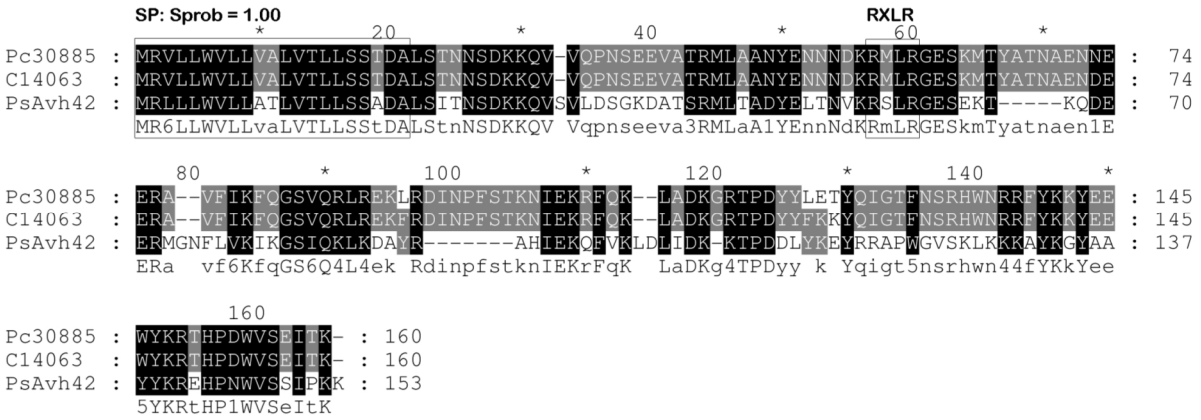
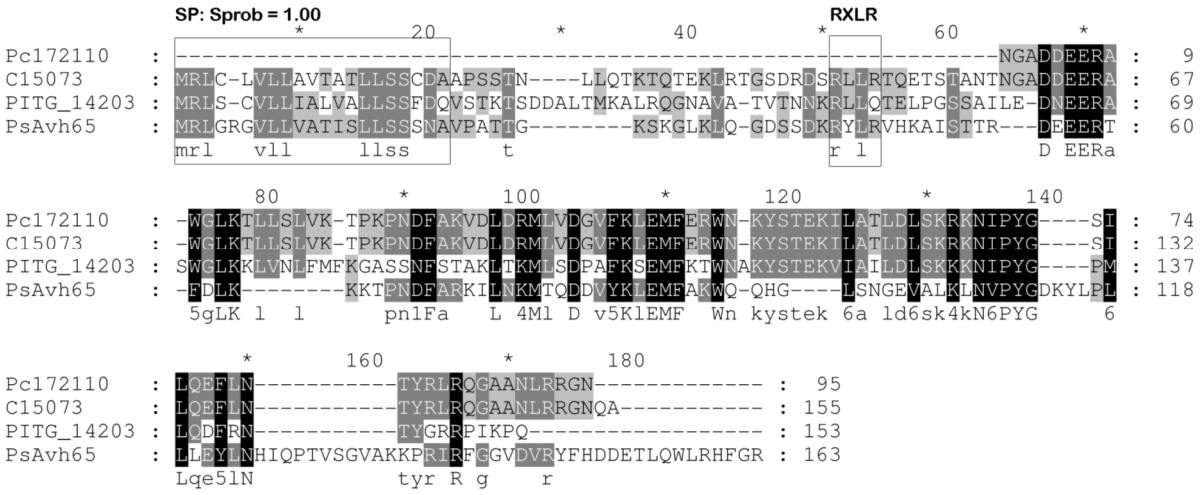


Figure 10. Functional classification of unigenes form the cyst and germinating cyst library of *Phytophthora cinnamomi* according to the Clusters of orthologous (COG) protein database after blastx analysis (E-value >1x10^{5}). Each bar represents the percentage unigenes (from unigenes that showed significant homology to *P. cinnamomi* and *Phytophthora infestans* protein models) that correspond to each of the 26 functional classes of the COG database. A single unigene may represent more than one functional class.

A



B



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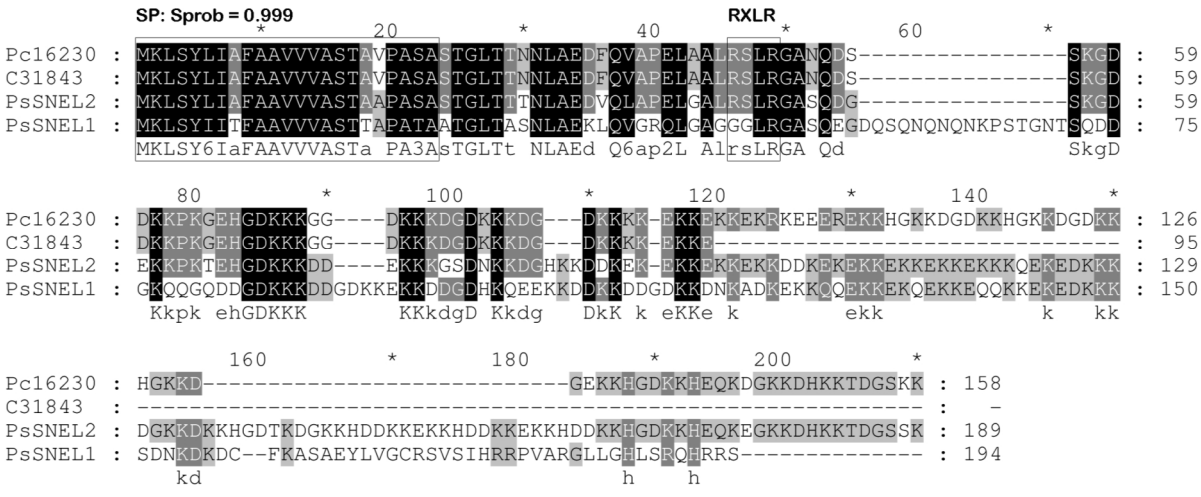

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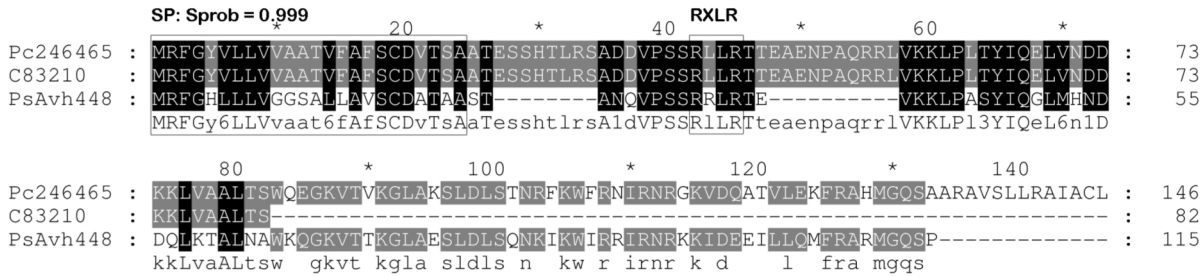

Figure 11. Protein sequence alignments of translated RXLR-motif containing unigenes to the best blastx hit in the genome of *Phytophthora cinnamomi* (top sequence) and RXLR protein sequences of *Phytophthora infestans* and/or *Phytophthora sojae* (bottom sequences). Conserved domain names are shown above the relevant domain sequence (blocks). Signal peptide Sprob value is indicated as determined from the sequence present in the *P. cinnamomi* contig. Shaded amino acids indicate amino acids that are similar between all aligned sequences (black) or only between two or three of the sequences (shades of grey). The consensus sequence is indicated underneath each alignment. A) C14063 aligned to jgi-Phyci1-30885 and PsAvh42 B) C15073 aligned to jgi-Phyci1-172110, PsAvh65 and PITG_14203 C) C31843 aligned jgi-Phyci1-16230 and PsSNEL2 D) C83210 aligned to jgi-Phyci1-246465 and PsAvh448. SP – signal peptide, *P. cinnamomi* JGI protein ID numbers (jgi-Phyci1-) are indicated as Pc- in the figure, PITG ID numbers are from the *Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/), PsAvh ID numbers are from predicted *Phytophthora sojae* RXLR proteins by Wang et al. (2011).

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CHAPTER 3

Expression analysis of selected pathogenicity genes across the *in vitro* life stages of *Phytophthora cinnamomi*

ABSTRACT

Phytophthora cinnamomi is a hemi-biotrophic oomycete pathogen, able to infect over 3 000 plant species, including economically important fruit and forest trees. The mechanism employed by this pathogen to successfully colonize plant tissue is not well understood. Successful infection of living hosts requires the rapid adaption of the pathogen to the environment and the host defence system. Investigating the temporal regulation of pathogenicity genes will provide valuable insight into *P. cinnamomi* pathogenesis. In this study we determined the expression patterns of three effector genes, one cell wall degrading enzyme (CWDE) and three genes involved in pathogen adhesion. Transcript abundance in the sporulating mycelia, zoospores and cyst and germinating cyst *in vitro* structures were compared to expression levels in the mycelial phase. The RXLR- and elicitin transcripts were significantly more abundant in zoospores and germinating cysts relative to mycelia, indicating that they may function in early infection. The necrosis-inducing *Phytophthora* protein 1 (NPP1) showed induction slightly earlier in *P. cinnamomi* (in the zoospore phase) than reported in *Phytophthora sojae* (upregulation in germinating cysts). NPP1 expression *in planta*, where it is thought to mediate the switch from biotrophy to necrotrophy, was not investigated, as infected plant material was not available. The cellulose binding elicitor lectin protein (CBEL) and adhesin transcripts were significantly upregulated in zoospores relative to mycelia, where they may function in pathogen adhesion to host surfaces during cyst germination. Putative alternative splicing was observed in an RXLR-motif containing unigene, but this remains to be verified. Alternative splicing may serve as a mechanism to increase functional variation within *P. cinnamomi* effector proteins. Large scale *in planta* expression analysis in the future will provide further insight into *P. cinnamomi* infection strategies.

INTRODUCTION

A plant-pathogen interaction is a very intimate, often hostile relationship between a host and a pathogen. The successful infection of a living host (biotrophy) requires the rapid adaption of the pathogen in reaction to host defence responses and to environmental queues. Genes that facilitate infection are termed pathogenicity genes and assist this process in various ways, ranging from protecting the pathogen against host attack, to manipulating host physiology. Expression analysis of genes involved in pathogenesis shows very specific expression patterns prior to- and during *in planta* infection (Kunjeti et al. 2011; Ye et al. 2011; Chen et al. 2013) suggesting that they are under strict temporal regulation.

Phytohthora cinnamomi Rands is a hemi-biotrophic, oomycete pathogen that is able to infect over 3000 plant species (Hardham 2005). The economic impact due to *P. cinnamomi* infestation is evident in the food industry (avocado, pineapple, peach, chestnut, macadamia) and the forest industry affecting eucalyptus, pine and oak trees (Zentmyer 1980; Zentmyer 1985). It is also the main pathogen on avocado worldwide resulting in Phytophthora root rot in California, Australia, Chile and South-Africa (Wager 1942; Allen et al. 1980; Aveling and Rijkenberg 1986; Sepúlveda-Chavera et al. 2013).

During its life cycle, *P. cinnamomi* has different phenotypical developmental structures, each with a specialized function. Expression analysis of various developmental stages of *Phytophthora* indicates that developmental structures are transcriptionally distinct (Judelson et al. 2008; Ye et al. 2011; Chen et al. 2013). An RNA-seq study by Ye et al. (2011) showed that zoospores were transcriptionally more similar to cysts, mycelia was more similar to sporulating mycelia and germinating cysts grouped alone (Ye et al. 2011). Here I discuss zoospores, cysts, germinating cysts, mycelia and mycelia containing sporangia in more detail with regard to their function and associated pathogenicity proteins.

The primary infection agents of oomycetes are motile, biflagellate zoospores that are released from sporangia (Hardham 2005). Their main function is to direct the spore to a suitable infection site by following chemotactic- and electrotactic signals emitted from living plant tissue (Hardham and Gubler 1990). The spore orientates itself with its ventral surface facing the plant (Hardham 2005) and attaches to the plant surface by secreting a mucous substance from internal vesicles (Gubler et al. 1989; Hardham and Gubler 1990). This secreted substance contains mucin and adhesin proteins (Görnhardt et al. 2000; Robold and Hardham 2005; Grenville-Briggs et al. 2010). Adhesion is further enhanced by cellulose

binding and elicitor lectin (CBEL) proteins, found in the cell walls of oomycetes that can bind to cellulose components of the host cell wall (Gaulin et al. 2002).

At this stage zoospores transform into cysts by the loss of flagella and the formation of a cell wall that allows the build up of turgor pressure within the cell (Hardham 2005). A germ tube emerges from the cyst and grows along the plant surface until a suitable point of entry is found (Hardham 2005). The objectives of germinating cysts are to penetrate host tissue (through specialized structure called appresoria) and simultaneously prepare the pathogen for direct interaction with the hosts' defence system. Host penetration is aided by the secretion of cell wall degrading enzymes (CWDE) that assist in breaking down this physical barrier of the host (Have et al. 2002) (lignified cell wall and tyloses deposited as a defence strategy by the host (Benhamou and Nicole 1999)). In a similar way proteins that inhibit host plant proteases (Misas-Villamil and van der Hoorn 2008) are released into the apoplastic space to protect the pathogen germling and colonizing hyphae from degradation.

Tissue colonization occurs after successful penetration, consisting of inter- and intracellular spread of hyphae, with haustoria frequently protruding into host cells (Hardham 2001). Haustoria are specialized intracellular biotrophic structures that serve as the front for nutrient, infection- and defence molecule exchange between host and pathogen (Latijnhouwers et al. 2003; Birch et al. 2008). Oomycetes secrete effector molecules into the apoplastic- and haustorial space that act by altering host cell structure and in this way facilitate infection and/or trigger defence responses (Hogenhout et al. 2009). Both elicitins and necrosis-inducing proteins are apoplastic effectors and owe their names to the response observed in the host in the presence of these proteins (Kamoun et al. 1993a; Kamoun et al. 1993b; Bae et al. 2006). The targets of cytoplasmic effectors are inside host cells. The main groups of cytoplasmic effector proteins include the RXLR gene family and Crinkling- and necrosis-inducing proteins (CRN). Members of both families have the ability to suppress host defence responses (Torto et al. 2003; Bos et al. 2010; Liu et al. 2010; Cheng et al. 2012; Chen et al. 2013).

Phytophthora propagates in the form of mycelia within host tissue and accordingly constantly faces attack by the host. The pathogen needs to rapidly respond to survive in this hostile environment. Mitogen-activated protein (MAP) kinases are critical for signal transduction and efficient communication during infection and have been implicated in pathogenesis, hyphal colonization and appresoria formation in fungal pathogens (Xu 2000). Protective proteins guard against the host basal defence response by aiding in the transport (ATP-binding cassette transporters) and detoxification (cytopchrome P450, peroxidases,

reductase enzymes etc.) of toxic compounds (including reactive oxygen species) released by the host.

Infection of adjacent plant material (or roots) is established by the generation of more zoospores from sporangia produced by hyphae on infected plant material. This occurs roughly two to three days after infection and rapidly increases the inoculum at a single site (Hardham 2005). The main objective of mycelia is to colonize host tissue, while sporulating mycelia forms part of both sexual and asexual reproduction.

Thus, a successful pathogen is one that can 1) locate suitable hosts, 2) successfully penetrate and colonize the host tissue, 3) effectively protect itself from the host defence system once detected, by either counteracting the effects and actively suppressing defence or lastly, completely escape the host surveillance systems. Pathogenicity genes enable successful infection by fulfilling various functions at specified times to achieve the above mentioned steps during infection. A study by Wang et al. (2011) showed that pre-mature expression of key *Phytophthora sojae* Kaufm. & Gerd. effectors (usually highly expressed only prior to infection or during infection), by DNA transformation, decreased the transformant's virulence on soya bean. Temporal regulation of pathogenicity related genes thus directly impacts on the success of infection and disease development.

In the previous chapter we employed RNA-seq analysis to identify over 2 000 pathogenicity genes within the *in vitro* infection structures of *P. cinnamomi*, cysts and germinating cysts. In this study we determined the expression pattern of seven genes over the four *in vitro* life stages of *P. cinnamomi* (mycelia, sporulating mycelia, zoospores and cysts and germinating cysts) with reverse transcriptase quantitative PCR (RT-qPCR).

MATERIALS AND METHODS

In vitro developmental stages of *P. cinnamomi*

Mycelia, sporulating mycelia, zoospores and cysts and germinating cysts were produced using an adjusted protocol of Chen and Zentmeyer (1970). Multiple 10 % V8 agar blocks (approximately 2 mm x 2 mm in size) containing a 3-day-old *P. cinnamomi* culture (isolate CNVDB29) were incubated in 5 % V8 broth (cleared V8 juice (Campbell Soup Company, USA), 0.1 g/L $CaCO_3$, 20 mg/L *B*-sitosterol, distilled water). V8 agar plates contained 17 g/L

agar in addition to the reagents present in V8 broth. After two days incubation under fluorescent light at 23 °C, mycelial plugs were washed with three changes of distilled water and harvested with a nylon sieve.

Sporangia formation was induced by incubating mycelial plugs under fluorescent light at 23 °C in river water (filtered twice through chromatography paper MN260 (Macherey-Nagel, Düren, Germany)) until sporangia developed (36 hours). Both mycelia and sporulating mycelia were harvested with a nylon sieve, frozen in liquid nitrogen, ground to a fine powder and stored at -70 °C.

Zoospore release from sporangia was induced by washing sporulating mycelia with two changes of distilled water at 4 °C, followed by incubation at 4 °C for 12 min. Zoospore release started approximately 1 hour later, while being incubated at 23 °C. The zoospores were separated from sporulating mycelia with a nylon sieve. Zoospores were allowed to encyst and were counted with the Superior haemocytometer (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany).

To enrich for germinating cysts, zoospores were vigorously shaken for 2 min to induce encystment and incubated in the dark at 19 °C for approximately 90 min. Both zoospores and germinating cysts were harvested via centrifugation (10 000 rpm at 4 °C for 5 min) in the Centrifuge 5810 R (Eppendorf, Hamburg, Germany). Pellets were immediately frozen in liquid nitrogen and stored at -70 °C. All *in vitro* developmental stages were photographed with the AxioCam MRc (Zeiss, Göttingen, Germany) coupled to the Axioskop microscope (Zeiss).

RNA extraction and cDNA synthesis

Total RNA was extracted from mycelia and sporulating mycelia using the CTAB Lithium-chloride method (Chang et al. 1993). Tri-Reagent (Ambion, Life technologies, USA) was used according to the manufacturer's instructions to obtain total RNA from cysts and germinating cysts. The RNA extraction methods were selected based on the quality and quantity RNA obtained from each type of developmental tissue using each method.

All RNA samples were treated with DNase I (Fermentas, Life Sciences, Hanover, USA) and purified using the Qiagen RNeasy cleanup kit (Qiagen, Valencia, California, USA). The presence of residual DNA in RNA samples was assessed by conventional PCR using the LPV3 primer pair (Kong et al. 2003). Each PCR reaction of 25 µl contained 2.5 µl 10X PCR

reaction buffer (Roche Diagnostics, Mannheim, Germany), 200 mM dNTPs (Bioline, London, UK), 3 mM $MgCl_2$, 0.2 µM of each LPV3 primer, 2 units Fast Start Taq DNA polymerase (Roche Diagnostics), Sabax water and 1 µl purified total RNA (199 – 7 444 ng/µl). As Fast Start Taq polymerase is a DNA dependent polymerase that lacks reverse transcriptase activity, amplification is only possible from a DNA template and no product will be produced from a RNA template. The PCR was performed on the 2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA) under the following cycling conditions: 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min, finishing with an 8 min incubation at 72 °C. PCR products were analysed with gel electrophoresis (2 % agarose gel, 80 V) yielding a product of 450 bp in size in the presence of DNA.

The purified total RNA concentrations and OD 260/280 and 260/230 ratios were measured with the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA). The cDNA synthesis system (ImProm-II Reverse Transcriptase) from Promega (Madison, Wisconsin, USA) was used according to the manufacturer's instructions, together with oligo poly-dT primers (Roche Diagnostics) to synthesize cDNA, used in RT-qPCR.

Selection of genes, primer design and evaluation

A total of 14 unigenes were selected for expression analysis based on their putative role in oomycete infection as indicated by literature. Two criteria were considered to gain confidence in selected unigene functional annotations: a) the quality of the unigene protein alignment to the protein sequence used for annotation (alignment length of >70 amino acids, >70 % protein similarity) and b) the presence of shared conserved motifs (NCBI conserved domain search tool, (Marchler-Bauer et al. 2011)) between the unigene and homologous protein sequence. Protein sequences were aligned with Mafft Version 7 (Katoh and Standley 2013), trimmed in MEGA 4 (Tamura et al. 2007) and visual outputs created with GeneDoc Version 2.7.000 (Nicholas et al. 1997). Unigene annotations were confirmed through blastx analysis (E-value >$1x10^5$) to *Phytophthora* proteins in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/), FungiDB database version 2.2 (Stajich et al. 2012), the *Phytophthora infestans* database (*Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/, downloaded February, 2013) and the *P. cinnamomi* genome v1.0 (http://genome.jgi.doe.gov/Phyci1/Phyci1.home.html, downloaded March, 2013).

Primers were designed to selected target unigene sequences using Primer3Plus version 2.3.5 (Untergasser et al. 2012) (amplicon size 70 – 150 bp, GC content 40-65 %, primer length 18-23 bp) and NetPrimer (PREMIER Biosoft, Palo Alto, CA, USA). Primer sequences directed against candidate endogenous control genes were obtained from the literature. This included primer pairs directed to ubiquitin-conjugated enzyme (Ubc), translation elongation factor 1 alpha (EF-1α), Beta-tubulin (β-tubulin) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes from King et al. (2010) and the WS041 primer pair from Narayan et al. (2010). All primers were synthesized by Integrated DNA Technologies (Coralville, United States). The target unigene name, primer sequences, annealing temperatures (Ta) used in RT-qPCR and the expected size of the products are provided in Table 1.

Primer specificity was evaluated with conventional PCR (as described above) and melting peak analysis via RT-qPCR using the Bio-Rad CFX Manager Software Version 1.5.534.0511 (Bio Rad laboratories, Hercules, California, USA). A dilution series (1:5, 1:10, 1:50, 1:100, 1:500 and 1:1000) of cDNA (containing small amounts of various cDNA samples from this study) was constructed to evaluate reference gene primer efficiency using the standard curve method. An additional dilution series (1:2, 1:5, 1:25, 1:50, 1:100 and 1:500) of DNA was used to determine the primer efficiency of primers directed to the target genes, as the amount of cDNA available was limited. RT-qPCR reactions were performed on the C1000 cycler CFX96 Real-Time System (Bio Rad laboratories). Each 20 µl reaction contained 10 µl 2x SensiMix SYBR No-ROX Master mix (Bioline, London, UK), 2 µl diluted cDNA (dilutions as described above), 1 µl of each forward and reverse gene specific primer (10 µM) and Sabax water. The reactions were performed in triplicate under the following conditions: 95 °C for 10 min, 42 cycles of 95 °C for 15 sec, Ta for 15 sec and 72 °C for 15 sec. The slope of the fitted regression line was used to determine the efficiency of each primer set.

Gene expression profiling using RT-qPCR

The expression pattern of seven pathogenicity genes (indicated with * in Table 3) was assessed across the *in vitro* life stages of *P. cinnamomi*. All reactions were performed in triplicate for four biological replicate samples of each life stage (mycelia, sporulating mycelia, zoospores, cysts and germinating cysts). Cycling conditions and reaction constitution were the same as described above, using 2 µl of cDNA (1:5) as template in each reaction. The expression stability of potential endogenous control genes was calculated with the Bio-Rad CFX Manager Software Version 1.5.534.0511 (Bio Rad laboratories), employing the geNorm method (Vandesompele et al. 2002). Gene expression levels of each target gene were

calculated by the same software, taking the determined primer efficiency into account and using three endogenous control genes (WS041, Ubc, β-tubulin) to normalize the data.

Statistical analysis

Significant differences in gene expression levels between life stages were determined with analysis of variance (ANOVA) followed by a Least Square Means Differences Student's t-test using the JMP '9 software ($p>0.05$) (SAS Institute, Cary, North Carolina).

RESULTS

RNA extraction and cDNA synthesis

Phytophthora cinnamomi (isolate CNVDB29) grew profusely in V8 broth producing mycelia, sporulating mycelia, zoospores, cysts and germinating cysts (Figure 1). On average, zoospore concentrations of $4x10^4$ spores/ml were obtained. Total RNA was successfully extracted from all developmental stages (Figure 2) and of sufficient quality to enable cDNA synthesis (Table 2). Conventional PCR (with a DNA dependent polymerase, lacking reverse transcriptase activity) confirmed the absence of residual DNA in all RNA samples (Figure 3).

Real-time RT-qPCR primer efficiency and specificity

Selection of candidate pathogenicity genes

Fourteen unigenes representing 11 pathogenicity gene groups (as described in Chapter 2 of this thesis) were selected for expression analysis (Table 3). These included two RXLR effector genes, one necrosis-inducing *Phytophthora* protein, one protease inhibitor, one elicitin, one small cysteine rich protein, four genes involved in pathogen adhesion and four cell wall degrading enzymes. All the selected unigenes showed significant homology to proteins predicted in the genome of *P. cinnamomi* (blastx analysis) (Table 4). The quality of the protein alignment, between the translated unigene and the protein sequence used for annotation, supported the functional annotation assigned to each unigene (requirements stipulated in the materials and methods section) (Table 5 and Figure 4). Of the 14 selected unigenes, nine contained conserved domains that are also present in the homologous

protein sequence. The five remaining unigenes shared at least 70 % similar amino acids with the homologous protein sequence over an alignment length of at least 75 amino acids.

Primer efficiency and specificity

The efficiency of each primer set (including primer pairs directed against five candidate endogenous control genes and 14 target genes) was evaluated using the standard curve method. A primer pair with 100 % efficiency will produce a slope of -3.32 during standard curve analysis. Thirteen primer pairs produced standard curves with slopes between -3.6 and -3.1 corresponding to primer efficiencies between 90 – 110 % (Figures 5 and 6). This included primer pairs amplifying reference genes Ubc, GAPDH, β-tubulin and WS041 and nine primer pairs directed to unigenes U8949, U15350, C31843, U6659, U2360, U6179, C83210, U4987 and U6809. The slopes of the standard curves generated for primer pairs amplifying the EF-1α gene and unigenes U17693, U6180, U8663, U9332 and U3104 were not within the accepted parameters (between -3.6 and -3.1). Accordingly the six above mentioned primer pairs and their corresponding genes were eliminated from further analysis.

The remaining 13 primer pairs were assessed with respect to primer specificity. Primer pair C31843 produced multiple peaks during melting curve analysis, suggesting that more than one product is amplified from cDNA by this primer pair (Figure 10B discussed later). Accordingly primer pair C31843 could not be used in expression analysis. The primer pairs amplifying endogenous control genes Ubc, GAPDH, β-tubulin and WS041 and eight primer pairs directed to unigenes U8949, U15350, U6659, U6179, C83210, U4987, U6809 and U2360 produced single PCR products as illustrated by gel electrophoresis analysis (Figure 7) and single trace signal during melting curve analysis (Figure 8). These 12 primer pairs were used in gene expression analysis as they were efficient and target specific.

Normalized expression analysis

Assessing the expression stability of candidate endogenous control genes

Real time RT-qPCR was used to determine the relative quantity of each transcript (WS041, Ubc, β-tubulin and GAPDH) across the *in vitro* life stages of *P. cinnamomi*. The M-value (gene stability value) for each candidate endogenous control gene was less than 0.5 (Table 6). The lower the M-value assigned to a gene, the more stable the expression is across the experimental conditions tested (Vandesompele et al. 2002), making it more suitable to act as

an endogenous control gene in a relative expression study. Genes WS041, Ubc and β-tubulin were selected to normalize the expression level of the target genes.

Gene expression profiling of target pathogenicity genes

Quantitative RT-PCR was used to assess the expression pattern of eight selected pathogenicity genes of *P. cinnamomi*. Great variation in cycle threshold values (Ct values differ >0.5) were repeatedly detected between technical replicates using primer pair U2360 during RT-qPCR analysis. Accordingly, the expression pattern of unigene U2630 could not be evaluated. Expression analysis was performed for the remaining seven unigenes (C83210, U15350, U4987, U6659, U6179, U8949 and U6809) across the *in vitro* life stages of *P. cinnamomi* (Figure 9).

Six of the seven genes assessed were significantly upregulated in the zoospore developmental stage in comparison to the mycelial phase. This included the effector genes (RXLR, NPP1 and elicitin genes), the polygalactorunase enzyme, CBEL and a mucin-like protein. The RXLR gene, the elicitin gene and the adhesion-like gene remained significantly induced in the cyst and germinating cyst phase relative to mycelia, although their expression levels were significantly lower than that in the zoospore developmental stage (Figure 9).

Transcript abundance of C83210 (RXLR-motif containing unigene) was 7.32 and 6.46 times higher in zoospores and cysts and germinating cysts respectively, relative to mycelial transcript levels (Table 7), while expression was significantly lower in sporulating mycelia (Figure 9). The NPP1 transcript accumulated to similar levels in mycelia, sporulating mycelia and cysts and germinating cysts, with a highly significant 5.79 fold induction in expression level (relative to mycelia) in the zoospore developmental stage. U4987 (elicitin gene) was significantly induced in the zoospore and cysts and germinating cyst phase (with respective 7.87 and 3.84 fold increase relative to the mycelia phase), with no noteworthy difference between mycelial and sporulating mycelial expression levels. The relative expression of the cell wall degrading enzyme, polygalacturonase (U6659) did not vary significantly between the developmental stages, except for a significant 1.63 fold induction observed in the zoospore phase, relative to mycelia. The CBEL transcript was significantly upregulated (8.81 fold) in zoospores relative to mycelia, with cysts and germinating cysts having similar expression levels than observed in mycelia. The mucin-like transcript (U8949) was significantly less abundant in zoospores and cyst and geminating cyst phases relative to mycelia. Lastly, an 18.69 fold increase was seen in the relative expression level of U6809 (the adhesin-like gene) in zoospores and a 6.63 fold increase in sporulating mycelia, relative

to mycelial levels. U6809 transcript abundance was significantly higher in germinating cysts (3.93 fold induction) than in mycelia.

Putative alternative splicing

Multiple peaks were detected during melting curve analysis of PCR products produced by the primer pair amplifying contig C31843 (RXLR-motif containing transcript) from *P. cinnamomi* cDNA (Figure 10B). Melting curve analysis of the PCR product produced from DNA by this primer pair indicated a single trace signal (at a melting temperature of 82 °C) (Figure 10A). In contrast, two melting peaks (at 82 °C and 85 °C) were detected when analysing the products amplified from cDNA of mycelia and sporulating mycelia by the same primer pair (Figure 10C and D), while melting curve analysis of the products from cDNA of zoospores and germinating cysts pointed to a single product similar to that produced from DNA (Figure 10E and F). Gel electrophoresis analysis suggests the presence of three products amplified from cDNA samples of the mycelial and sporulating mycelial phases from the same *P. cinnamomi* isolate (estimated sizes of 89 bp, 200 bp and 230 bp). The intensity of the larger sized bands was very low in comparison to the band of the primary product of 89 bp (Figure 11).

DISCUSSION

As a wide host range pathogen against which effective control strategies are lacking, investigation into the molecular basis of *P. cinnamomi* pathogenicity is critical. In this chapter, the expression pattern of seven putative pathogenicity genes was assessed across the *in vitro* developmental stages of *P. cinnamomi* (mycelia, sporulating mycelia, zoospores and cysts and germinating cysts) with the aim of gaining a better understanding of the infection strategy. This included expression profiling of three effector genes (RXLR-, NPP1- and elicitin genes), one cell wall degrading enzyme gene and three genes involved in pathogen adhesion.

The RXLR-, elicitin and adhesion-like genes showed elevated expression in *P. cinnamomi* cysts and germinating cysts, relative to the mycelial phase. The majority of the genes evaluated (six), however were most abundant in the zoospore stage. Microarray analysis of *Phytophthora infestans* (Mont.) de Bary during the same *in vitro* life stages suggested that genes involved in a specific developmental phase (e.g. haustoria formation) are already

induced in the stage preceding it (i.e. germinating cysts stage) (Judelson et al. 2008). Effector genes are accordingly expected to be induced in germinating cysts, pathogen adhesion molecules to be upregulated in zoospores and the necrosis-inducing protein to be expressed directly prior to the necrotrophic phase (during *in planta* infection). Based on this assumption, elevated expression in zoospores could indicate that the products of these genes are functionally important in the cysts and germinating cysts phase.

An alternative explanation involves the normalization factor (based on relative quantity of three endogenous control genes) used to normalize the expression level of each gene at each developmental stage. Suitable endogenous control genes are selected based on a high gene expression stability value, indicating that they are constitutively expressed at a constant level, independent of the treatment (or developmental stage). The geNorm algorithm, used to determine the endogenous control gene expression stability, calculates the gene stability value based on the principle that the expression ratio between various stable expressed genes would remain constant across different treatment groups. A situation may arise in which the expression ratio between endogenous control genes remains constant between different treatment groups (developmental stages) (resulting in a high gene stability value), but the relative quantity of all the endogenous control genes differ between treatments (resulting in different normalization factors for each treatment group). This may be possible in samples that are transcriptionally incomparable, as indicated by the large transcriptional shifts that occur between the various *in vitro* developmental stages of *Phytophthora* (Ye et al. 2011; Chen et al. 2013). If this is the case it may explain the unexpected high expression levels indicated in zoospores of most pathogenicity genes investigated in this study. RNA-seq analysis, where individual transcripts are counted as opposed to the measure of cycle threshold values, may verify the above mentioned hypothesis.

We amplified an RXLR-motif containing unigene (U31843) from DNA and cDNA of *P. cinnamomi* using a gene specific primer pair. Single trace signal indicated the amplification of a single product from DNA by this primer pair, while multiple products were detected in cDNA templates after melting curve analysis (two peaks) and gel electrophoresis (three bands). Different sized products with the same GC content will dissociate at the same temperature during melting curve analysis. This may explain the observation of only two melting peaks, while three bands were visible in gel electrophoresis analysis. The single product produced from DNA indicates that the primer pair is gene specific. The different sized transcripts present in cDNA may be explained by alternative splicing of mRNA transcripts. Alternative splicing has been reported in *Pseudoperonospora cubensis* (Berk. &

M.A. Curtis) Rostovzev and *P. sojae* (Shen et al. 2011; Savory et al. 2012). It is interesting that alternative splicing of transcript U31843 seemed to be restricted to the mycelial and sporulating mycelial phases as it was not detected in the zoospore and cysts and germinating cysts phases of *P. cinnamomi*. Evidence of stage dependent alternative splicing was found in at least 20 % of genes expressed in sporangia of *Ps. cubensis* and during early and late infection of *Cucumis sativus* (Cumbie 2013). The same may be true for transcript U31843 of *P. cinnamomi*. Alternative splicing may act as a mechanism to increase functional variation within the rapidly adapting effector repertoire of pathogens. The different products produced from cDNA need to be sequenced to confirm that they result from alternative splicing and not from non-specific amplification from cDNA by this primer pair.

The expression patterns of three effector genes were assessed including one RXLR (Arg-any amino acid-Leu-Arg) motif containing gene, one elicitin gene and one gene showing homology to the necrosis–inducing *Phytophthora* protein 1 (NPP1). Transcript levels of C83210, homologous to *P. sojae* Avh448 (RXLR protein), accumulated to higher levels in zoospores and germinating cysts than in *P. cinnamomi* mycelia. This is consistent with the expression pattern observed for most RXLR genes from oomycete species, where RXLR genes are more induced in the germinating cysts and/or appresorial developmental stages in comparison to mycelia (Judelson et al. 2008; Kebdani et al. 2010; Chen et al. 2011; Ye et al. 2011). The bulk of *P. infestans* RXLR gene expression is restricted to the biotrophic phase, coinciding with the presence of haustoria (Whisson et al. 2007). The upregulation of this *P. cinnamomi* RXLR gene in the germinating cyst phase suggests that it might function during early infection. Individual RXLR gene expression patterns show various temporal profiles. Some RXLR transcripts are present in pre-infection structures, others are restricted to plant infection, few are induced in the necrotrophic phase, while others are expressed during all the mentioned stages with higher induction at some points (Whisson et al. 2007; Wang et al. 2011; Chen et al. 2013). This differential regulation of RXLR family members implies that RXLR proteins function at different times during the infection process. Functional characterization of numerous RXLR proteins has illustrated their diverse functions in manipulating host physiology. The Avr3a effector from *P. infestans*, for example, stabilizes host E3 ligase CMPG1 (Bos et al. 2010) to suppress CMPG1-dependent cell death triggered by recognition of various pathogen elicitors (Bos et al. 2006; Gilroy et al. 2011). *Phytophthora sojae* effector Avr3b has ADP-ribose/NADH pyrophosphorylase activity and may act as a negative regulator of host plant immunity (Dong et al. 2011). Much research is required to speculate about the function of the product produced from transcript C83210, but its elevated expression in the germinating cysts phase suggest a role during early infection.

U4987, an elicitin-like gene, shows homology to the same gene from clade 3a (INL3a-like) of *P. infestans.* This gene family is divided into 17 clades containing elicitins and elicitin-like (ELL) genes based on the positions of the conserved cysteine residues within the elicitin domain (Jiang et al. 2006). Recognition of elicitin proteins induces a hypersensitive response (HR) in some plant species (Kamoun et al. 1993a; Kamoun et al. 1993b; Bonnet et al. 1996), associating this family with pathogenesis. This protein family is suggested to function in sterol acquisition in *Phytophthora* species (Vauthrin et al. 1999; Jiang et al. 2006; Yousef et al. 2011), but their role in pathogenisis remains unclear. Elicitins from the same clade show similar expression patterns. Clade 3 elicitins from *P. infestans*, *P. sojae* and *P. capsici* Leonian were more abundant in zoospore and germinating cysts phases than in mycelia (Qutob et al. 2003; Jiang et al. 2006; Chen et al. 2013). The data generated in this study supported these previous findings, indicating increased U4987 transcript accumulation in the zoospore and cysts and germinating cysts phases of *P. cinnamomi*. RNA-seq analysis of 46 *P. sojae* elicitin genes indicated high transcript abundance during pre-infection as well as during infection, with elevated expression levels in mycelia and zoospores (Ye et al. 2011). The precise role of elicitins in *Phytophthora* biology is unknown, but their expression pattern and elicitor activity suggest that they function in germinating cysts and during early infection.

Necrosis inducing proteins (NIPs) induce cell death in the host as part of the infection strategy (Bae et al. 2006), and is thought to facilitate the switch between trophic phases in hemi-biotrophic pathogens. It is therefore interesting that the NPP1-like (U15350) unigene in this study is upregulated during the zoospore phase of *P. cinnamomi*. Similar to this, necrosis-like proteins (NLP) of *P. sojae* showed two main induction points, one in germinating cysts and a second one at 12 -24 hpi of soya bean (start of necrotrophy) (Ye et al. 2011; Dong et al. 2012). Therefore the same proteins are expressed in *P. sojae* and *P. cinnamomi* pre-infection, but the time of expression varies. It is possible that the NIPs are suppressed by other effector proteins expressed in pre-infection- and infection structures (Dong et al. 2012), until the pathogen is ready to live off dead plant material. Kelley et al. (2010) showed that *P. infestans* suppressor of necrosis protein 1 (SNE1) is co-ordinately expressed with other necrosis inducing proteins (NIPs and Avr proteins) and is able to suppress their necrotic effect within *Solanum lycopersicum.* The authors suggest this antagonistic action as a regulatory mechanism to control the switch between biotrophy and necrotrohpy (Kelley et al. 2010). The same control mechanism may function within *P. cinnamomi* in the zoospore phase to suppress necrosis, although this remains to be determined. In contrast to the NIP expression observed in pre-infection structures of *P. cinnamomi*, other investigations did not detect NIP induction in germinating cysts, but only

during plant infection (and in mycelia in the case of *P. sojae* and *Phytophthora megakarya*) (Qutob et al. 2002; Bae et al. 2005; Haas et al. 2009). A study by Dong et al. (2012), detected varied expression patterns of 13 NIPs of *P. sojae* via RT-PCR, where some NIPs had two induction points, while others were only induced in germinating cysts or during late *in planta* infection of soya bean. Together, the data suggest differential regulation of NIP genes.

Plant pathogens secrete hydrolytic enzymes that degrade the plant cell wall (Have et al. 2002), a physical barrier in plant defence. Polygalacturonase (an enzyme which degrades pectin) is one of the first hydrolytic enzymes secreted by many plant pathogens during infection (Jones et al. 1972), exposing other components of the cell wall to different CWDE. The *P. cinnamomi* polygalacturonase (U6659) expression profile showed a significant increase in transcript abundance in zoospores relative to mycelia, while the expression in the other *in vitro* stages remained constant and at lower levels. The expression level of transcript U6659 in the cysts and germinating cysts phase did not differ significantly from the transcript abundance in zoospores, although it was not significantly higher than in mycelia. This profile suggests that the product of transcript U6659 may be functionally active in the cysts and germinating cysts phase of *P. cinnamomi*, aiding in plant cell wall hydrolysis once the pathogen is in contact with the host surface. In contrast to the detected regulation of the polygalacturonase of *P. cinnamomi* in this study, the mean transcript abundance of 24 polygalacturonase genes of *P. sojae* revealed a different expression profile (Ye et al. 2011). The *P. sojae* polygalacturonase family had a higher transcript abundance in mycelia, germinating cysts and during the biotrophic phase of soya bean infection, with decreased expression in zoospores (Ye et al. 2011). The average expression level of this group of genes was lower than that of other CWDE (serine protease, cysteine protease and glucanase enzyme genes) of *P. sojae* (Ye et al. 2011). Polycalacturonase genes exist as large families in *Phytophthora* species (with 3 to 19 members as listed in FungiDB database version 2.3) and *P. cinnamomi* has at least 17 members (Götesson et al. 2002). A cumulative effect may be achieved if all members are expressed at low levels simultaneously. Alternatively individual members of the gene family may be induced during different developmental stages, explaining the varied expression patterns observed between the single polygalacturonase of *P. cinnamomi* and the gene family of *P. sojae*.

CBEL proteins are family 1 carbohydrate binding molecules (CBM1) that are specific to the oomycete lineage (Larroque et al. 2012). It is functionally implicated in cell wall deposition and adhesion to cellulose substrates (Gaulin et al. 2002). The cellulose binding domain of the protein is a pathogen-associated molecular pattern (PAMP), eliciting host defence

responses (Gaulin et al. 2006). The CBEL protein transcript (U6179) from *P. cinnamomi* accumulated to highest levels in the zoospore phase and was not significantly induced in cysts and germinating cysts relative to mycelial levels (0.23 fold). This is similar to the expression pattern observed for the *P. infestans* CBEL transcript (Grenville-Briggs et al. 2010). CBEL was also highly expressed in sporulating mycelia of *P. infestans* relative to mycelia, while transcript abundance was even lower in sporulating mycelia in this study of *P. cinnamomi*. Since *P. infestans* sporangia detach and disperse (Gerrettson-Cornell 1989), it is not unexpected to observe adhesion related transcripts expressed in this developmental structure. *Phytophthora cinnamomi* sporangia are non-papillate and thus the pathogen relies on zoospores to locate the host surface. Accordingly, the expression of CBEL in *P. cinnamomi* zoospores, the stage preceding the cyst developmental phase where cysts attach to host surfaces, support the role of CBEL proteins in recognizing and binding to cellulose in plant cell walls (Gaulin et al. 2002). The opposite expression pattern was observed in *P. sojae* (non-papillate sporangia), where the average transcripts level of five CBEL genes decreased in zoospores and germinating cysts and were highly induced in mycelia, sporulating mycelia and *in planta* (Ye et al. 2011). It may be possible that different CBEL genes are expressed during different pre-infection phases.

Other genes involved in pathogen adhesion include adhesion-like genes (Robold and Hardham 2005) and mucin-like genes. The adhesion-like gene investigated (U6809) in this study was expressed during all *in vitro* growth stages of *P. cinnamomi*, but was highly induced in zoospores. It is interesting that this adhesin-like transcript is more abundant in sporulating mycelia than in germinating cysts. None the less, increased expression in zoospores and germinating cyst relative to mycelia suggest that this protein might function during early infection. In contrast to this, the relative expression level of transcript U8949 (mucin-like protein) was higher in mycelia and sporulating mycelia of *P. cinnamomi* than in zoospores and germinating cysts. This reflects the same expression pattern as observed from the average expression analysis of seven mucin-like genes from *P. sojae,* also indicating high transcript accumulation during soya bean infection (Ye et al. 2011). In contrast to this, the expression of three mucin-like proteins from *P. infestans* was restricted to germinating cysts as determined by Northern blot analysis (Görnhardt et al. 2000). Mucins are large, threonine, cysteine and serine rich glycoproteins that are either secreted or associated with the cell membrane (Perez-Vilar and Hill 1999; Hicks et al. 2000). They act as lubricants, signal molecules and protectants by covering surfaces and preventing dehydration (Perez-Vilar and Hill 1999). The expression pattern observed for the mucin-like transcript in this study suggests that oomycete mucins rather act as protective agents than in facilitating pathogen adhesion.

CONCLUSION

This is the first study to investigate pathogenicity related gene expression in *P. cinnamomi.* The expression patterns of seven pathogenicity related genes, identified from an RNA-seq library of the cyst and germinating cyst phase of *P. cinnamomi*, were evaluated across four *in vitro* developmental stages. The majority of the genes assessed were highly induced in the zoospore developmental stage, indicating that they are functionally important in the subsequent phase, namely germinating cysts and may be involved in the infection process. Analysis of individual gene expression profiles revealed similar expression patterns as indicated for other oomycete species. Even though the *in vitro* expression pattern of genes provide insight into their potential function, expression analysis within a specific *in planta* interaction is necessary to obtain a more complete understanding of infection. Putative alternative splicing was observed in a RXLR-containing gene. Alternative splicing may serve as a mechanism to increase variation among the effector repertoire with regard to function and the ability to evade host detection, and will be interesting to investigate further among other effector genes.

Acknowledgements

This work was supported by funding from the National Research Foundation THRIP Grant TP 2011060300010, The Hans Merensky Foundation and Westfalia.

TABLES

Table 1. Sequences of primers to be used in RT-qPCR analysis, targeting pathogenicity related genes of *Phytophthora cinnamomi*. The annealing temperature (Ta) used in reactions and the expected product size are indicated for each primer pair.

Target	Forward Primer 5'-3'	Reverse primer 5'-3'	PCR product (bp)	Ta (°C)
C31843	GATGACAAGAAGCCCAAG	TTGTCTCCGTCCTTCTTC	89	58
C83210	TACCGAGGCTGAAAATCC	CTTCTTGTCGTCGTTCAC	88	58
U15350	GTTCCAAGACCTCATCAC	TCTGTATCCTTCCTCTCC	102	58
U17693	TCTGACGGGATGACATAC	TCGTGTGCTTTGGTAATAG	83	58
U2360	TTCCATCAAGAACACGTTC	TCCTTGGACAGCGTAATG	75	58
U3104	GGTGGTGTCGGCTGTATC	GACGAAGGCTTTGGACTGAG	75	61
U4987	ACAACGGGCATATTCAAG	GTTAGTAGCTCCCATCAG	75	58
U6179	TGGTACTCGTTGAAGAAAG	AAGACTGTGTACGGTTTG	92	58
U6180	CACGATACCAGTGACGAC	CGACCAAAACGAGAAGAG	80	58
U6659	GTCACCATCACAGGAAAC	TGCTGAAGACCGTGTTAG	79	58
U6809	TCATCCAAGAAGTCATCG	TTCCAGTTCAGTCAGTTC	78	58
U8949	GCTCCAGTCTTGATCTTC	CCACGAGAACCTGTCTAG	84	58
U8663	CAAGTGCGTGCTGATCTC	CTCGGCATCTTGATCTTCTC	75	58
U9332	GATCAATTCCACACTATG	AACTCTGTGTACTTACTG	86	58
Primers directed to candidate reference genes from the literature:				
Ubc [a]	CTGAACATCTACTTCCCGGCC	CGTTGGCATTGATGTTGCAG	70-150	61
β-tubulin [a]	TGACCCAGCAGCAGTTCG	CATCGACCTCCTTCGTGCTC	117	61
GAPDH [a]	CCATCCGTGTGTTCAACGAG	GTGAAGGCACCCGTGGAC	70-150	61
EF-1 α [a]	CGAGAAGTCGGGCAACATGC	TTGTCAACCGGGCGC	90	61
WS041 [b]	GCTGACCAACAACGGCACG	GATCACCTCCGTACCTGCG	178	61

[a] King et al. (2010)

[b] Narayan et al. (2010)

Table 2. Quality and concentration of purified total RNA from *Phytophthora cinnamomi in vitro* life stages.

Life stage	Biological replicate	RNA concentration (ng/µl)	OD 260/280	OD 260/230
Mycelia	1	741.47	2.18	2.18
	2	424.53	2.15	2.26
	3	660.8	2.16	2.1
	4	402	2.15	1.96
Sporulating mycelia	1	391.67	2.14	2.17
	2	781.43	2.2	2.35
	3	532.97	2.2	2.25
	4	707.9	2.17	1.38
Zoospores	1	1540.4	2.2	2.2
	2	2214.37	2.08	2.02
	3	1352.87	2.15	2.28
	4	459.867	2.13	2.13
Cysts and germinating cysts	1	199.27	2.15	1.95
	2	7444.87	2.18	2.44
	3	914.43	2.18	2.31
	4	4225.73	2.08	2.03

Table 3. Unigenes from the *Phytophthora cinnamomi* cyst and germinating cyst cDNA library selected for expression analysis. The best *Phytophthora* protein match during blastx analysis and its functional description is indicated for each unigene.

Unigene ID	Protein ID of best blast hit	Species	Putative function	Bit Score	E-value
C31843	Physo_298800 [a]	*P. sojae*	SNEL2	55.84	2.40E-13
C83210*	Phyra_97565 [a]	*P. ramorum*	Pr_Avh387	32.73	1.42E-06
U15350*	PITG_19936	*P. infestans*	NPP1-like protein	111.69	5.04E-26
U17693	PITG_07096	*P. infestans*	protease inhibitor Epi11	66.24	3.00E-12
U2360	EGZ23444.1 [b]	*P. sojae*	cellulase-1	165.24	6.39E-45
U3104	PITG_15968	*P. infestans*	mucin-like protein	114.78	5.80E-27
U4987*	PITG_06370	*P. infestans*	elicitin INL3a-like protein	55.84	3.23E-09
U6179*	PITG_03640	*P. infestans*	CBEL putative	167.16	5.69E-47
U6180	PITG_12361	*P. infestans*	cutinase, putative	338.96	3.39E-100
U6659*	AAN05466.1 [b]	*P. cinnamomi*	polygalacturonase	192.2	1.14E-57
U6809*	PITG_16183	*P. infestans*	adhesin-like protein	200.29	3.25E-52
U8663	PITG_16145	*P. infestans*	small cysteine rich protein SCR108-like protein	194.13	1.10E-50
U8949*	PITG_09760	*P. infestans*	mucin-like protein	157.15	8.95E-39
U9332	PITG_17947	*P. infestans*	chitinase, putative	196.82	4.68E-51

SNEL2 - suppressor of necrosis 1-like protein 2, avh – avirulence homologue, NPP1 – necrosis-inducing *Phytophthora* protein 1, CBEL – cellulose binding elicitor lectin

* Unigenes for which the expression pattern was determined

[a] Protein ID numbers from FungiDB database version 2.2

[b] Protein ID numbers from NCBI database

PITG ID numbers are from the *Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)

Table 4. Unigenes from the *Phytophthora cinnamomi* cyst and germinating cyst cDNA library, with the best protein match from the *Phytophthora cinnamomi* genome indicated as determined through blastx analysis.

Unigene ID	Protein ID of best blast hit	E-value	Bit Score	% Identity	% Similarity	% Gaps	Alignment length (aa)
C31843	jgi-Phyci1-16230	1.00E-14	65.47	100	100	0	95
C83210*	jgi-Phyci1-246465	3.09E-35	133.26	100	100	0	82
U15350*	jgi-Phyci1-501748	8.28E-64	228.79	98	99	0	109
U17693	jgi-Phyci1-107067	8.44E-15	160	40	52	23	115
U2360	jgi-Phyci1-227507	1.14E-48	178.33	100	100	0	100
U3104	jgi-Phyci1-324897	1.52E-23	94.36	39	52	28	54
U4987*	jgi-Phyci1-96866	6.11E-35	132.11	86	86	13	66
U6179*	jgi-Phyci1-89138	2.57E-51	186.81	100	100	0	86
U6180	jgi-Phyci1-6902	8.44E-115	399.82	83	83	16	217
U6659*	jgi-Phyci1-98110	7.95E-56	192.2	6	11	57	91
U6809*	jgi-Phyci1-98652	6.63E-76	270.01	100	100	0	187
U8663	jgi-Phyci1-96960	4.28E-65	233.42	96	96	3	126
U8949*	jgi-Phyci1-103247	1.25E-45	170.63	35	35	62	144
U9332	jgi-Phyci1-280221	3.90E-54	197.98	52	60	27	159

* Unigenes for which the expression pattern was successfully determined

Table 5. Amino acid identity scores of the protein alignment between selected unigenes from the cyst and germinating cyst cDNA library of *Phytophthora cinnamomi* and the most homologous protein from other *Phytophthora* species. The presence and name of conserved domains predicted in the unigene sequences are indicated.

Unigene	Annotation protein ID	% Identity	% Similarity	% Gaps	Alignment length (U to blast hit)	Shared domain	Domain description
C31843	Physo_298800	68	72	17	115	Yes	RXLR**
C83210*	Phyra_97565	43	53	16	83	Yes	RXLR**
U15350*	PITG_19936	50	69	1	109	Yes	NPP1 ∓
U17693	PITG_07096	28	37	43	147	Yes	KAZAL FS ǂ
U2360	EGZ23444.1	92	95	0	100	No	NA
U3104	PITG_15968	69	76	0	76	No	NA
U4987*	PITG_06370	40	57	14	65	Yes	Elicitin
U6179*	PITG_03640	87	94	0	86	Yes	APPLE Factor XI like
U6180	PITG_12361	70	75	17	215	Yes	Cutinase
U6659*	AAN05466.1	35	39	47	289	Yes	Glyco-hydro 28
U6809*	PITG_16183	65	78	3	184	No	NA
U8663	PITG_16145	80	87	5	125	No	NA
U8949*	PITG_09760	69	76	9	394	No	NA
U9332	PITG_17947	45	53	39	131	Yes	Hydrolase

* Unigenes for which the expression pattern was successfully determined

** RXLR – Arg-any amino acid-Leu-Arg motif, ∓ NPP1 – necrosis inducing *Phytophthora* protein 1 motif, ǂ Kazal FS - Kazal type serine protease inhibitors and follistatin-like domains

Table 6. Analysis of uniform expression of candidate reference genes (WS041, Ubc, β-tubulin and GAPDH) across the various *in vitro* life stages of *Phytophthora cinnamomi*. The gene stability (M-value) and coefficient of variance is indicated for each gene.

Target	Coefficient Variance	M-Value
Ubc	1.6204	0.1420
B-tub	1.6761	0.0805
GAPDH	1.8466	0.1860
WS041	1.9400	0.0029

Table 7. Expression fold change of pathogenicity genes in sporulating mycelia, zoospores and cyst and germinating cyst developmental stages of *Phytophthora cinnamomi* relative to mycelial expression levels, as determined with RT-qPCR.

		Expression fold change relative to mycelia expression level		
Unigene ID	Function	Sporulating mycelia	Zoospores	Cysts and germinating cysts
C83210	RXLR	0.33	7.32	6.46
U15350	NPP1	0.26	5.79	0.40
U4987	Elicitin	0.78	7.87	3.84
U6659	Polygalacturonase	1.40	1.63	1.21
U6179	CBEL	0.09	8.81	2.11
U8949	Mucin-like protein	1.27	0.02	0.12
U6809	Adhesin-like protein	6.63	18.69	3.93

FIGURES

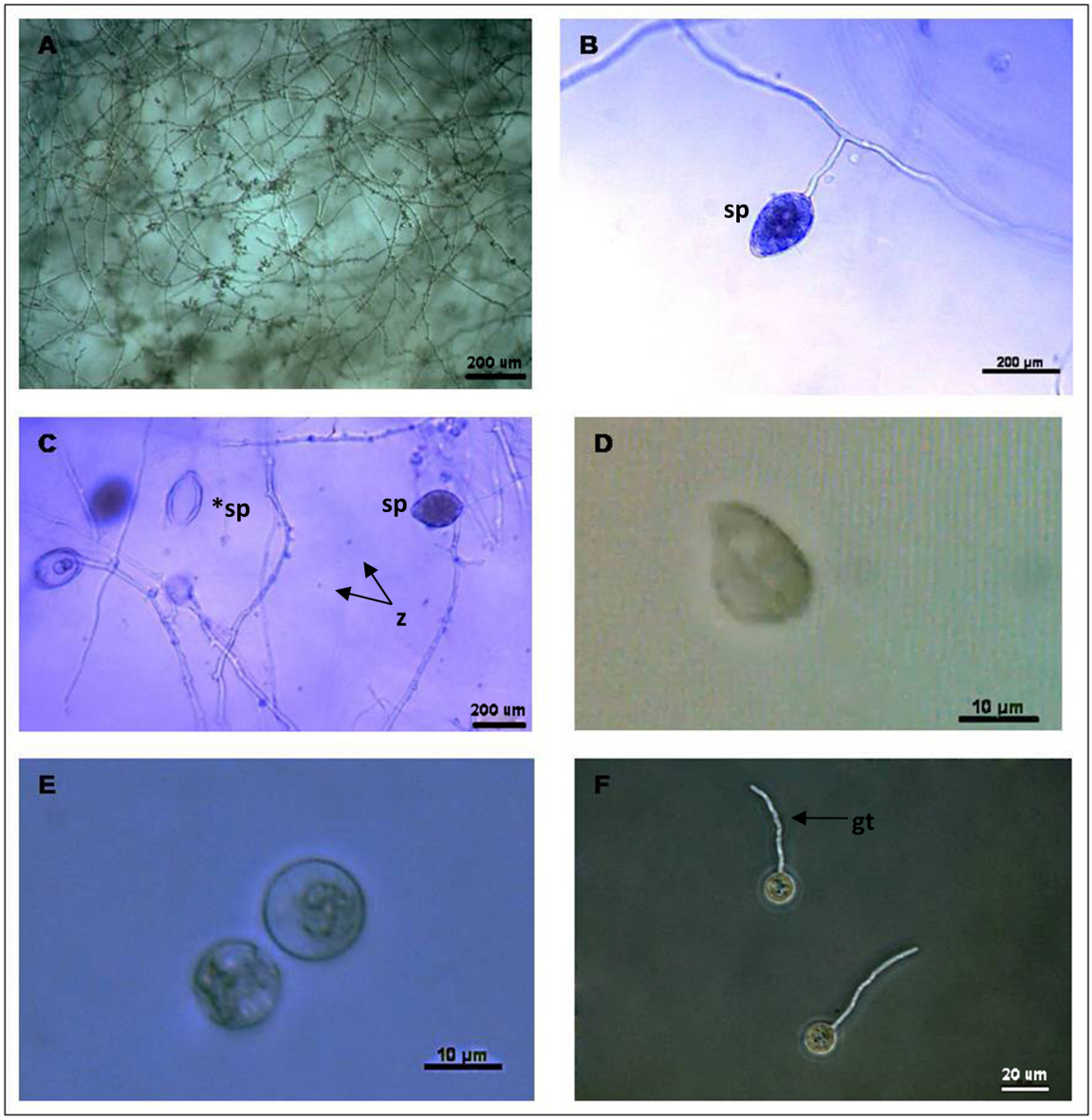


Figure 1. Stereo microscopy images of the *in vitro* life stages of *Phytophthora cinnamomi.* A) Mycelia in 5% V8 broth B and C) Sporulating mycelia D) A single zoospore E) Cysts F) Germinating cysts. sp – sporangium, *sp – sporangium that released zoospores, z – zoospores, gt – germ tube emerging from a cyst. Scale bars in A to C = 200 µm, in D and E = 10 µm, in F = 20 µm

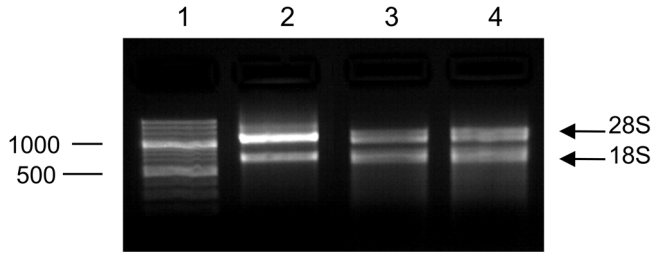


Figure 2. Agarose gel electrophoresis image of selected total RNA samples from *Phytophthora cinnamomi*. The 28S and 18S ribosomal subunit rRNA bands are clearly visible. 1) O'GeneRuler 100 bp DNA ladder 2) RNA from mycelia 3 and 4) RNA from zoospores.

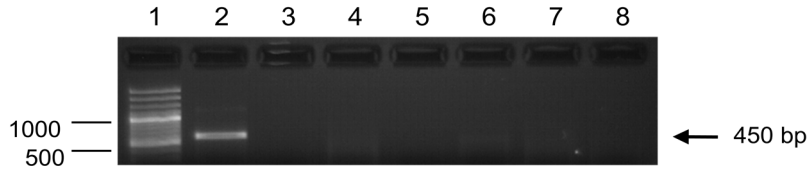
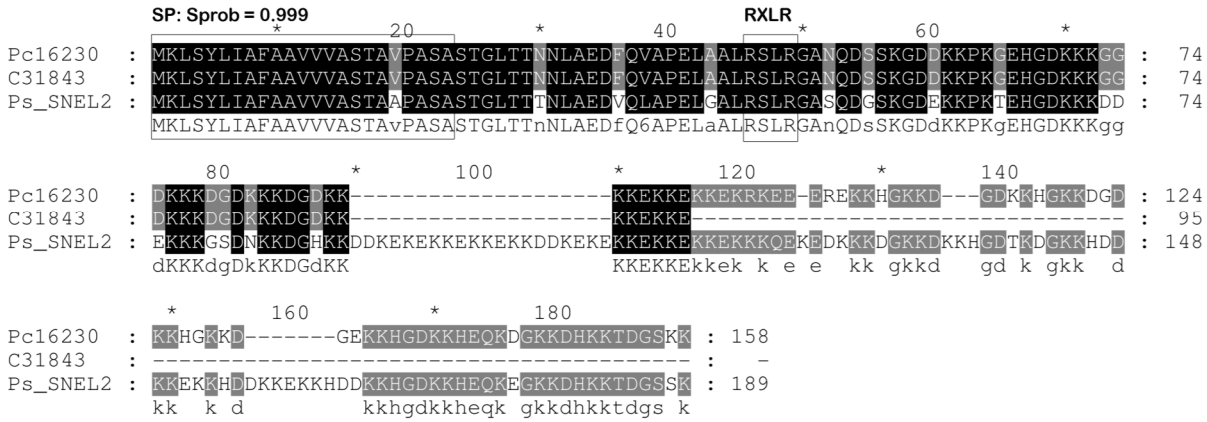


Figure 3. Conventional PCR, with the LPV3 primer pair, to confirm the absence of residual DNA in *Phytophthora cinnamomi* RNA samples. As the DNA-dependent polymerase used in the PCR reaction lacks reverse transcriptase activity, no amplification occurs in the absence of a DNA template. Lane 4-6 contained purified total RNA as template in the PCR reaction originating from sporulating mycelia (lane 4) and cysts and germinating cysts (lane 5 to 8). Lane 1) O'GeneRuler 100 bp DNA ladder 2) Positive control (DNA from a *P. cinnamomi* isolate CMW33388 as template) 3) Negative control.

A



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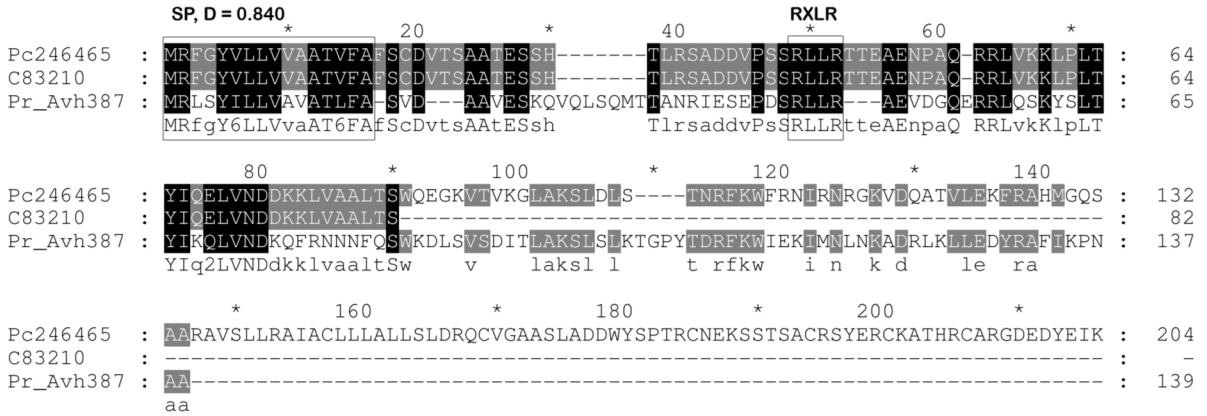

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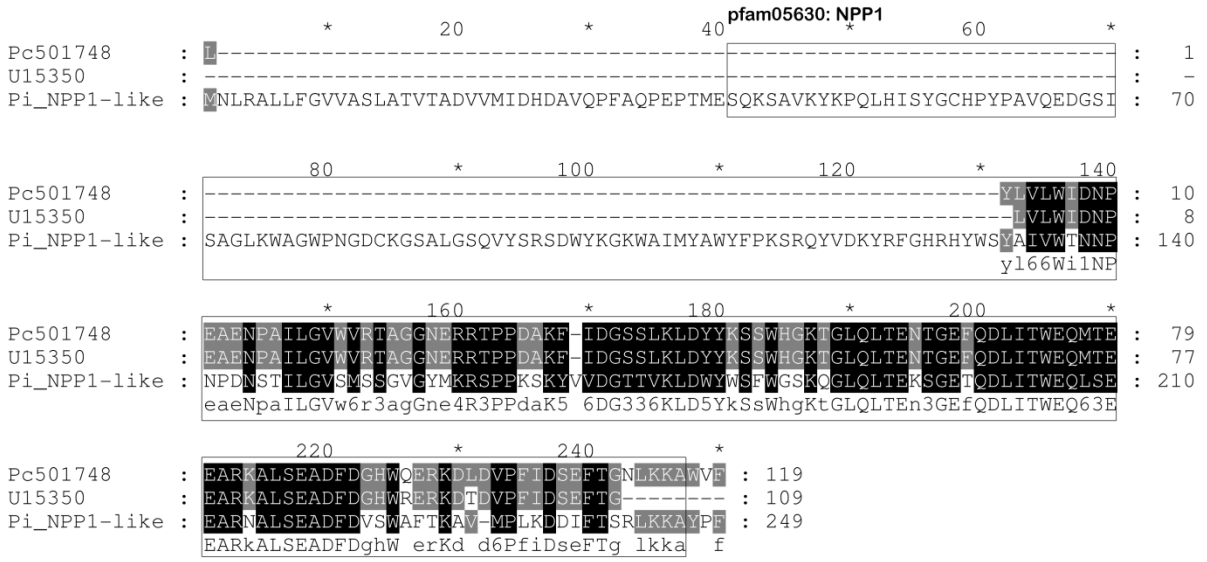

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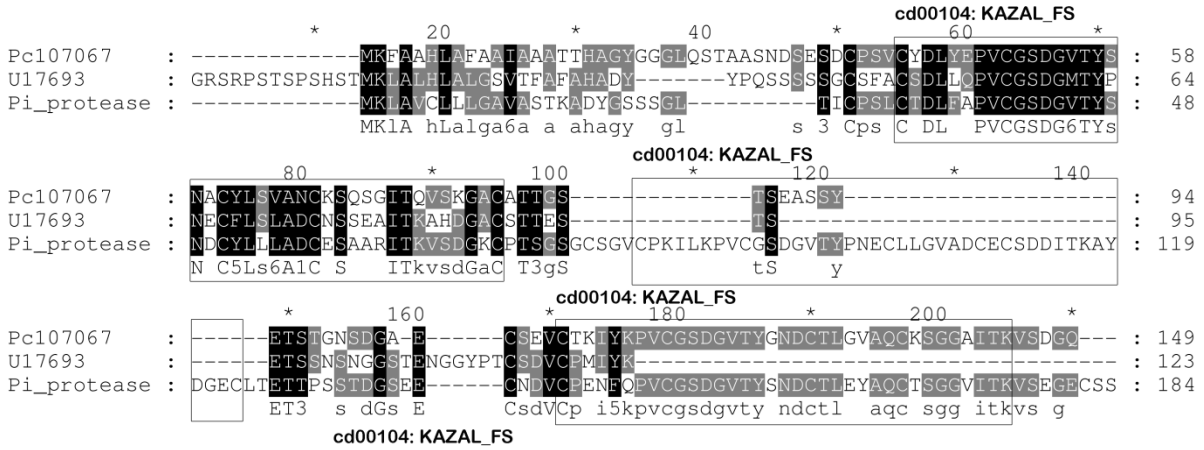

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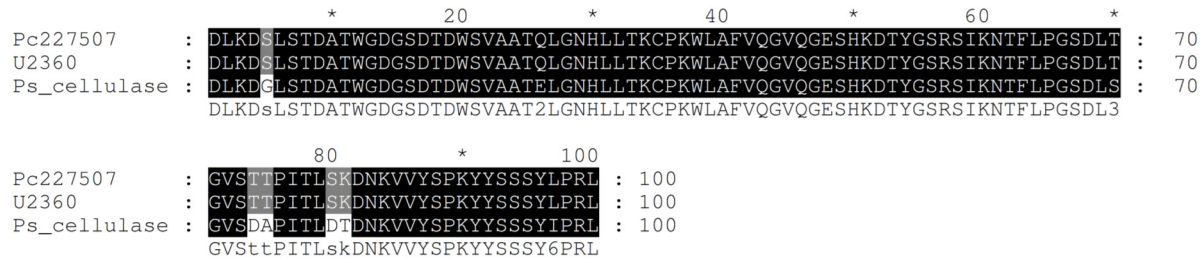

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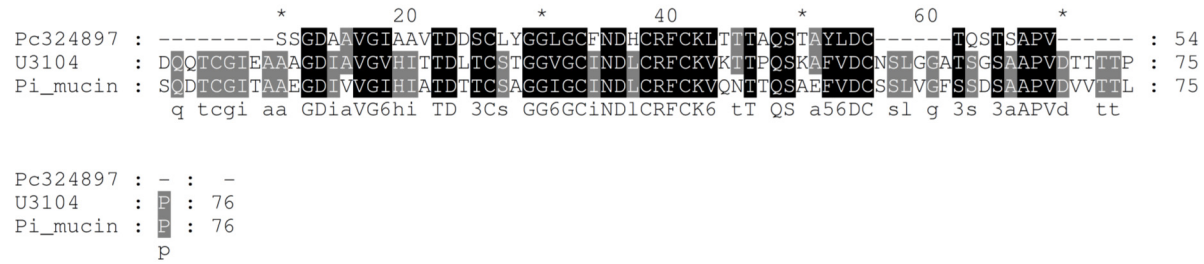

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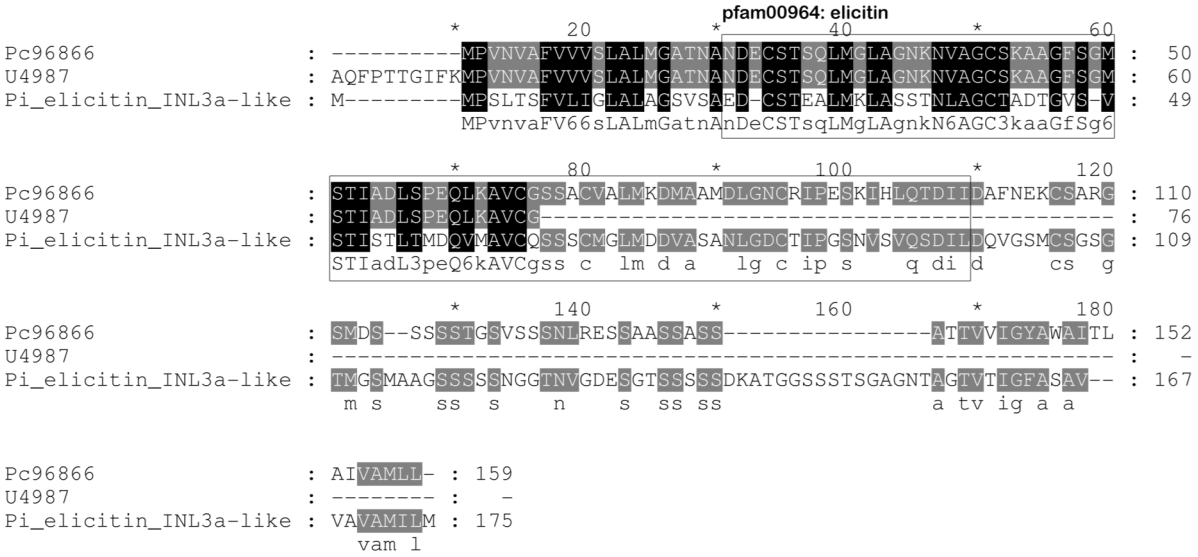

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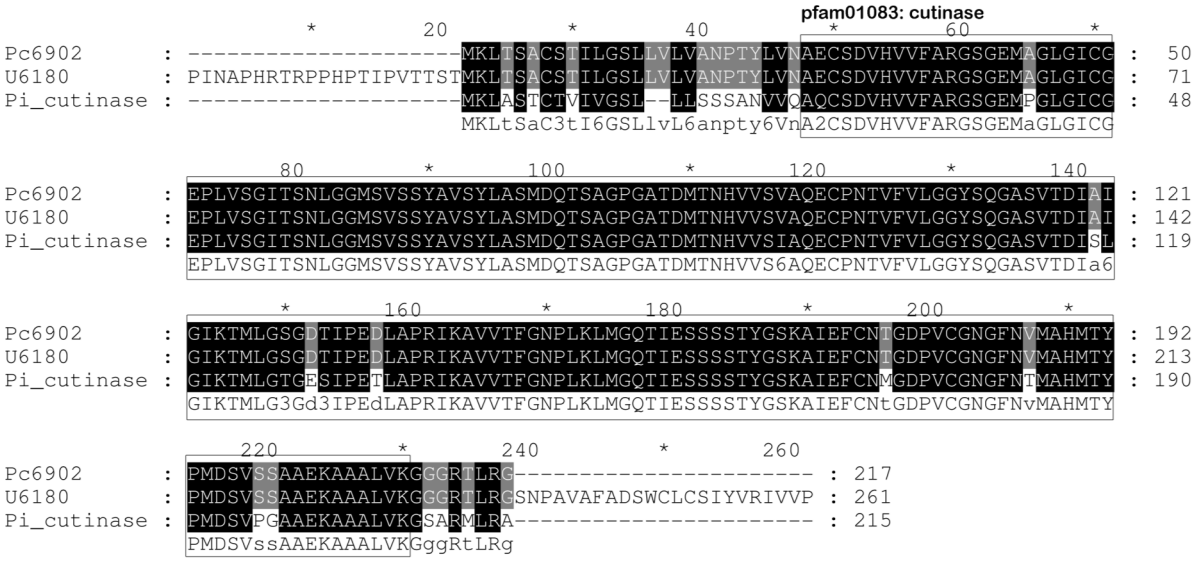

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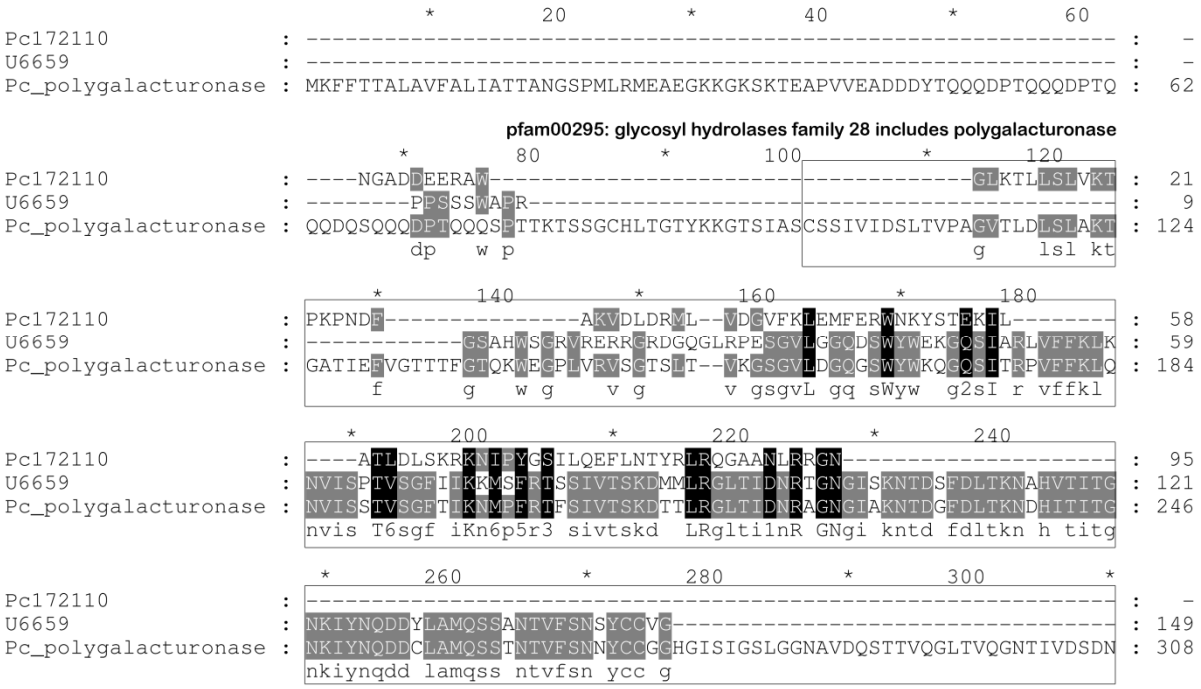

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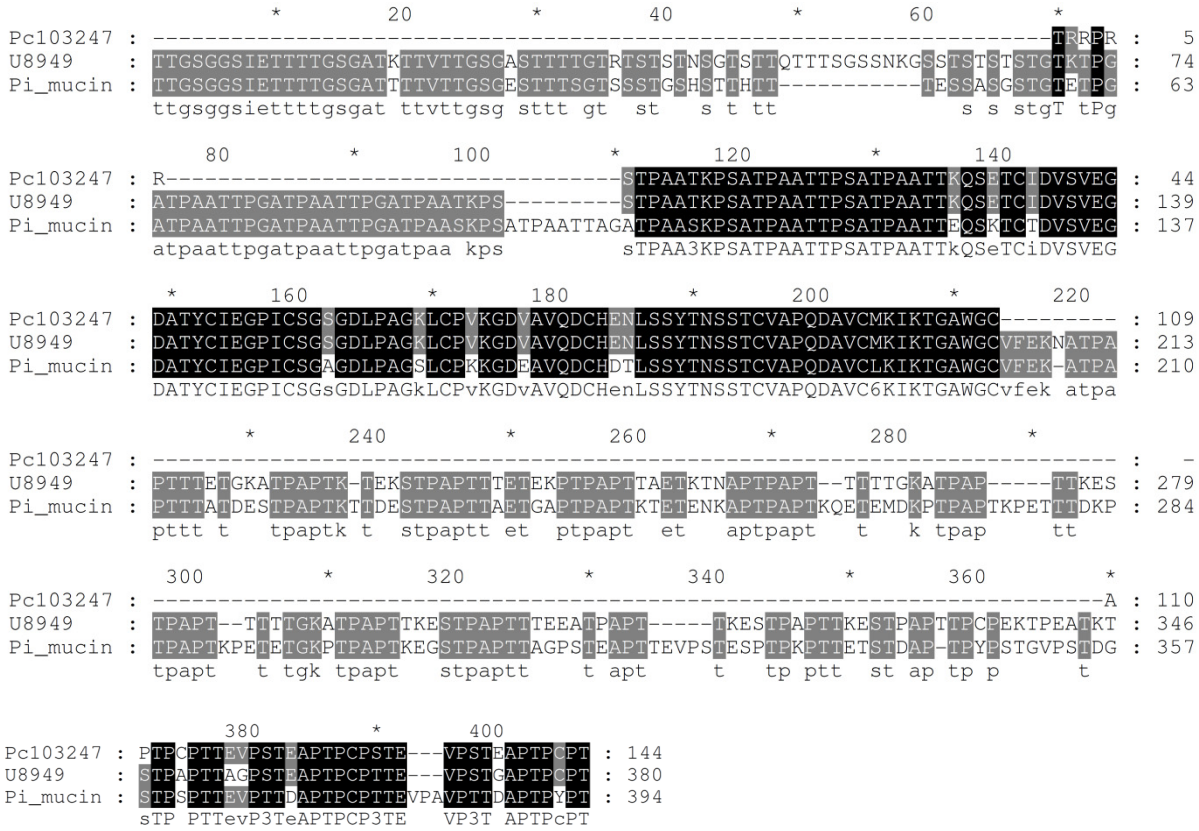

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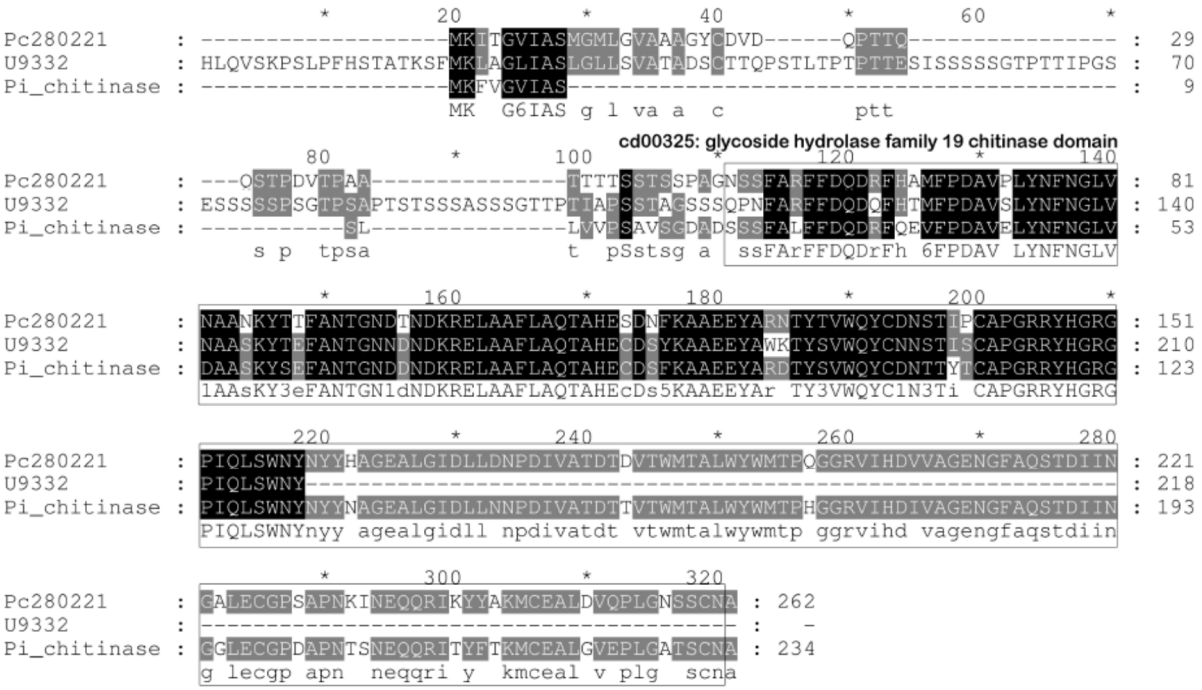

Figure 4. Protein sequence alignment of unigenes from the cyst and germinating cyst cDNA library of *Phytophthora cinnamomi* selected for expression profile analysis, to the best blastx hit in the genome of *P. cinnamomi* (top sequence) and the *Phytophthora* protein sequence used to annotate the selected unigene. Conserved domain names are shown above the relevant domain sequence (blocks). Shaded amino acids indicate amino acids that are similar between all three aligned sequences (black) or only between two of the sequences (grey). The consensus sequence is indicated underneath each alignment. Note only the complete unigene sequence is shown in the figure but not the whole protein sequence of the protein it is aligned to. A) C31843 aligned to jgi-Phyci1-16230 and Physo_298800 B) C83210 aligned to jgi-Phyci1-246465 and Phyra_97565 C) U15350 aligned to jgi-Phyci1-501748 and PITG_19936 D) U17693 aligned to jgi-Phyci1-107067 and PITG_07096 E) U2360 aligned to jgi-Phyci1-227507 and EGZ23444.1 F) U3104 aligned to jgi-Phyci1-324897 and PITG_15968 G) U4987 aligned to jgi-Phyci1-96866 and PITG_06370 H) U6179 aligned to jgi-Phyci1-89138 and PITG_03640 I) U6180 aligned to jgi-Phyci1-6902 and PITG_12361 J) U6659 aligned to jgi-Phyci1-98110 and AAN05466.1 K) U6809 aligned to jgi-Phyci1-98652 and PITG_16183 L) U8863 aligned to jgi-Phyci1-96960 and PITG_16145 M) U8949 aligned to jgi-Phyci1-103247 and PITG_09760 N) U9332 aligned to jgi-Phyci1-280221 and PITG_17947.

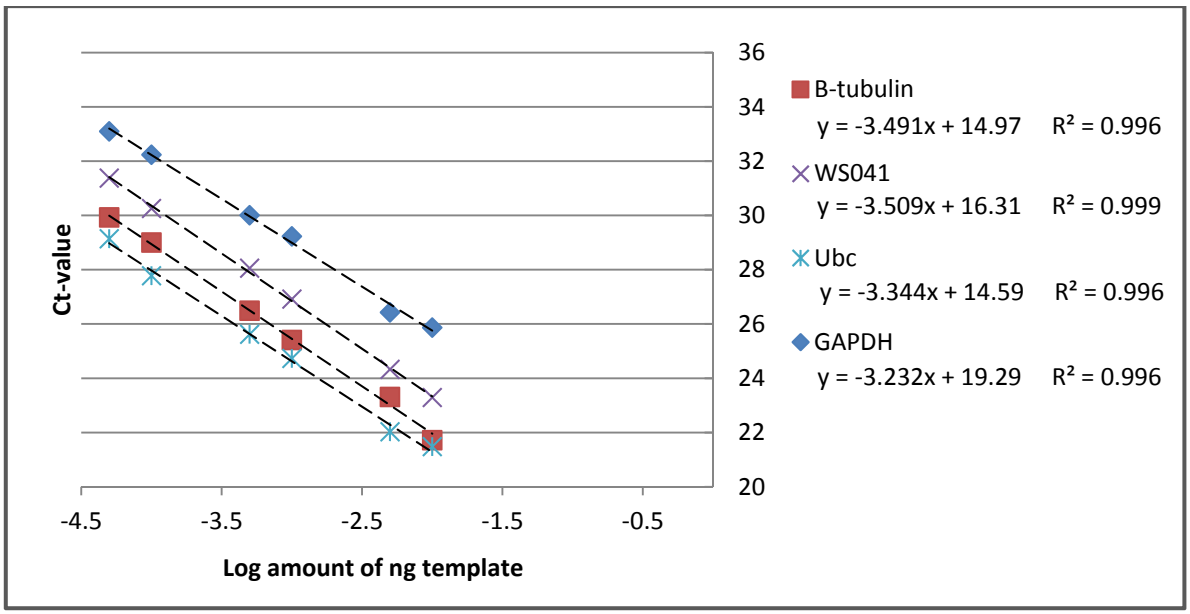

Figure 5. Standard regression curves for primers directed to four candidate endogenous control genes to be used in a relative transcript expression study in *Phytophthora cinnamomi*. This included analysis of primer pairs ubiquitin-conjugated enzyme (Ubc), beta-tubulin (β-tubulin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and WS041. A dilution series of a mixture of cDNA (1:5, 1:10, 1:50, 1:100, 1:500 and 1:1000) was used to construct standard curves. The mean Ct (threshold cycle for each trace) value of 3 technical repetitions was plotted against the log [10] function of the concentration of input template for each primer pair. A linear trend-line was fitted to the data. The equation for each fitted regression line, as well as the R^2 value (as an indicator of the ability of the model to predict a trend) for each gene is indicated. An R^2 value of more than 0.98 and a slope between -3.6 and -3.1 are considered acceptable for efficient primers.

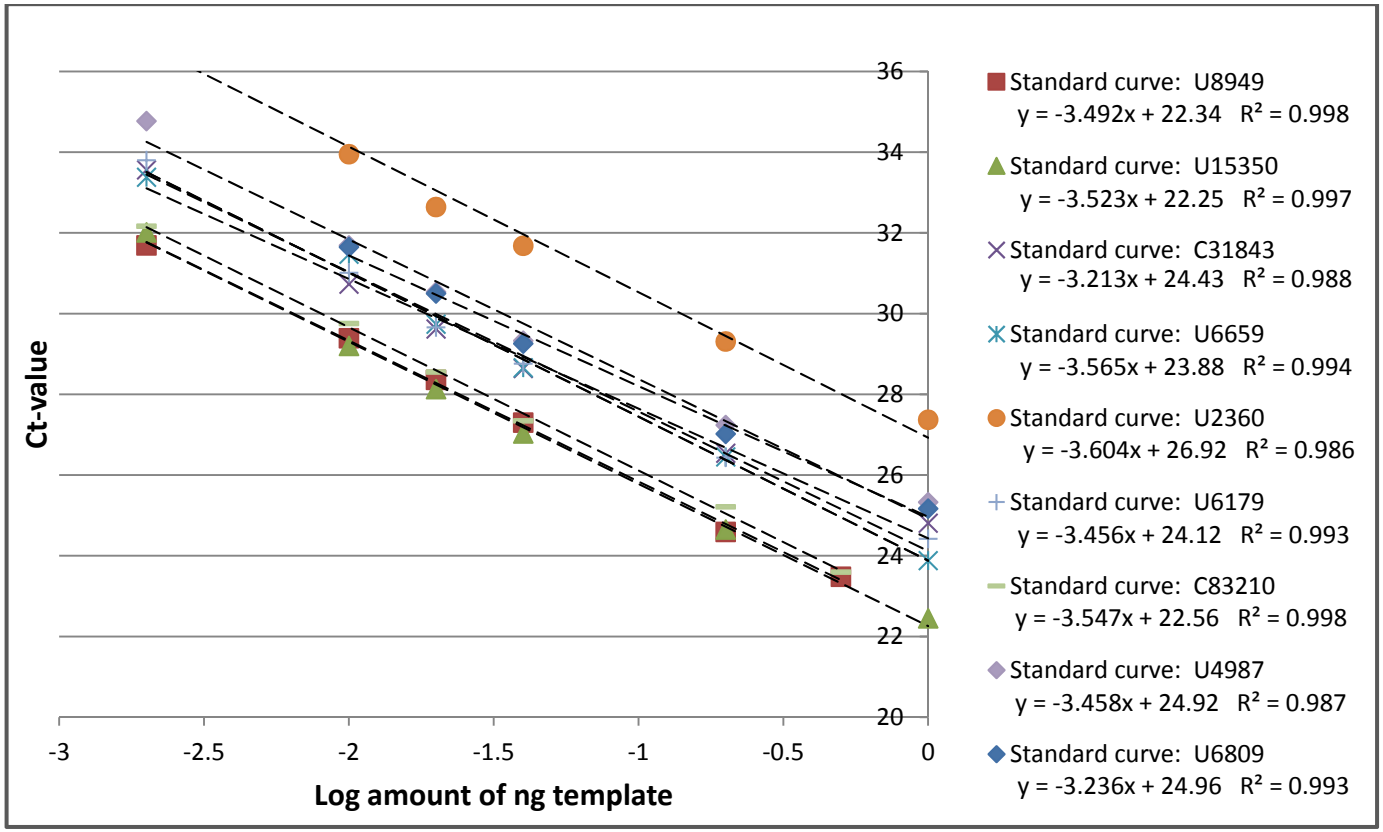

Figure 6. Standard regression curves for primers directed to nine target genes to be used in a relative expression study in *Phytophthora cinnamomi*. This included analysis of primer pairs U8949, U15350, C31843, U6659, U2360, U6179, C83210, U4987 and U6809. A dilution series of DNA (1:2, 1:5, 1:25, 1:50, 1:100 and 1:500) was used to construct standard curves. The mean Ct (threshold cycle for each trace) value of 3 technical repetitions was plotted against the log [10] function of the concentration of input template for each primer pair. A linear trend-line was fitted to the data. The equation for each fitted regression line, as well as the R^2 value (as an indicator of the ability of the model to predict a trend) for each gene is indicated. An R^2 value of more than 0.98 and a slope between -3.6 and -3.1 are considered acceptable for efficient primers.

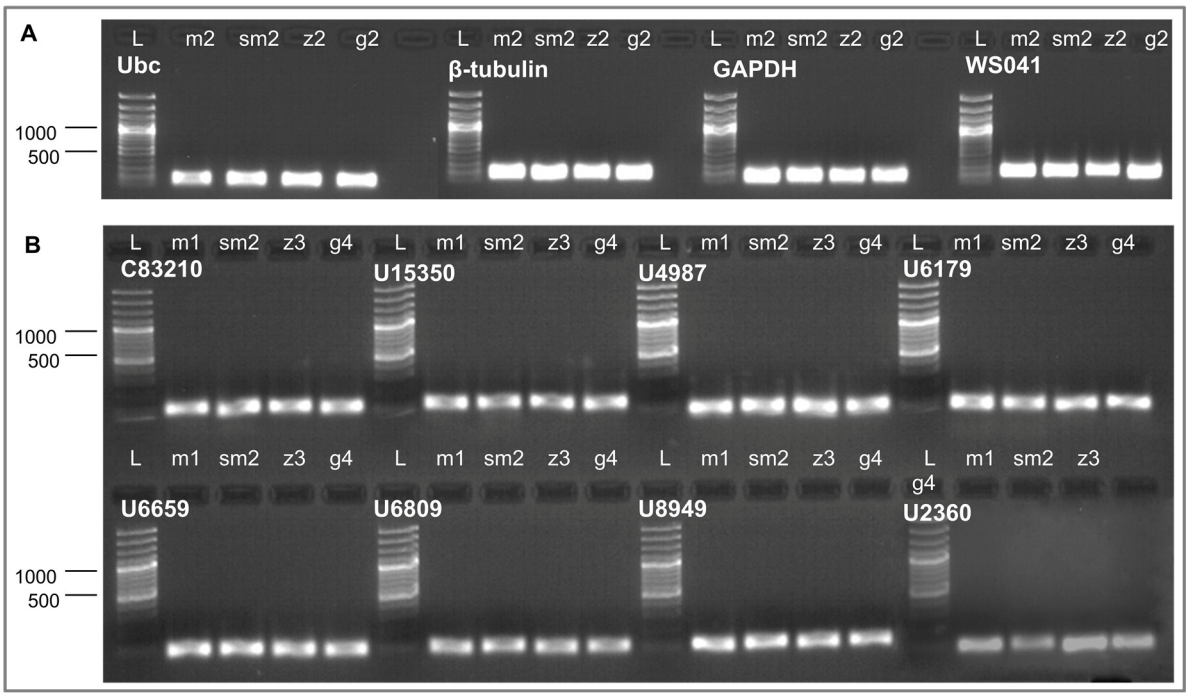

Figure 7. Agarose gel electrophoresis image confirming single products produced by primer pairs to be used in RT-qPCR analysis in *Phytophthora cinnamomi*. A) PCR products produced from cDNA by primer pairs targeting four candidate endogenous control genes namely ubiquitin-conjugated enzyme (Ubc), Beta-tubulin (β-tubulin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and WS041 B) PCR products produced from cDNA by primer pairs directed to eight target genes including C83210, U15350, U4987, U6179, U6659, U6809, U8949 and U2360. L - O'GeneRuler 100 bp DNA ladder, m – mycelia, sm – sporulating mycelia, z – zoospore, g – germinating cysts.

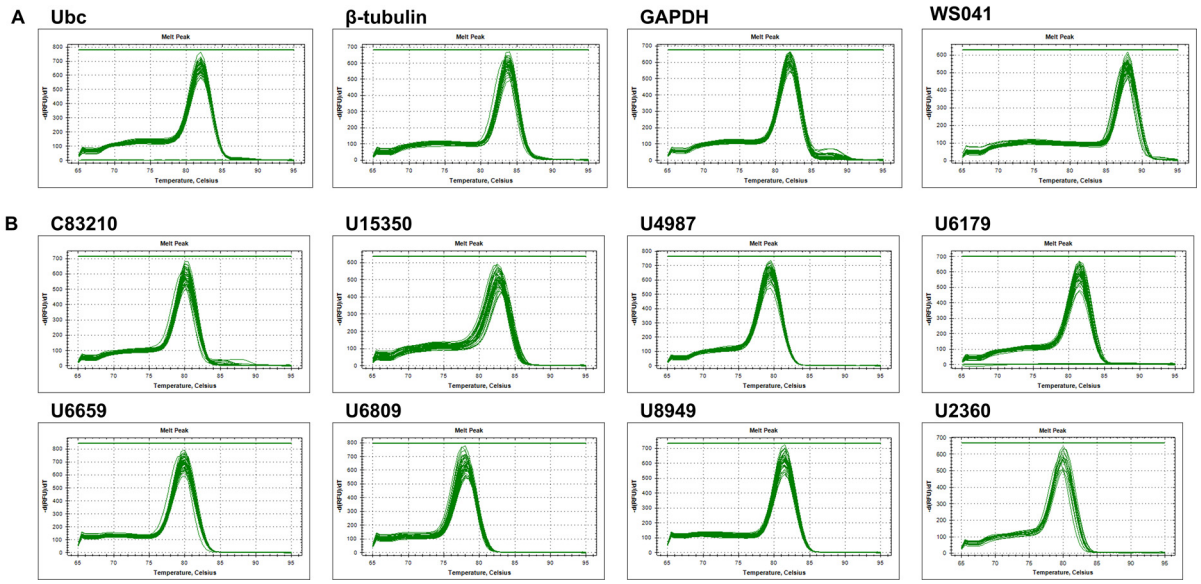

Figure 8. Melting curve analysis of the products produced by primer pairs to be used in expression analysis in *Phytophthora cinnamomi*. A) Melting curves produced from PCR products produced by primer pairs targeting four candidate endogenous control genes namely ubiquitin-conjugated enzyme (Ubc), Beta-tubulin (β-tubulin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and WS041 B) Melting curves produced from PCR products produced by primer pairs directed to eight target genes including C83210, U15350, U4987, U6179, U6659, U6809, U8949 and U2360.

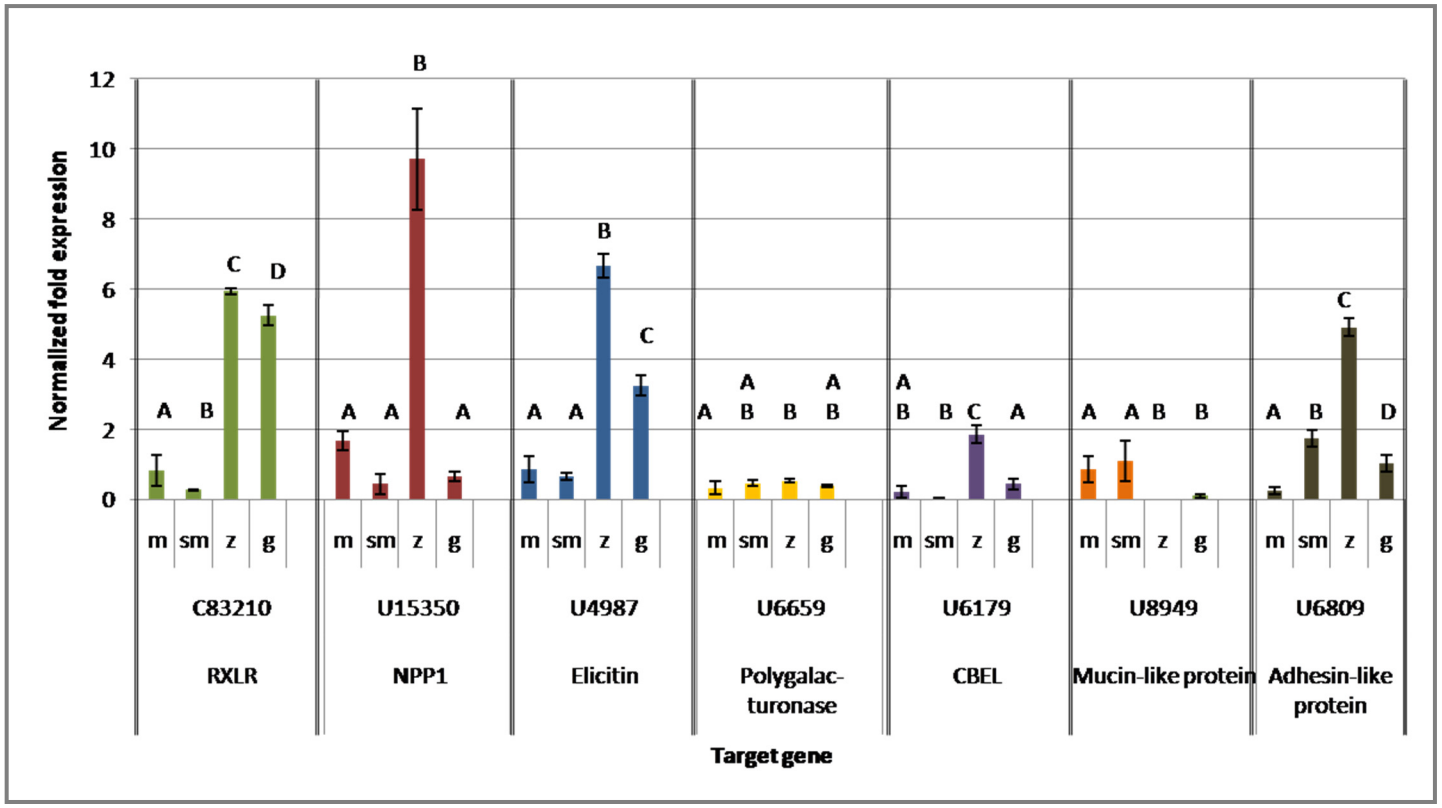

Figure 9. Expression profiles of seven target pathogenicity genes across the *in vitro* developmental stages of *Phytophthora cinnamomi*. The unigene number and putative function of each target gene are indicated at the bottom of each profile. Gene expression levels of each target gene were calculated considering determined primer efficiencies and using three endogenous control genes (WS041, Ubc, β-tubulin) to normalize the data. Error bars represent standard deviation and are indicated for each data point. Analysis of variance (ANOVA) followed by a Least Square Means Differences Student's t-test ($p>0.05$) was performed considering each target gene individually. Accordingly, this analysis does not allow comparison of gene expression levels between genes. For each individual target gene, bars containing the same letters do not differ significantly ($P = 0.05$). m – mycelia, sm – sporulating mycelia, z – zoospore, g – cysts and germinating cyst, NPP1 – necrosis-inducing *Phytophthora* protein 1, CBEL – cellulose binding elicitor lectin

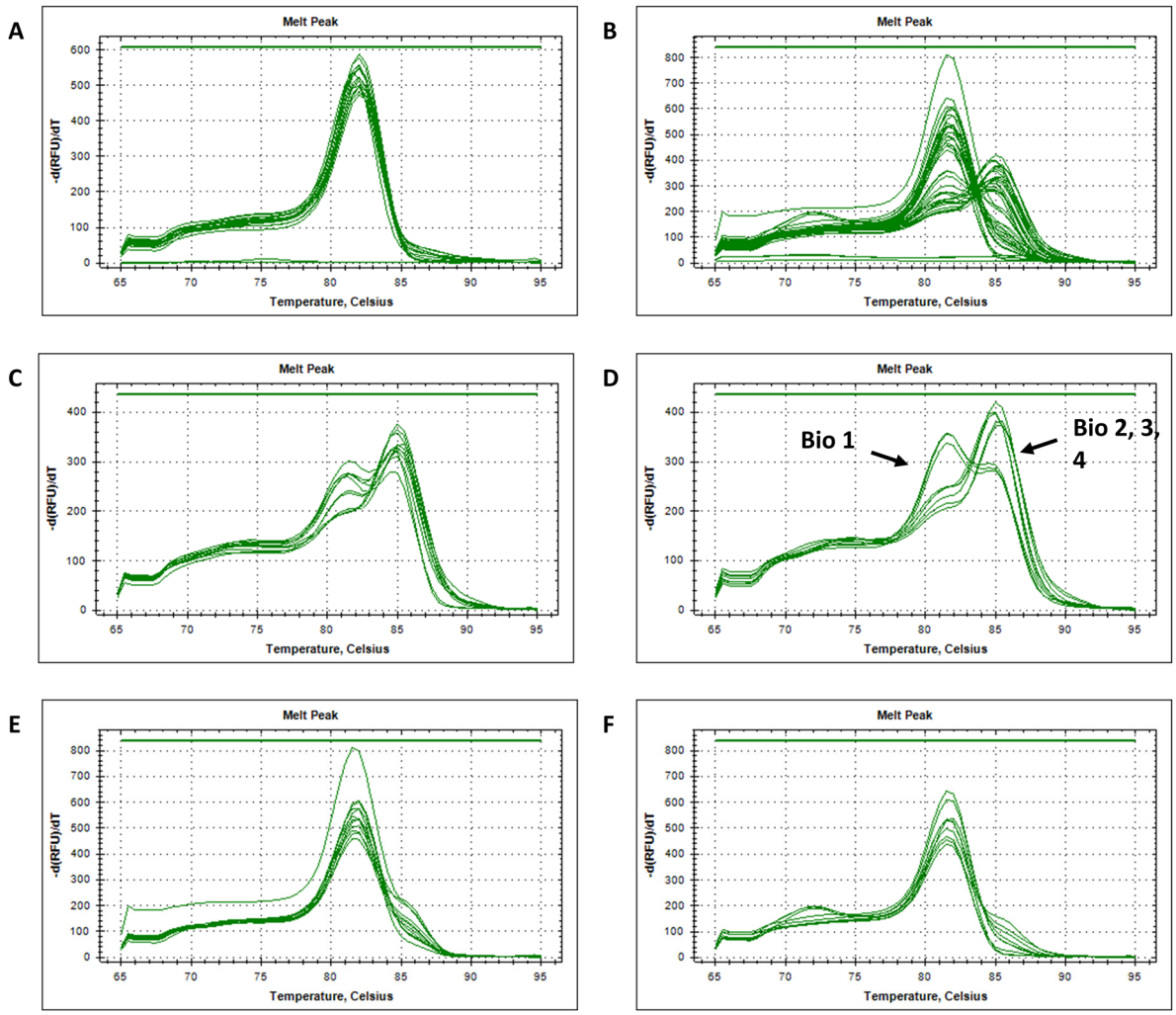

Figure 10. Melting curve analysis of PCR products produced by the primer pair directed to contig C31843 of *Phytophthora cinnamomi*. Each graph shows the negative rate of change in relative fluorescence units (RFU) as the temperature changes. The products produced from the following templates were assessed: A) *Phytophthora cinnamomi* DNA B) cDNA from all four *in vitro* life stages C) cDNA from mycelia D) cDNA from sporulating mycelia (the different biological replicates are indicated) E) cDNA from zoospores D) cDNA from germinating cysts. Two melting peaks at 82 °C and 85 °C are visible in C and D.

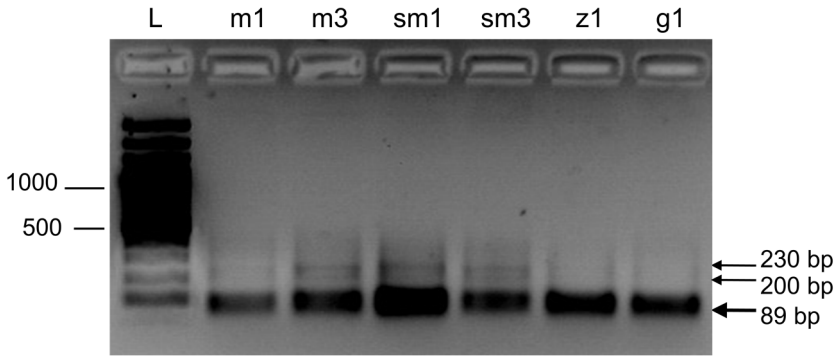

Figure 11. Agarose gel electrophoresis image showing the products produced from *Phytophthora cinnamomi* cDNA by the primer pair directed to contig C31843. A single high intensity band of 89 bp in size is observed in each sample. Multiple low intensity bands of bigger sized products are visible in mycelia- and sporulating mycelia samples. The image is over exposed to enable visualization of low intensity bands. L - O'GeneRuler 100 bp DNA ladder, m – mycelia, sm – sporulating mycelia, z – zoospore, g – germinating cysts. The number following each stage indicates the biological replicate from which the template cDNA originated.

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CHAPTER 4

General discussion

DISCUSSION

Phytophthora cinnamomi Rands is a devastating pathogen responsible for great economic losses in many crop and forest industries. Except for the application of phosphite that has proved helpful in combating *P. cinnamomi* in some plant species (Darvas et al. 1984; Shearer and Fairman 2007; Eshraghi et al. 2011; Akinsanmi and Drenth 2013), there is no effective control measure for this pathogen. Many industries are moving to organic farming practises to limit the negative effects of pesticides on the environment. This involves the use of resistant crop cultivars, biological control agents, crop rotation, green manure and compost (Li et al. 2013). As crop rotation is not feasible for a wide host range pathogen such as *P. cinnamomi* (Mbaka 2011) and mulching alone does not effectively control disease, resistant crop cultivars seem the only durable solution to *P. cinnamomi* disease. As the public become more accepting of genetically modified plants, molecular knowledge of a specific plant-pathogen interaction will be invaluable in speeding the process towards disease resistant crop- and forest trees.

This study aimed to identify pathogenicity genes of *P. cinnamomi* in order to gain a better understanding of the infection process of this pathogen. The application of next generation sequencing allowed the identification of more than 2 000 unigenes with a role in infection, from cysts and germinating cysts of *P. cinnamomi*. Three different approaches were used to identify pathogenicity genes. The first approach was based on significant protein sequence homology to proteins that were previously implicated in pathogenicity in the literature. Unigenes annotated to the protein models from the genome of *Phytophthora infestans* (Montagne) de Bary was used to achieve this. As only previously characterized proteins were considered, it did not allow identification of novel pathogenicity factors. This approach is valid for proteins that are subject to selective pressure to maintain conserved functional motifs, with little amino acid variation expected between homologous genes. Polygalactorunases, for example, do not show signs of diversifying selection, with the ratio between non-synonymous and synonymous mutations being one or less within conserved regions between homologous genes (Götesson et al. 2002). The majority of the pathogenicity genes identified in this study was successfully obtained with the homology based method.

Protein families that are subject to diversifying selection, like RXLR effector genes, are more difficult to identify with homology based approaches as sequence conservation is less apparent among orthologs and paralogs (Win et al. 2007; Jiang et al. 2008). Effector genes

are under selective pressure to evolve protein variants that are able to evade detection by the host surveillance system (Birch et al. 2006). The second approach, therefore, relied on the identification of conserved functional protein motifs within RXLR effector genes. We employed Hidden Markov Models (HMM) to identify unigenes containing the conserved RXLR-motif (Arg-any amino acid-Leu-Arg). In this way previous unidentified RXLR-effector transcripts and RXLR transcripts specific to *P. cinnamomi* could be discovered. Functional characterization of RXLR proteins from other oomycete species has illustrated, through gene silencing, that specific RXLR proteins are essential for virulence (Bos et al. 2010). Their direct interaction with host resistance proteins (Bos et al. 2006; Dou et al. 2008) and the observation of allelic variants able to evade host recognition (Armstrong et al. 2005) makes them interesting candidates for further investigation. It will be interesting to compare the RXLR proteins identified in *P. cinnamomi* to RXLR proteins in other pathogenic oomycetes.

Putative alternative splicing was detected in an RXLR-motif containing transcript (C31843) that showed homology to *Phytophthora sojae* suppressor of necrosis 1-like protein 2 (SNEL2). Alternative splicing may serve as a mechanism to increase functional variation in effector proteins at the protein level. Melting curve analysis suggested that the splicing is stage dependent. This implies that transcript C31843 produces functionally different products in the mycelial and sporulating mycelial phases than in zoospores and germinating cysts of *P. cinnamomi*. Confirmation of the splicing event and the implication thereof will be an interesting topic to investigate in the future.

Other effector families also require a motif-based approach for proper identification. This includes the Crinkling- and necrosis inducing (CRN) protein family that was annotated based on sequence homology to *P. infestans* CRN proteins in this study. Stam et al. (2013) designed a bioinformatic pipeline for the identification of the CRN protein family in *Phytophthora capsici* (Leonian), utilizing the HMM for the LFLAK-motif used by Haas et al. (2009). This pipeline can be applied to the *P. cinnamomi* dataset to investigate CRN proteins in the future. Functional annotation of other pathogenicity proteins can also be verified by the presence of conserved functional motifs. The identity of protease inhibitor proteins can be confirmed by the presence of Kazal domains (Laskowski and Kato 1980) and necrosis inducing *Phytophthora* proteins (NPP1) by the presence of a NPP1 motif (Gijzen and Nürnberger 2006).

Expression analysis of the NPP1 family of *P. cinnamomi* within a specific pathosystem may clarify the confusion regarding the trophic classification of *P. cinnamomi.* If *P. cinnamomi* does indeed behave like a hemi-biotroph, phenotypic data together with the temporal

regulation of NPP1 transcripts may provide the time frame in which the switch to necrotrophy occurs and whether the phases alternate as suggested by Shearer and Crane (2012).

Another motif based method (the third approach applied in this study to identify pathogenicity genes) was employed to define the secretome of the *P. cinnamomi* cyst and germinating cyst dataset (110 unigenes). Many pathogenicity proteins are secreted and have specific targets in the apoplast or inside host cells during infection. Accordingly, any secreted protein has the potential to act as a virulence factor. Characterization of secreted proteins with unknown function (as in the case of the 9 unigenes identified in this study) can potentially reveal novel pathogenicity factors. The amount of secreted proteins identified in the *P. cinnamomi* dataset is likely an underestimation as many unigenes did not represent the N-terminal region of proteins and could not be assessed for the presence of a signal peptide.

The expression pattern of seven pathogenicity genes was evaluated across the *in vitro* developmental stages of *P. cinnamomi*. In theory genes are upregulated directly prior to the stage where their functional products are required (Judelson et al. 2008). This implies a functional role for the RXLR-, elicit and adhesion-like proteins (upregulated in cysts and germinating cysts) in early infection and the necrosis-inducing *Phytophthora* protein 1 (NPP1), polygalactorunase and cellulose binding elicitin lectin (CBEL) protein in cysts and germinating cysts (induced in zoospores). The expression patterns observed were consistent with previous reports in the literature for each transcript. Although, much can be inferred from the biological processes active in a sample by expression analysis, all transcripts are subject to post-transcriptional regulation (e.g. miRNA, siRNA). Therefore, transcriptional data should be interpreted with caution.

The ability of RNA-seq analysis to accurately quantify transcript level enables the identification of differentially expressed transcripts between biological samples (Strickler et al. 2012). Novel pathogenicity factors can be identified based on significantly higher transcript abundance in infection structures in relation to non-infection structures. Not only does this approach allow novel transcript identification (Strickler et al. 2012), it also enables the study of large scale transcriptional changes between pathogen life stages and during *in planta* infection (Ye et al. 2011; Chen et al. 2013). The cyst and germinating cyst library of *P. cinnamomi* generated in this study was successfully utilized for pathogenicity gene identification, but did not provide quantitative data to prioritize pathogenicity transcripts, nor did the design include a reference library to compare to. This dataset can be utilized for novel transcript identification and the investigation of alternative spliced transcripts in the future. Ultimately, expression analysis of pathogen genes during host infection provides the

most relevant information as the pathogen responds to the host environment and defence system. The success of *in planta* expression analysis of pathogen genes relies on the sequencing depth (allowing detection of low abundance transcripts) and the ability to differentiate between pathogen- and host derived transcripts. The recent release of the *P. cinnamomi* genome will enable transcriptional analysis of specific *P. cinnamomi*-host interactions in the future.

This project generated the first transcriptomic data available for cysts and germinating cysts of *P. cinnamomi*. The identification of various pathogenicity genes, including 46 RXLR motif containing unigenes is a significant contribution to our current knowledge of *P. cinnamomi* pathogenicity. Functional characterization of candidate pathogenicity genes proposed in this study will help to gain a better understanding of the mechanisms employed by *P. cinnamomi* to manipulate host physiology and will aid in identifying pathogen molecules that can serve as targets for disease control. The available sequence data can further be exploited to predict the likely effectiveness of certain pesticides on *P. cinnamomi*, based on the presence or absence of genes involved in specific metabolic pathways, as well as indicate the mode of action of chemicals on *P. cinnamomi* in subsequent transcription experiments. Ultimately, scientists want to exploit knowledge of plant-pathogen interaction to develop more effective and novel control strategies to combat pathogens.

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