Establishment of a compatible pathosystem between *Eucalyptus nitens* and *Phytophthora cinnamomi* to determine host transcriptional responses

by

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DECLARATION

I, Febé Elizabeth Wilken, declare that the thesis, which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Febé Elizabeth Wilken

December 2013

This thesis is dedicated to my mother

Ria Wilken

who has not only taught me how to be a woman of noble character in words,

but shows it in the way she lives and forgives.

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PREFACE

Phytophthora species are “plant destroyers” capable of devastating a variety of economically important crop and tree species. Cold tolerant *Eucalyptus* species, such as *Eucalyptus nitens*, may succumb to infection by the root rot pathogen, *Phytophthora cinnamomi* leading to die-back and death. This thesis discusses the establishment of a pathosystem between *Eucalyptus* and *Phytophthora* and the subsequent use of the pathosystem to profile *E. nitens* host responses to *P. cinnamomi* using RNA-sequencing.

Chapter 1 is a literature review which includes background pertaining to *Eucalyptus* genomics and the use of RNA sequencing as a tool to dissect host transcriptional responses. The focus of the review is the host defence responses in different *Phytophthora*-host pathosystems studied to date, with an emphasis on host responses to *P. cinnamomi*.

Chapter 2 encompasses the search for a suitable *Phytophthora* species to infect *E. nitens*, and characterizes the pathosystem by confirming the presence of hyphae within inoculated stem tissue.

Chapter 3 describes the use of the aforementioned pathosystem to obtain tissue for whole transcriptome profiling through RNA sequencing. The analysis and prominent trends from the transcriptome sequencing is discussed. The chapter has been written in the format of an article aimed at *BMC Genomics* and the final manuscript is in the preparation phase.

The outputs of the research presented in this thesis was undertaken from February 2009 to December 2013 in the Department of Genetics at University of Pretoria. The project was supervised by Dr. Sanushka Naidoo, and Prof. Zander Myburg and Dr. Noëlani van den Berg co-supervised it. Prof. Dave Berger regularly acted in an advisory role.

Conference proceedings from the research include:

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Chapter 1

Literature review

Host responses to *Phytophthora* spp. with a focus on *Eucalyptus* and *P. cinnamomi*

Febé E. Wilken, Noëlani van den Berg, Sanushka Naidoo

1.1 Introduction

Eucalyptus is a diverse genus composed of several hundred species, some of which have been adapted for commercial forestry (Potts and Pederick, 2000; Turnbull, 2000). Due to its commercial importance, the study of *Eucalyptus* has not lagged far behind in the field of genomics. With the availability of high-throughput next-generation sequencing (NGS), it has become viable to perform whole transcriptome analysis of *Eucalyptus* species at a relatively low cost, and the release of an annotated genome for *E. grandis* eliminates the need for *de novo* transcriptome assembly (Myburg, 2012; Ward *et al.*, 2012). This technology has now enabled transcriptome profiling of *Eucalyptus* host responses upon challenge with various pathogens with greater ease.

Phytophthora root rot is a well-known disease in *Eucalyptus* plantations which has remained problematic in South Africa for the last three decades at least (J. Roux, personal communication; Wingfield and Knox-Davies, 1980). Though there are resistant species available, the cold-tolerant species are highly susceptible (Gardner and Swain, 1996; Swain and Gardner, 2003). *Phytophthora* spp. are hemi-biotrophs responsible for damage to many different crops around the world, and can be devastating to natural ecosystems as well. One of the most destructive species is *P. cinnamomi*, which has an abnormally large host range of over 3500 species (Erwin and Ribeiro, 1996; Hardham, 2005; Hardham and Blackman, 2010; Shearer *et al.*, 2004).

A large amount of research has focussed on the virulence mechanisms employed by *Phytophthora* spp. and as a result pathogen associated molecular patterns (PAMPs) and effectors specific to this genus and other oomycetes have been discovered (Bozkurt *et al.*, 2012; Hein *et al.*, 2009; Kamoun, 2006). PAMPs include elicitins and Pep-13, a conserved thirteen amino acid peptide (Brunner *et al.*, 2002; Tyler, 2002). Effectors contain an RXLR domain that is required for translocation of the effectors into the host cell (Whisson *et al.*, 2007). Resistance genes and their corresponding avirulence genes in *Phytophthora* spp. have been well characterized in potato (Vleeshouwers *et al.*, 2011).

The knowledge concerning *Phytophthora* virulence factors has provided a good platform for numerous studies which aim to uncover the molecular defence mechanisms of host species infected with *Phytophthora. Arabidopsis thaliana* and some non-model hosts have also been studied in an attempt to unravel the signalling networks and mechanisms that are activated upon challenge with species from this genus. PAMP triggered immunity (PTI) and effector triggered immunity (ETI) are triggered in hosts upon challenge with *Phytophthora* (Hein *et al.*, 2009). Both mitogen activated protein kinase (MAPK) signalling cascades and calcium

ion fluxes are involved in resistance to *Phytophthora* spp. The timing and extent of the hypersensitive response (HR) and reactive oxygen species (ROS) seem to play a critical role in establishment of resistance by a host. Jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) responses are dependent on the specific pathosystem under investigation. An emerging hypothesis concerning this area of defence is that regulation of these hormones needs to change in accordance with the stage of infection (biotrophic or necrotrophic) (Attard *et al.*, 2010; Moy *et al.*, 2004). Additionally, it has been noted that *Phytophthora* spp. may manipulate these hormones, and certain pathogenesis related (PR) proteins or host targets in order to ensure favourable conditions depending on the specific infection phase it finds itself in (Belhaj *et al.*, 2009; Gilroy *et al.*, 2011; Schlink, 2009). There is a great deal of recent work with regards to elucidating host targets of *Phytophthora* effectors. A recurring theme in *Phytophthora*-host interactions is the role of phytoalexins and various products of the phenylpropanoid pathway. These substances seem to be important in exerting successful defence and certainly warrant more detailed investigation. It is noteworthy that many similar trends are found in the defence mechanisms of hosts challenged with various *Phytophthora* spp.

There is not much information concerning the interaction between *Eucalyptus* and *Phytophthora* at the molecular level. The emergence of NGS technology has provided an opportunity to study the whole transcriptome of this interaction. RNA-seq data from a compatible interaction between *Eucalyptus* and *Phytophthora* will afford us with a basis for understanding which defence mechanisms are employed by the tree in order to attempt evasion of infection.

The focus of this review is on the molecular (specifically gene level) responses of various host species upon challenge with *Phytophthora*. There are several other reviews dealing with the topic. (Cahill *et al.*, 2008) devoted a review subsection to resistance mechanisms against *P. cinnamomi* and recently Hein *et al.* (2009) have compiled a review describing PTI and ETI in light of oomycete interactions. However, there is a paucity of reviews of molecular host responses to infection with *Phytophthora*. Hardham and Cahill (2010), as well as Hardham and Blackman (2010) have provided reviews summarizing findings concerning *Phytohpthora* PAMPs, as well as the molecular cytology of *Phytophthora*-plant interactions.

1.2. *Eucalyptus*

1.2.1 Biology

Eucalypts are hardwood evergreen trees belonging to three genera within the family Myrtaceae (Johnson and Briggs, 1984; Potts and Pederick, 2000). These genera are *Angophora*, *Corymbia*, and *Eucalyptus*, with the latter genus comprising over 600 species. The bulk of eucalypt species are mainly endemic to Australia, New Guinea and surrounding islands (Ladiges *et al.*, 2003). Despite this, several species from the genus *Eucalyptus* have been widely commercialized and are grown as exotics in numerous other countries.

A noteworthy characteristic of *Eucalyptus* is the broad genetic diversity within the genus and at species level (Potts and Pederick, 2000). This can be noted by differences in morphology, with heights ranging from one to a hundred metres, diversity in bark and leaf morphology and varying susceptibility to certain diseases between and within species (Potts and Pederick, 2000). The genetic variety within the genus is brought about by self-incompatibility mechanisms which prevent inbreeding and as a result eucalypts are primarily outcrossers (Gaiotto *et al.*, 1997; Horsley and Johnson, 2007). Eucalypt breeders have exploited this rich gene pool and have been able to produce hybrid clones that combine numerous desirable traits such as growth, adaptability, wood properties and disease resistance (de Assis, 2001).

1.2.2 Economic importance of *Eucalyptus*

Eucalypts have been integral to the lifestyle of indigenous Australians for a long time as a source of fuel, building materials and medicine (Turnbull, 2000). The western world became aware of the potential eucalypts had and took note of their rapid growth as well as their ability to produce straight stems (Turnbull, 2000). The development of eucalypts as a commercially valuable species has since progressed considerably. They were at first planted for windbreaks, leaf-oil, and along railway lines as a source of fuel for wood-burning locomotives (Turnbull, 2000). Eucalypts are now utilized for sawn timber, mine props, poles, firewood, pulp, chemical cellulose, charcoal, essential oils, honey and tannin (Hillis and Brown, 1983). Some favourable characteristics that have promoted the use of the eucalypts include their adaptability to different soils and climates, high growth rates and good wood properties. Furthermore, eucalypts can be planted as exotic species since they are fairly intolerant to competition from surrounding plants and tend not to spread as weeds (Potts and Pederick, 2000; Turnbull, 2000).

The eucalypt industry forms a significant component of the world's forestry sector. In 2005, 224 million hectares of productive forest were under cultivation, and 8% (18 million ha) of this was comprised of *Eucalyptus* (FAO, 2006, 2007b). Prominent commercially planted

Eucalyptus spp. include *E. grandis*, *E. globulus* and *E. nitens* (565, 442 and 266 thousand hectares respectively) (FAO, 2006). Brazil, India and China are the leading producers of *Eucalyptus*, with countries in South/South-east Asia as well as Chile, Spain, Portugal, Australia and South Africa also contributing significantly to the total cultivated area (Iglesias-Trabado and Wilstermann, 2008).

Forestry is a prominent sector in South Africa's economy. Close to half a million jobs are created either directly or indirectly as a result of this sector, and it is estimated that it facilitates the livelihood of 1.8 million people (Godsmark, 2009). It is especially important in KwaZulu-Natal, where the sector facilitates 30 400 jobs (Godsmark, 2009). South Africa is mainly a producer of *Pinus* spp. (52% of plantations are pine trees), with the other major part of the sector being supported by *Eucalyptus* spp. (39% of plantations) (Godsmark, 2009). Primarily produced species are *E. grandis* and *E. grandis* hybrids as well as the cold tolerants *E. nitens, E. smithii* and *E. dunnii* (FAO, 2006, 2007b; Foelkel, 2008).

Importantly, *Eucalyptus* is used for the production of pulpwood for paper production, which is critical for the functioning of many industries and businesses worldwide. South Africa's paper and pulp industry ensures that minimal pulp and paper imports are required. In 2008 South Africa's pulp production was 1.93 million tonnes, and consumption reached 1.8 million tonnes (FAO, 2011).

1.2.3 Genetics

Efficient improvement and management of the trees cultivated is important for optimal productivity. Target traits for breeding are those directly related to industry such as volume growth, wood density and pulp yield. Traits relating to biotic and abiotic stresses become significant when they negatively influence the primary traits (Grattapaglia and Kirst, 2008). In the past, investment in research and tree improvement has benefited the industry by resulting in the introduction of clones, which has nearly doubled the yield of commercially grown eucalypts (Bertolucci *et al.*, 1995). The use of these genetically identical trees has proved to be helpful in capturing valuable traits obtained from hybrid breeding (Grattapaglia and Kirst, 2008). Further investment in research regarding the genetic composition of *Eucalyptus* is crucial to make breeding more effective and for taking full advantage of the genetic potential and sought-after traits within the genus.

An alternative avenue to plant breeding for improvement of *Eucalyptus* spp. is that of transgenic technology. This could greatly benefit the forestry industry in introducing novel traits that are not found within the broad spectrum of genetic diversity of the eucalypts

(Grattapaglia and Kirst, 2008). However, before an effective breeding program or genetic modification is embarked upon, it is important to have an in-depth understanding of the biological mechanisms surrounding target traits such as wood quality or disease resistance, along with the necessary genomic analysis tools.

A focus area for *Eucalyptus* research is the development of markers, as well as the construction of genetic and physical linkage maps associated with them. Markers are not only used for verifying clonal identity within industry, but are needed for marker assisted selection during the breeding process (Grattapaglia and Kirst, 2008). Some examples of markers developed in *Eucalyptus* spp. include RAPDs, AFLPs, RFLPs, microsatellites - reviewed by Grattapaglia and Kirst (2008). Recently, two studies in SNP discovery have been performed (Külheim *et al.*, 2009; Novaes *et al.*, 2008), and a DArT microarray with 7680 markers was developed (Sansaloni *et al.*, 2010). The DArT markers have already been used to perform an in-depth phylogenetic analysis between 94 *Eucalyptus* spp., and to construct a high-density linkage map in conjunction with microsatellites (Kullan *et al.*, 2012; Steane *et al.*, 2011). Additionally, several QTL studies have generated data for traits such as wood properties, as well as fungal and frost tolerance in *Eucalyptus*, which could be useful tools in selecting for desirable traits [reviewed in Grattapaglia and Kirst (2008) and Neale (2011)].

One of the most prominent *Eucalyptus* resources available to date is the annotated reference genome sequence of *E. grandis BRASUZ1* with eight times coverage which is available at www.phytozome.net/eucalyptus.php. The genome consists of 640 Mbp, with 44 974 *ab initio* predicted protein-coding loci on its 11 chromosomes, and the availability of this sequence greatly expands the horizon of possibilities within the sphere of *Eucalyptus* research (Myburg, 2012).

Genomic and transcriptional sequence data is essential for efficient progress in *Eucalyptus* research. Transcriptional profiling aids enhanced understanding of genes controlling certain traits under the influence of particular environmental, developmental or biotic conditions. Until recently, expressed sequence tags (ESTs) and microarrays have been the primary techniques employed to elucidate information from transcriptomes. Numerous *Eucalyptus* ESTs have been generated and used for mapping, although a lot of the EST data is not publicly available due to the commercial value of the information (Grattapaglia and Kirst, 2008; Keller *et al.*, 2009). Several EST and microarray profiling studies have been performed in *Eucalyptus* to investigate wood properties (Qiu *et al.*, 2008a; Ranik *et al.*, 2006; Solomon

et al., 2010), as well cold stress (Keller *et al.*, 2009) and biotic stresses (Barbosa-da-Silva *et al.*, 2005; Duplessis *et al.*, 2005).

Though microarrays have been useful for expression profiling in the past, the development of NGS technology, with high throughput and lowered sequencing costs per megabase, has ushered in a new era in transcriptome studies (Wang *et al.*, 2010). Microarrays are limited by the need for sequence data from the species under investigation, and often genes and altenative transcripts are missing from the arrays (Bräutigam and Gowik, 2010). Another drawback to microarray technology is the fact that the results are expressed as relative ratios. RNA-seq eliminates these problems since the entire transcriptome is sequenced and therefore absolute quantification of transcript amounts is possible (Bräutigam and Gowik, 2010).

With the availablility of NGS technology, researchers are now looking to the future, where integration of the "omics" namely genomics, transcriptomics, proteomics and metabolomics would provide the opportunity to gain a holistic view of plants, as well as an opportunity to model interactions with various microbes (Liberman *et al.*, 2012; Schenk *et al.*, 2011). NGS platforms include three current technologies, namely the Roche 454 Life Science platform, which produces longer reads (about 400 bp) and short read (approximately 35-76 bp) technologies such as the Illumina Solexa platform and SOLiD from Applied Biosystems (ABI). The short read technologies produce sequencing data in the order of several gigabases at relatively low costs. These technologies, and third generation single molecule sequencing technology are reviewed by Metzker (2009) and Brautigam and Gowik (2010).

NGS technology provides a more feasible way to approach both new and old questions. Some of the applications of this technology include sequencing and resequencing of genomes, polymorphism discovery, mutant mapping, protein-DNA interactions through ChIP-seq and DNA methylation mapping. Entire transcriptomes can be sequenced (referred to as RNA-seq), and expression levels of transcripts can be quantified absolutely. The discovery of alternative splice variants, previously unannotated transcripts and small RNAs (smRNAs) is also facilitated by this technology (Lister *et al.*, 2009).

The NGS field is still in its infancy, but it has already proven useful for providing a holistic overview of a pathogen-responsive transcriptome. RNA-seq has been used to study responses to pathogen challenge in well-studied model organisms such as soybean, wheat and *Arabidopsis thaliana* (Dowen *et al.*, 2012; Tremblay *et al.*, 2012; Xu *et al.*, 2012). Dowen *et al.* (2012) exploited NGS to investigate the influence of *Pseudomonas syringae* and SA on

the DNA methylome of *A. thaliana* and to profile the gene and smRNA expression under these conditions. It is noteworthy that there are several studies investigating pathogen interactions with non-model species. These species include cotton, citrus, cucumber and raspberry and potato (Gao *et al.*, 2013; Martinelli *et al.*, 2012; Savory *et al.*, 2012; Ward and Weber, 2012; Xu *et al.*, 2011). Transcriptomes of non-model species can be sequenced using NGS and assembled *de novo* or mapped to the closely related reference genomes. Ward *et al.* (2012) compared two approaches for obtaining expression profiles of the transcriptome of *Phytophthora*-infected raspberry. There was good correlation between results from *de novo* assembly of the transcriptome and results from mapping to a close relative (strawberry) reference genome. This provides a basis for transcriptomes from non-model species to be mapped to genomes of close relatives, such as different *Eucalyptus* spp. being mapped to the *E. grandis* reference genome.

RNA-seq has already been performed on *Eucalyptus*. An *E. grandis* x *E. urophylla* transcriptome was assembled *de novo* (Mizrachi *et al.*, 2010), and recently Villar *et al.* (2011) used 454 sequencing to compare the responses of two *Eucalyptus* hybrids to water stress. Importantly, the stage has been set to use RNA-seq to study transcriptional responses to pathogens in *Eucalyptus*. Already, the transcriptome of *E. grandis* has been sequenced and used to establish host responses to an important gall-forming pest, *Leptocybe invasa* (C. Oates, personal communication).

1.2.4 Threats to the *Eucalyptus* industry

The *Eucalyptus* and, more generally, the forestry industry is vast and its products are essential to many spheres of life. Therefore it is important to assess the risks that could negatively influence its productivity. Though fire is prominent due to reports in the media, insect pests and diseases are primarily responsible for the vast majority of disturbances in forests (FAO, 2007b). The threat posed by insect pests and diseases on the African continent has been highlighted in a survey by the FAO (Food and Agriculture Organization of the United Nations) where it was stated that there is “no doubt that increasing problems with invasive insects, diseases and woody species have affected the productivity and vitality of African forests” (FAO, 2007b).

Globally, 2.8% of indigenous and planted forest area is affected by insects pests and diseases. Plantations are monocultures and sometimes even consist of clones. The implications are that large areas of forest are genetically predisposed to the rapid spread of a pathogen due to small or no genetic variation that can offer resistance to a pathogen and

slow or prevent its spread. Therefore the statistics regarding disturbances specific to plantations are likely to be much more concerning (FAO, 2007b).

The *Eucalyptus* industry suffers from a large range of pests and pathogens. Problems with diseases can start while plants are still in the nursery, and pathogens may cause stem and foliar diseases, cankers, and root rot. In South Africa particularly, several insect pests of concern on *Eucalyptus* spp. include *Coryphodema tristis* (cossid moth), *Gonipterus scutellatus* (*Eucalyptus* snout beetle) and *Thaumastocoris peregrinus* (bronze bug) (FAO, 2007a). *Ralstonia solanacearum* and *Pantoea ananatis* cause bacterial wilt and blight respectively on various *Eucalyptus* spp. Additionally, several important fungal pathogens present in South Africa pose a threat to commercial propagation of *Eucalyptus*. These include *Chrysoporthe austroafricana*, *Coniothyrium zuluense* and *Botryosphaeria* spp. which are able to produce stem cankers and *Mycospaerella nubilosa* which causes leaf blight (FAO, 2007a; Wingfield *et al.*, 2011). *Phytophthora* spp. are soil borne, and able to cause root and collar rot of cold-tolerant eucalypts. In South Africa, four of these species have been observed on *Eucalyptus*, namely *P. cinnamomi*, *P. nicotianae*, *P. alticola* and *P. frigida* (Linde *et al.*, 1994a; Linde *et al.*, 1994b; Maseko *et al.*, 2001; Maseko *et al.*, 2007; Wingfield *et al.*, 2011).

One of the *Phytophthora* species, *P. cinnamomi,* has been described by Newhook and Podger, 1972 as “the most destructive plant pathogen ever recorded in this (southern Australia) and possibly any region”. Though it is present in South African plantations, the planting of resistant cultivars of *Eucalyptus* such as pure *E. grandis* and *E. grandis* hybrids reduces problems associated with the disease. However, its presence prevents the use of extremely susceptible cultivars with excellent pulping characteristics, such as *E. fraxinoides* and *E. fastigata* (Wingfield and Knox-Davies, 1980; Zwolinski and Bayley, 2001). Other cold-tolerant species such as *E. smithii*, *E.nitens* and *E. dunnii* are still cultivated and succumb to Phytophthora root rot under stress (J. Roux, personal communication). Numerous studies investigating control measures and mechanisms that influence disease, as well as the spread of the pathogen, have been performed on the *Eucalyptus* - *P. cinnamomi* interaction (Shearer and Smith, 2000). This knowledge, along with the availability of resistant and susceptible species, serves as a good platform for further study of defense mechanisms against *Phytophthora* in eucalypts.

1.3. *Phytophthora*

1.3.1 Biology and taxonomy

The oomycetes are a diverse group that include pathogens of plants, vertebrates, fish and insects (Govers and Gijzen, 2006). Within this group, resides the hemi-biotrophic genus *Phytophthora* (meaning 'plant destroyer') that is responsible for numerous plant diseases such as cankers, root rots, damping off, rots of lower stems and tubers, fruit and bud rots, as well as blights on foliage, young twigs and fruits (Agrios, 2005; Govers and Gijzen, 2006). Other oomycete plant pathogens include *Pythium* species, downy mildews and white rusts (Agrios, 2005). These plant pathogens are very similar to fungi with regards to the mycelial growth form, propagation via spores and the infection structures and cell wall degrading enzymes used during infection (Govers and Gijzen, 2006; Judelson and Blanco, 2005; Latijnhouwers *et al.*, 2003). However, they differ considerably when their physiology, biochemistry and genetics are compared (Govers and Gijzen, 2006). Oomycetes have biflagellate zoospores and cellulose cell walls (as opposed to chitin in fungi; Hardham 2005). *Phytophthora* consists of over a hundred species (Brasier, 2009; Érsek and Ribeiro, 2010) that have been divided into ten clades based on internal trancriber spacer (ITS) regions and mitochondrial and nuclear genes (Blair *et al.*, 2008; Cooke *et al.*, 2000; Kroon *et al.*, 2004).

Phytophthora spp. may produce asexual spores in the form of chlamydospores or zoospores or oospores, which are produced during sexual reproduction (Erwin and Ribeiro, 1996). Chlamydospores and oospores of *P. cinnamomi* are capable of persisting in soil or in dead plant material, where it could act as a saprophyte (Weste, 1983; Zentmyer, 1983; Zentmyer and Mircetich, 1966). Subsequent to successful germination and penetration of a host, hyphae are formed under favourable conditions such as high humidity and moderate temperature, and the pathogen amplifies inoculum by repeating the asexual life cycle numerous times. When sporangia of *P. cinnamomi* form, they are cleaved to produce twenty to thirty zoospores each. These zoospores encyst, germinate and penetrate the plant, whereafter more hyphae are formed that produce sporangia within two to three days (Hardham, 2005).

Disease development by *P. cinnamomi* is favoured by fairly cool, wet conditions. In *E. marginata*, the optimal temperature for infection is between 25°C and 30°C, with a minimum of 5°C and a maximum temperature of 34°C (Shearer *et al.*, 1987). Under drought stress, *E. sieberi* and *E. marginata* have been found to be less susceptible to *P. cinnamomi* infection (Smith and Marks, 1985, 1987). Moisture plays a very important role in establishment of infection, spread and survival of this soil-borne pathogen (Hardham, 2005). Water and temperature do not only influence the host, but are the main physical factors that affect

germination of sporangia. In many cases, direct contact with water is required for germ tube formation or zoospore release from sporangia (Duniway, 1979, 1983).

1.3.2 Importance of *Phytophthora* species

Every year, the genus *Phytophthora* is responsible for the loss of billions of dollars in the United States alone (Erwin and Ribeiro, 1996; Stokstad, 2006). *Phytophthora* species are diverse with respect to their hosts and the size of their host ranges. In contrast to most *Phytophthora* species, *P. infestans* and *P. sojae* have narrow host ranges (Attard *et al.*, 2008), but still have devastating effects on their hosts. The causal agent of late blight of potato, *P. infestans*, is a foliar pathogen that is capable of dispersing inoculum in dry air, a characteristic not often seen within the genus (Waterhouse, 1974). This pathogen was responsible for the Irish potato famine in 1845 that resulted in millions of the Irish population emigrating and dying of starvation (Bourke, 1965; Woodham-Smith, 1962) and is still a major threat to potato crops more than a 100 years later (Fry and Goodwin, 1997). Though other *Phytophthora* species have not been responsible for such large demographic changes, there are several other species that are responsible for severe economic losses or ecological damage, and a few are highlighted below (see Figure 1.1).

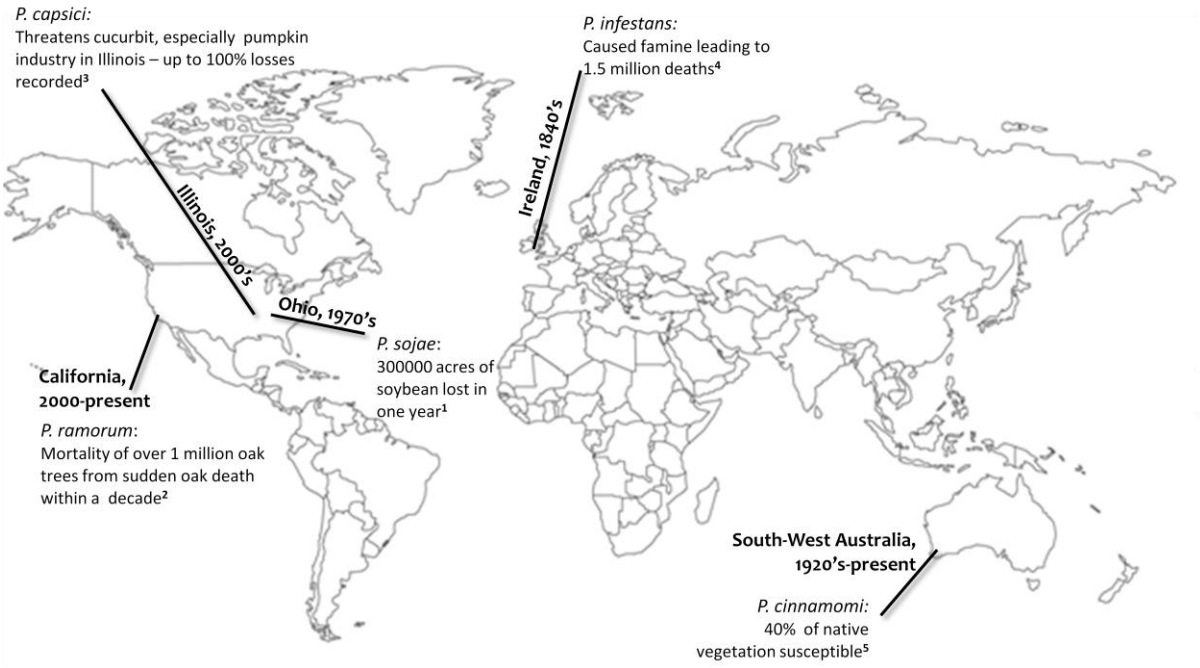

Figure 1.1 Selected epidemics by several *Phytophthora* spp. which highlight the potential for negative economic and ecological impacts by this genus. [1]Dorrance 2007; [2]Parke 2008; [3]Babadoost 2005; [4]Bourke 1965; [5]Shearer 2004

Phytophthora sojae is responsible for root rot of soybean, which results in vast expenditure for control measures and causes losses amounting to between one and two billion US dollars annually (Erwin and Ribeiro, 1996; Tyler, 2007). *Phytophthora capsici* is capable of infecting over fifty hosts, and is an important pathogen on several vegetable crops such as

the cucurbits and some solanaceous crops such as pepper and eggplant. Since it is able to survive in the soil for several years, crop rotation strategies are of little use, and outbreaks of this pathogen may cause heavy crop losses (Babadoost, 2005). In 2001 a new species, *P. ramorum*, the causal agent of sudden oak death in California, was described by Werres *et al.* (2001). Over a million trees have been killed in Oregon and south-west California in the last decade (Meentemeyer *et al.*, 2011), and the spread of the pathogen in Europe on Japanese larch is a major cause for concern (Brasier and Webber, 2010). The devastation caused by this pathogen has resulted in the sequencing of its genome, which is now available along with the genomes of *P. sojae* and *P. infestans* (Govers and Gijzen, 2006). Another species of note is *P. parasitica*. It is widespread, with a broad host range that infects over 60 plant families, in particular the Solanaceae (Erwin and Ribeiro, 1996). Attard *et al.* (2008) consider it to be a good model organism for the genus since it is has a broad host range and infects roots, representing the majority of *Phytophthora* species.

The *Phytophthora* with the widest reported host range and some of the most far-reaching effects, is *P. cinnamomi*. According to Hardham (2005), *P. cinnamomi* is a devastating pathogen that causes "extensive economic losses in agriculture, horticulture and forestry and is a major threat to natural ecosystems and biodiversity." It is able to infect over 900 species (Erwin and Ribeiro, 1996; Zentmyer *et al.*, 1980), and it is estimated that over 3500 species are compatible hosts (Hardham and Blackman, 2010). The majority of hosts are native Australian species, such as *Eucalyptus*. *P. cinnamomi* was first described after isolation from cinnamon trees (Rands, 1922), but since then it has been identified on other crops such as avocado, pineapple, chestnut, peach, macadamia, oak and pine (Erwin and Ribeiro, 1996; Hardham, 2005; Zentmyer, 1983; Zentmyer *et al.*, 1980). It has caused extensive damage to ecosystems in jarrah (*E. marginata*) forests in western Australia and understorey vegetation in some south-west Australian forests (Podger, 1968; Shearer, 1990). Close to 2300 plant species (40% of species present) in this region are estimated to be susceptible to *P. cinnamomi* (Shearer *et al.*, 2004) and it continues to cause disease and threaten the existence of numerous endangered species (Cahill *et al.*, 2008). This epidemic is responsible for motivating a considerable amount of research on the interaction between *P. cinnamomi* and eucalypts (Shearer and Smith, 2000). *P. cinnamomi* is present in South Africa, and causes disease on *Eucalyptus* spp., *Pinus* spp. (Linde *et al.*, 1994a; Linde *et al.*, 1994b), some proteaceae (Van Wyk, 1973; Von Broembsen and Brits, 1985), and is problematic on avocado orchards (Darvas and Bezuidenhout, 1987). South Africa was hypothesized to be the centre of origin (Von Broembsen, 1985), but at present Papua New Guinea is the likely origin based on the high genetic diversity within populations (Hardham, 2005; Linde *et al.*, 1999; Old *et al.*, 1984). Currently, *P. cinnamomi* has a worldwide

distribution (Zentmyer, 1976), and it is poised to increasingly affect host species at raised temperatures due to global warming (Bergot *et al.*, 2004; Brasier and Scott, 1994).

1.3.3 Control of *Phytophthora*

Due to the destructive effects of *Phytophthora* species on many crops, several mechanisms have been established to minimize infection and crop losses. A logical control mechanism is to plant resistant cultivars of a particular crop. Accounts of general (horizontal) resistance and single-gene (vertical) resistance are available for several crop species (Tyler, 2007; Umaerus *et al.*, 1983; Vleeshouwers *et al.*, 2011). In *Eucalyptus* plantations in South Africa, *P. cinnamomi* has mostly been successfully controlled by utilization of resistant species (Wingfield and Knox-Davies, 1980). However, there are some valuable species like the cold-tolerants *E. fastigata* and *E. fraxinoides*, which succumb to Phytophthora root rot easily (Wingfield and Kemp, 1994) and can therefore not be cultivated anymore.

Several fungicides have been developed to control *Phytophthora* with success. This includes a well-researched class of chemicals, called phosphite (Erwin and Ribeiro, 1996; Hardham, 2005). Though phosphite's mode of action is unclear, it appears to either stimulate plant defence responses, decrease pathogen virulence, or directly inhibits the pathogen (Eshraghi *et al.*, 2011; Guest and Grant, 1991; Hardy *et al.*, 2001; Jackson *et al.*, 2000; Pilbeam *et al.*, 2011; Shea and Broadbent, 1983). It has been effective when used as a spray on plant communities in Western Australia (Hardy *et al.*, 2001). Further control practices may include the use of ectomycorrhizal fungi which are capable of conferring a certain extent of protection to the roots of plants (Shea and Broadbent, 1983). Sanitation of vehicles and humans and quarantine measures are also critical for containment of disease outbreaks (Hardham, 2005).

1.4. *Phytophthora* virulence mechanisms and establishment of infection

Considerable research has been conducted on the interactions of oomycete plant pathogens, particularly *Phytophthora*, with their hosts [reviewed in Birch *et al.*, (2009); Hardham (2007); Hardham and Blackman (2010); Hardham and Shan (2009); Kamoun (2006); Tör (2008); Tyler (2002); Tyler (2009)]. In the last decade, there has been substantial progress in characterizing virulence factors involved in *Phytophthora* infection, as well as the plant host responses. Correct and timely recognition of certain pathogen associated molecules activates various defence mechanisms.

1.4.1 Establishment of infection by *Phytophthora*

Recognition of a suitable host and initial contact are important for successful invasion of a plant. With regards to *P. cinnamomi*, motile zoospores are essential to this process since they are capable of finding suitable infection sites and attaching to the host (Hardham, 2005). Zoospores are asexual spores without cell walls that are derived from sporangia. Two flagella enable them to swim considerable distances relative to their size - *P. cinnamomi* spores have been reported to swim up to six centimetre in flooded soil (Carlile, 1983).

The first interaction of *Phytophthora* with a host is observed even before there is any physical contact. Zoospores are capable of sensing chemical and electrical signals from their environment and adjusting the direction in which they are headed accordingly. The most noteworthy chemical signals are root exudates from plants that may contain various amino acids which attract *Phytophthora* species in a nonspecific manner (Carlile, 1983). Root exudates can also play an important role in host recognition and promoting specificity by the pathogen. Soybean root exudates contain two isoflavones: daidzein and genistein. These compounds are capable of attracting *P. sojae* zoospores at concentrations as low as 0.1 nM, while other *Phytophthora* species are not attracted even at 30 µM (Morris and Gow, 1993; Tyler *et al.*, 1996). *P. sojae* is attracted by other phenolics as well and may have several receptors for sensing of the chemical environment (Tyler, 2002; Tyler *et al.*, 1996). In addition to this host-specific chemotaxis by zoospores, encysted zoospores are capable of attracting other zoospores in order to increase the probablility of a successful infection at a site. Calcium released from the encysted zoospores may play a role in this attraction (Irving *et al.*, 1984; Reid *et al.*, 1995). Besides releasing chemicals, plant roots have a weak electric field around them, particularly at tips, wound and branch points which are vulnerable to infection (Morris and Gow, 1993). Electrotaxis based on the polarity of flagella has been displayed *in vitro* in *Pythium* and *Phytophthora* species. Using this mode of environmental sensing, zoospores can possibly distinguish dead and live plant material, and target optimal infection sites (Morris and Gow, 1993).

Once a zoospore senses close proximity to the root surface, a switch in behaviour to allows for encystment (Donaldson and Deacon, 1993; Morris and Gow, 1993; Tyler, 2002). Zoospores are orientated with the flagella-bearing (ventral) side towards the plant to facilitate direct penetration of hyphae that emerge from this side of the encysted zoospore (Deacon and Donaldson, 1993). Encystment is a rapid process, wherein flagella are shed and a temporary cell wall is synthesized within 10 minutes (Carlile, 1983). During the encystment process in *P. cinnamomi*, dorsal vesicles secrete a mucilage-like coating over the cyst's surface that possibly plays a role in preventing dessication (Gubler and Hardham, 1988).

Ventral vesicles secrete a protein, PcVsv1, between the plant cell and cyst that is most likely responsible for the adhesive properties of the cyst (Hardham and Gubler, 1990). PcVsv1 contains domains showing homology to adhesins from animals and malarial parasites (Adams and Tucker, 2000; Tomley and Soldati, 2001).

Approximately 20-30 minutes subsequent to cyst formation, *P. cinnamomi* germination takes place (Hardham, 2005). Germination may be stimulated by substances present in the rhizosphere, such as nutrients and calcium (Tyler, 2002). In *P. sojae*, isoflavones stimulate encystment and germination (Morris *et al.*, 1998; Morris and Ward, 1992), so this is another step at which these secreted substances play a role in host specificity. Germination consists of the formation of a germ tube from the ventral side of the cyst that grows towards the anticlinal cell walls of epidermal cells for penetration. Penetration may occur at periclinal cell walls as well, where it is usually accompanied by an appressorium-like swelling of the germ tube (Hardham, 2001, 2005). Turgor pressure within this swelling may be regulated by accumulation of proline (Ambikapathy *et al.*, 2002). Once the hyphae have entered the apoplastic space, a haustorium is formed to mediate nutrient uptake from the host cell. Shan *et al.* (2006) proposed that H^+-ATPases generate an electrochemical gradient across the plasma membrane that may direct compounds from the plant cell to the pathogen. Subsequent to penetration of the host root, colonization of susceptible plants proceeds rapidly and sporangia are visible on the root suface within two to three days (Hardham, 2005).

1.4.2 *Phytophthora* PAMPs

Upon contact with a host, pathogens secrete or display molecules on their surface called PAMPs. They are usually substances that pathogens cannot do without, otherwise selective pressure would eliminate their expression (Nürnberger *et al.*, 2004). PAMPs possess highly conserved regions that are recognized by the plant's pattern recognition receptors (PRR) in the plasma membrane. This recognition can lead to subsequent defence activation (Jones and Dangl, 2006). Examples of PAMPs are found in the major classes of plant pathogens. Bacterial PAMPs include flagellin, lipopolysaccharide and elongation factor Tu (Nürnberger *et al.*, 2004; Zipfel *et al.*, 2006), whereas fungal PAMPs include chitin, β-glucan and ergosterol (Ingle *et al.*, 2006; Nürnberger and Lipka, 2005).

The PAMPs identified in oomycetes to date are secreted in the apoplast. Studies by Gaulin *et al.* (2002) identified a protein from *P. parasitica* that is involved in attaching the pathogen to the host cell by two cellulose binding domains. This protein was named the cellulose binding elicitor lectin (CBEL). Further investigations revealed its ability to induce expression

of defence genes and form necrotic lesions on tobacco when expressed by *Escherichia coli*. Synthetic peptides of this protein activate defence responses in *A. thaliana* and tobacco, so it has been concluded that it contains the molecular patterns responsible for activation of PAMP triggered immunity (PTI), and is therefore classified as a PAMP (Gaulin *et al.*, 2006; Gaulin *et al.*, 2002).

In fungi, cell walls are composed primarily of chitin, which acts as a PAMP. In oomycetes, the cell walls are composed mainly of cellulose, but fragments derived from cell walls have also been observed to trigger immunity in plants and are therefore also classified as a PAMP. This has been studied in several *Phytophthora* pathosystems, including one between *P. cinnamomi* and *Cinchona robusta* (a medicinal, alkaloid-containing tree) (Ramos-Valdivia *et al.*, 1997; Tyler, 2002). In *P. sojae*, *β*-glucans are released from the cell wall by germinating cysts, and as a result of soybean glucanases (Ham *et al.*, 1991; Ham *et al.*, 1997; Waldmüller *et al.*, 1992). *β*-glucans are capable of inducing various defence responses in soybean (Daxberger *et al.*, 2007; Mithöfer *et al.*, 1997; Mithöfer *et al.*, 1999; Mithöfer *et al.*, 2001; Yamamizo *et al.*, 2006). A protein subunit with the ability to bind these *β*-glucans has been cloned and has the ability to confer resistance to *Phytophthora* (Ebel and Mithöfer, 1998; Tyler, 2002).

Another cell-wall associated PAMP that is conserved in *Phytophthora* species, is a highly conserved 13 amino acid peptide (Pep-13) from calcium dependant transglutaminase (Brunner *et al.*, 2002). Pep-13 from soybean is able to elicit production of phytoalexins, generate ROS and elevate expression of various defence genes in parsley (Nürnberger *et al.*, 1994; Sacks *et al.*, 1995). It is suggested by Brunner *et al.* (2002) that *Phytophthora* calcium dependant transglutaminases have an essential epitope that plant receptors are capable of recognizing and therefore acts as a PAMP.

Small cysteine-rich proteins form another class of pathogen derived molecules involved in triggering defences in the host. These proteins consist of peptides less than 150 amino acids in length that have an even number of cysteine residues with disulfide bridges in between the cysteine pairs (Kamoun, 2006). *Peronospora parasitica* - *A. thaliana* (Ppat), *P. cactorum* - *Fragaria* (Pcf, a possible toxin) and Pcf-like belong to this class (Bittner-Eddy *et al.*, 2003; Bos *et al.*, 2003; Liu *et al.*, 2005; Orsomando *et al.*, 2001), but the most extensively studied molecules are the elicitins. Elicitins are able to induce the HR and other defences in plants. It has been noted that some elicitin deficient strains of *P. parasitica* are more virulent on *Nicotiana benthamiana* than those producing elicitins (Tyler, 2002). This may be due to the fact that strains lacking this PAMP cannot be recognized as a pathogen and therefore do not

initiate PTI. Highly similar elicitin sequences have been sequenced from various *Phytophthora* spp. and these are designated class I elicitins (Tyler, 2002). Two elicitins, α – and *β*-cinnamomin, have been observed in *P. cinnamomi* (Perez *et al.*, 1999; Pernollet *et al.*, 1993). Class I elicitins are sterol-binding proteins and are therefore hypothesized to acquire sterols from the host, since *Phythophthora* is unable to produce its own sterols (Hendrix, 1970). The most comprehensively studied elicitin is INF1 from *P. infestans* (Kamoun, 2007). Tyler (2002) speculated that expression of an elicitin receptor should confer broad-spectrum resistance to all *Phytophthora* species. This has not been performed to date, but Kanzaki *et al.* (2008) found a lectin-like receptor kinase (NbLRK1) in *N. benthamiana* that interacts with INF1 and is required for HR-mediated cell death in tobacco. More recently NgLRK1 from *N. glutinosa* has been identified to interact with the *P. capsici* elictin (Kim *et al.*, 2010).

Another type of PAMP found in *Phytophtora* spp. is the Nep1-like proteins (NLPs), which are found in fungi, bacteria and oomycetes. These proteins play a role in inducing necrosis of host tissue (Pemberton and Salmond, 2004) and are capable of triggering defence responses in susceptible and resistant plants. It is thought that these proteins may be involved in inducing necrosis of host cells during the necrotrophic phase of *P. infestans* and *P. sojae* since they are expressed during the late stages of infection (Kamoun, 2006). *P. parasitica* has an NLP called NPP1, which is able to trigger defence responses similar to that of Pep-13 in parsley cells (Fellbrich *et al.*, 2002).

1.4.3 *Phytophthora* effectors

Besides PAMPs, another class of proteins secreted by pathogens is that of effectors. These proteins are able to manipulate or suppress defence by targeting specific host factors involved in defence mechanisms such as signalling (Jones and Dangl, 2006). Other than the PAMPs which are secreted into the apoplast, effectors mostly function inside the cell (Jones and Dangl, 2006). However, *Phytophthora* species are known to secrete effectors into both the apoplast and the cytoplasm, where they can suppress PTI-related defences (Haas *et al.*, 2009; Whisson *et al.*, 2007).

A class of apoplastic effectors are the glucanase inhibitor proteins (GIPs). These have been identified in *P. sojae* and possibly in *P. infestans* (Kamoun, 2006). They play a role in preventing degradation of pathogen cell walls and release of cell wall components that may act as PAMPs (Kamoun, 2006) by inhibiting plant glucanases that aim to disrupt the cellulose wall of invading *Phytophthora* hyphae.

In addition to the glucanase inhibitors, serine protease inhibitors and cysteine protease inhibitors play a role as effectors in counter-defence. The serine protease inhibitors, extracellular protease inhibitor 1 (EPI1) and EPI10, as well as extracellular protease inhibitor with cystatin-like domain 1-4 (EPIC1-4), EPIC1 and EPIC2 from *P. infestans* have been proven to inhibit the various PR-7 proteases from tomato (Tian *et al.*, 2005; Tian *et al.*, 2004; Tian *et al.*, 2007). The suppression of PTI is discussed in more detail later.

Two classes of cytoplasmic effectors are found in *Phytophthora* and oomycete species. These classes include the RXLR-dEER domain avirulence (Avr) proteins and the crinkling- and necrosis inducing (CRN) proteins (Morgan and Kamoun, 2007; Torto *et al.*, 2003). CRN proteins are not well characterized, but it is known that they bring about crinkling and necrosis of leaves in *N. benthamiana* and tomato (Torto *et al.*, 2003). Their function in host infection has not been determined, but it has been shown that they are probably capable of entering host cells since they trigger necrosis regardless of expression inside host cells or secretion outside by the pathogen (Kamoun, 2006; Torto *et al.*, 2003). CRN proteins have RXLR motifs, like the Avr proteins, but lack the dEER domain. Instead, they have a FLAK motif that overlaps the RXLR motif (Win *et al.*, 2007).

It is generally accepted that effectors interact indirectly or directly with NB-LRR proteins (also often termed resistance, or R proteins) and that this results in detection of the pathogen by the plant and an incompatible interaction as a result of effector triggered immunity (ETI) [reviewed in Dangl *et al.* (2001) and Jones and Dangl (2006)]. Several oomycete Avr effectors have been characterized and reported (Table 1.1). Avr1b, Avr3a, ATR1, and ATR13 trigger an interaction with the product of an *R* gene when expressed in host cell cytoplasm, and are therefore functional inside the cell (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Dou *et al.*, 2008a; Rehmany *et al.*, 2005).

Table 1.1 Summary of characterized Avr effectors secreted by *Phytophthora* spp.

Avr effector	Pathogen	Host	Reference
Avr1b	*P. sojae*	Soybean	(Shan *et al.*, 2004)
ATR1	*Hyaloperonospora arabidopsis*	*Arabidopsis*	(Allen *et al.*, 2004)
ATR13	*H. arabidopsis*	*Arabidopsis*	(Rehmany *et al.*, 2005)
Avr3a	*P. infestans*	Potato	(Armstrong *et al.*, 2005)
Avr4	*P. infestans*	Potato	(Van Poppel *et al.*, 2008)
Avr-blb1	*P. infestans*	Potato	(Vleeshouwers *et al.*, 2008)

An RXLR-EER motif has been found directly after a signal peptide in all the oomycete Avr proteins (Birch *et al.*, 2008). This highly conserved sequence is required for the transport of effectors across the cell membrane into the cytoplasm (Dou *et al.*, 2008b; Whisson *et al.*, 2007). Whisson *et al.* (2007) replaced the amino acids in the motif with KMIK-DDK, thereby still maintaining the sequence's chemical properties, but effectors could not be delivered into the host cell. Effector proteins in malaria parasites possess a motif, RXLXE/D/Q, that is needed for entering host blood cells, and the motif is similar in function and sequence to that from the oomycete effectors (Birch *et al.*, 2009; Hiller *et al.*, 2004; Marti *et al.*, 2004).

Avr3a has been characterized by Bos *et al.* (2006) in *N. benthamiana*. Avr3a is detected by the R protein R3a and is able to suppress PTI-related cell death triggered by the elicitin, INF1, in this host (Birch *et al.*, 2009). Bos *et al.,* (2006) determined that the C-terminal half of the protein is important for virulence, whereas the N-terminal half containing the RXLR-EER motif is not required for suppressing programmed cell death (PCD). These results are confirmed by Dou *et al.*, (2008b) who demonstrated that Avr1b is able to suppress PCD triggered in yeast. They also identified conserved K, W and Y motifs in the C-terminal halves of various predicted Avr effectors that are essential for interaction with host targets and functioning of these proteins. Many putative Avr effectors have been identified based on various *Phytophthora* genome sequences available (Birch *et al.*, 2009). Birch *et al.* (2009) hypothesize that, similar to prokaryotic effectors secreted by type III and IV secretion systems, effectors from *Phytophthora* could also target the host ubiquitin proteosome system for manipulation of host defences. Therefore, by inhibiting PCD processes, the biotrophic stage can be established successfully by the pathogen.

The ETI that occurs as a result of recognition between a host *R* gene and a pathogen effector is important for preventing infection by pathogens that are adapted to the host and contain effectors that may manipulate certain host functions. In potato, though *R* gene-mediated resistance may break down rapidly and has been proved fallible, the search for *R* genes continues due to the strong resistance phenotype they confer (Fry, 2008). Numerous *R* genes have been identified in two commercially important *Phytophthora* hosts – eleven from wild potato (Van Der Vossen *et al.*, 2003) and fifteen from soybean (Burnham *et al.*, 2003; Imsande *et al.*, 1992; Lohnes *et al.*, 2001; Lohnes and Schmitthenner, 1997; Sandhu *et al.*, 2004; Weng *et al.*, 2001). Additionally, various *R* genes and their matching *Avr* gene counterparts have been successfully elucidated (Vleeshouwers *et al.*, 2011). All cloned *R* genes involved in mediating resistance against *Phytophthora* spp. encode NB-LRR domain proteins (Hardham and Shan, 2009).

The resistance to the *P. sojae* (*Rps1k*) gene, is used commercially to protect soybean. Additionally, wild solanaceae (such as *S. demissum*) contain *R* genes that show promise, such as *R1*, *R3a*, *Rpi-blb1* and *Rpi-blb2*, which are able to prevent invasion by *P. infestans* (Ballvora *et al.*, 2002; Foster *et al.*, 2009; Huang *et al.*, 2005; Lokossou *et al.*, 2009; Pel *et al.*, 2009; Song *et al.*, 2003; Van Der Vossen *et al.*, 2003; Vleeshouwers *et al.*, 2008). Despite the apparent promise of the *R* gene approach, it is evident that no single *R* gene has been capable of conferring resistance (Fry, 2008). One approach being applied in combating *P. infestans* currently is to stack *R* genes in one cultivar to avoid resistance breaking down so fast (Kim *et al.*, 2012; Zhu *et al.*, 2012). Even so, *P. infestans* is notorious for its ability to overcome introduced *R* genes (Fry, 2008), and therefore more durable mechanisms of defence need to be employed. An example of this is the broad spectrum resistance gene: resistance to *P. infestans* from *S. bulbocastanum* 1 -*Rpi-blb*1 (*RB*), which was cloned from *S*. *bulbocastanum* (Song *et al.*, 2003). It does not confer classic vertical resistance, but rather durable field resistance. *RB* has been transformed into commercial lines of potato, and field trials with these lines proved that the gene is capable of conferring resistance without assistance from fungicides (Bradeen *et al.*, 2009). This resistance is not mediated by HR, as with the other *R* genes (Song *et al.*, 2003). Such broad spectrum resistance mechanisms may prove valuable in combating the disease. Additionally, research into host responses that provide protection against *Phytophthora* will provide valuable knowledge that can contribute to correct selection of cultivars/species for cultivation, or the creation of *Phytophthora*-resistant transgenics.

1.5 *Phytophthora*-host interactions

For some time now the model dicotyledon, *A. thaliana*, has afforded scientists with a glimpse of the molecular mechanisms at play in other plant species. In fact, for the area of plant defence, *A. thaliana* has provided not only a glimpse, but a broad knowledge base of plant defence against various types of pathogens. Research mainly in *A. thaliana*, but also in *N. benthamiana* and other plant species, has resulted in generally accepted models of defence cascades that describe the interaction of a plant with a pathogen. This "general knowledge" of plant defence provides a basis from where studies can examine the dynamics of the interaction between *Phytophthora* spp. and their hosts. In this way, mechanisms specific to defence against *Phytophthora* can be identified. The literature related to general host defence is discussed in this section, along with *Phytophthora*-specific examples. Since *Phytophthora* is a diverse genus, the focus is narrowed in section 5, which only encompasses host responses to *P. cinnamomi*.

1.5.1 Pre-existing defences

Plants possess defence mechanisms which are present even in the absence of pathogen challenge. These include physical factors such as the thickness of cell walls, wax layers on leaves, the structure of stomata that may guard against pathogen entry, and the structure of components of the vascular tissue (Agrios, 2005). There are also certain chemicals present prior to challenge that may act in a protective capacity. These include certain exudates, which are toxic to pathogens, as well as hydrolytic enzymes such as glucanases and chitinases which may act to break down pathogen cell walls. Additionally, antimicrobial compounds called phytoanticipins, may be present within plant cells and act to directly inhibit potential microbial pathogens (Agrios, 2005).

Though these constitutive defences have immense value, this is only a small part of what is known of the plant defence artillery. The majority of defence mechanisms available to the plant, are only induced upon pathogen challenge. These pathways may either be involved in general metabolism, and then regulated in response to the pathogen, or they may be undetectable in the absence of a pathogen and only induced upon perception (Van Loon *et al.*, 2006). This has a two-fold benefit: energy is conserved by not sustaining constant activation of defence pathways, and efficacy of defence is optimized, since defence pathways appropriate to a certain pathogen may be set in motion upon perception. The rest of this defence overview is focused on inducible defences, specifically those pertaining to microbial pathogens.

1.5.2 Inducible defences

1.5.2.1 PAMP- and Effector Triggered Immunity

In order for a plant to launch defence against a pathogen, some key events are required. Firstly, the pathogen needs to possess, secrete or release a compound (a PAMP or effector) that reveals its presence, or interact with some part of the host in order to trigger recognition. Secondly, the host needs to be capable of perceiving the abovementioned compounds. The result of perception of these molecules is defence activation within the host in the form of PTI and ETI (Chisholm *et al.*, 2006).

According to a model proposed by Jones and Dangl (2006), the first line of defence in a plant is PTI. Pattern recognition receptors (PRRs) are activated by PAMPs and then signal the activation of various downstream defence mechanisms, which is denoted as PTI. Though PTI is sufficient to prevent entry by most microorganisms, some pathogens possess a mechanism to overcome this basal defence in the form of effectors. Effectors target specific host factors involved in defence mechanisms such as signalling, and attempt to control or manipulate the host responses (Jones and Dangl, 2006). If these effectors are not detected, a compatible reaction may result due to delayed defence by the host. Effectors have been studied extensively in bacterial systems, but eukaryotic effectors have not been characterized well (Jones and Dangl, 2006).

The second line of defence in plants is triggered by direct or indirect recognition of effectors by receptors (encoded by *R* genes). Dangl and Jones (2001) proposed that R proteins may 'guard' effector targets and therefore detect interference by the pathogen and initiate appropriate defence signalling. These R proteins may be localized in the cell membrane and perceive extracellular effectors, or intracellular R proteins recognize effectors that have been delivered into the host cell. R proteins may differ in structure, but generally possess two domains: a nucleotide binding (NB) and a leucine rich repeat (LRR) domain (Dangl *et al.*, 2001).

In a recent review, Tsuda and Katagiri (2010) discuss the different roles of PTI and ETI and the common immune responses utilized by both. These include generation of ROS, the HR, mitogen activated protein kinase (MAPK) signalling, and hormone signalling through SA, JA and ET. Though these defences mechanisms are common between PTI and ETI, they are utilized differently. PTI-associated responses tend to be transitory in nature and are generally weaker than those displayed during ETI. Different components of the host defence artillery are discussed below, with an emphasis on their function in response to challenge with *Phytophthora*.

1.5.2.2 Mitogen activated protein kinase and calcium signalling

MAPK phosphorylation cascades and Ca^{2+} signalling play a central role in mediating the activation of various defence mechanisms such as hormone signalling, the oxidative burst and HR, as well as the production of phytoalexins and other antimicrobial compounds or proteins.

A general model for MAPK signalling is that a plasma membrane receptor in the plant (such as a PRR) perceives a signal, which initiates a phosphorylation cascade. The signal activates a MAP kinase kinase kinase (MAPKKK). The MAPKKK activates a MAP kinase kinase (MAPKK), which in turn activates a MAPK (Boller and He, 2009; Rodriguez *et al.*, 2010). The MAPKs may target certain molecules such as enzymes or transcription factors in the cytoplasm or nucleus. This elaborate network of signal transduction is integral to plant defence, and plays a role in PTI, as well as ETI (Rodriguez *et al.*, 2010). Flagellin, a well-characterized bacterial PAMP is known to activate MAPK cascades (Chinchilla *et al.*, 2007; Rodriguez *et al.*, 2010). MAPK signalling may also involve certain WRKY-domain transcription factors, which aid in regulation of downstream defence activation. An example of this is the regulation of phytoalexin deficient 3 (*PAD3*), which is responsible for phytoalexin (camalexin) biosynthesis in *A. thaliana*. Regulation of *PAD3* occurs through WRKY33 as part of a MAPK cascade (Andreasson *et al.*, 2005; Qiu *et al.*, 2008b; Rodriguez *et al.*, 2010). Other defence mechanisms regulated through MAPK cascades include the oxidative burst and SA signalling (Rodriguez *et al.*, 2010).

Various *Phytophthora* PAMPs may give rise to MAPK activation, such as Pep-13, and NPP1 from *P. parasitica*, which activate a parsley ortholog of salicylic acid-induced protein kinase (SIPK) (Fellbrich *et al.*, 2002). Additionally, heptaglucoside β-glucans from *P. sojae* activate two MAPK orthologs and one MAPKK ortholog in soybean, which may be involved in signalling subsequent to recognition of the PAMP (Daxberger *et al.*, 2007). The importance of this signalling is evident in a potato transgenic with pathogen inducible MAPK cascade activation, which confers broad spectrum resistance. This MAPK cascade confers increased resistance to *P. infestans* through the activation of defence genes and NADPH oxidase, which results in HR and ROS generation (Yamamizo *et al.*, 2006). However, even though MAPK signalling mediates induction of various defence mechanisms, it is not solely responsible for providing protection against *Phytophthora* invasion. In *Capsicum chinense* (Habanero pepper), inoculation with *P. capsici* resulted in strongly induced transcript levels of two MAPKs, but the plants were highly susceptible and died despite this (Nakazawa-Ueji *et al.*, 2010). It would seem that correct and timely activation of defence mechanisms subsequent to MAPK signalling is required for successful defence, and other signalling

components such as cytoplasmic Ca^{2+} concentration ([Ca^{2+}]) may influence the outcome of a plant's interaction with *Phytophthora*.

Various environmental signals can give rise to changes in cytosolic [Ca^{2+}], by way of Ca^{2+} influx from the apoplast or organelles, such as the endoplasmic reticulum or vacuoles (Dodd *et al.*, 2010). Signals from the [Ca^{2+}] increase are interpreted by Ca^{2+}- binding proteins, such as calmodulin (Batistic and Kudla, 2004; Dodd *et al.*, 2010; Luan *et al.*, 2002; Sanders *et al.*, 2002). One of the roles of Ca^{2+} signalling is in pathogen perception and subsequent defence regulation. An increase in nuclear [Ca^{2+}] has been observed in response to a PAMP (Dodd *et al.*, 2010; Lecourieux *et al.*, 2005). Additionally, Ca^{2+}- dependant activation of SA and wound induced–MAPKs (SIPKs and WIPKs) has also been noted (Dodd *et al.*, 2010; Lecourieux *et al.*, 2005; Ma and Berkowitz, 2007). Ca^{2+} signalling may also negatively regulate certain defence mechanisms, such as the repression of enhanced disease susceptibility (*EDS1*) (Dodd *et al.*, 2010; Du *et al.*, 2009).

In parsley cells, NPP1 and Pep-13 are not only responsible for activation of MAPK cascades, but also for an increase in cytoplasmic Ca^{2+} concentration (Fellbrich *et al.*, 2002). Interestingly, both of these signalling mechanisms are involved in phytoalexin production in parsley (Jabs *et al.*, 1997). An influx of Ca^{2+} may also be required for phytoalexin production in soybean, where ion channel blockers prevent the normal *β*-glucan responsive transient cytosolic Ca^{2+} increase, along with the induction of phytoalexins (Mithöfer *et al.*, 2001). A CBEL from *P. parasitica* causes a transient calcium flux in tobacco, and this is required for defence activation (Gaulin *et al.*, 2006). Further evidence for the importance of calcium signal transduction in defence against *Phytophthora* is found in the induction of molecules involved in perception of Ca^{2+}. Calmodulin and a calmodulin binding protein are induced in a compatible and incompatible *P. infestans*-potato interaction respectively (Beyer *et al.*, 2001; Ronning *et al.*, 2003), and a calcium ion binding protein is upregulated in *A. thaliana* upon infection with *P. parasitica* (Wang et al., 2011). Clearly, application of PAMPs and challenge with *Phytophthora* spp. results in MAPK and calcium signalling related events in host cells. These signal transduction pathways do not provide protection against *Phytophthora* spp. directly, but are central to the activation of other defence mechanisms which inhibit pathogens, such as phytoalexin and pathogenesis related (PR) protein induction, hormone signalling, as well as the oxidative burst and HR-related cell death.

1.5.2.3 The hypersensitive response and oxidative burst

The hypersensitive response (HR) is a term denoted for a programmed cell death reaction in plants in response to an avirulent pathogen. At the site of attempted infection by a pathogen,

an area of cell death with sharp boundaries may be found (Mur *et al.*, 2008). This is thought to be a resistance mechanism to prevent the establishment of biotrophs, since they require living tissue (Glazebrook, 2005). Jones and Dangl (2006) proposed that there is a threshold for the HR to occur, which is not reached by PTI, but by ETI. However, the bacterial PAMP flg22 is able to elicit HR (Naito *et al.*, 2008) and several *Phytophthora* PAMPs such as elicitins and NLPs are responsible for HR and are said to push the PTI response above the HR threshold (Hein *et al.*, 2009; Kamoun, 2006). Tsuda *et al.* (2010) hypothesize that HR is mediated weakly by PTI, and more strongly by ETI. Signalling of the HR is promoted by [Ca^{2+}] fluxes, ROS, as well as nitric oxide (NO), and SA. Though these compounds are involved in HR, they are also able to act independently to mediate defence responses (Mur *et al.*, 2008).

The oxidative burst is associated with the accumulation of oxidative compounds called ROS, such as the hydroxyl radical (OH^-) and the superoxide anion (O^{2-}), which is converted to the more stable peroxide molecule (H_2O_2) (Lamb and Dixon, 1997). This usually occurs in a biphasic manner: upon initial recognition of the pathogen a first transient burst occurs, followed by a second more sustained oxidative burst that is usually responsible for the HR (Grant and Loake, 2000; Piedras *et al.*, 1998). ROS are secreted into the apoplast, but intracellular ROS are also important for defence. The respiratory burst oxidase (RBOH), NADPH oxidase, is mainly responsible for ROS production, but ROS may come from other sources as well. ROS production may be signalled through [Ca^{2+}] flux, or MAPK signalling (Asai *et al.*, 2008; Asai and Yoshioka, 2008; Kobayashi *et al.*, 2007; Mittler *et al.*, 2011; Ogasawara *et al.*, 2008).

ROS production acts as a defensive component of host cells, due to its role in initiating the HR, but also since these species may directly inhibit the pathogen (Chen and Schopfer, 1999), or reinforce the cell wall through the cross-linking of glycoproteins or lignin and suberin (Bradley *et al.*, 1992; Hückelhoven, 2007). ROS mediate the formation of certain lipid derivatives such as cyclic oxylipins, which are JA-type signalling molecules (Montillet *et al.*, 2005). Additionally, ROS may result in production of inhibitory compounds to the pathogen, such as phytoalexins and secondary metabolites (Thoma *et al.*, 2003).

Many studies investigating host responses upon challenge with various *Phytophthora* spp. report the presence of ROS, or enzymes associated with ROS, as well as the induction of a HR. One of the major differences between a resistant and susceptible interaction may be the speed of HR onset, as well as the extent of the HR. This is evident in a study by Vleeshouwers *et al.* (2000), where solanaceous hosts (including potato-*S. tuberosum*)

ranging from susceptible to fully resistant and non-host, were challenged with *P. infestans*. The level of resistance was correlated with the nature of the HR, measured by aniline – and trypan-blue-stained tissue. Non-hosts and fully resistant plants had an HR of one to three cells, and the HR was completed within 22 hours. In partially resistant interactions, the HR was slower (16-46 hours post inoculation), consisted of 5 or more cells undergoing PCD, and the pathogen was sometimes able to escape the confines of the necrotized tissue to establish a biotrophic interaction. The HR was rarely observed in fully susceptible interactions. Therefore susceptibility to *Phytophthora* may very well depend on the ability of the pathogen to escape the limits of the HR lesion and establish its biotrophic phase in living tissue (Chen and Halterman, 2011; Smart *et al.*, 2003).

An ability to manipulate HR-related cell death allows the pathogen to establish biotrophy in the early stages of pathogenesis. An example of this is an RXLR effector, AVR3a, that interacts with and stabilizes a host E3 ubiquitin ligase, CMPG1. This interaction abolishes CMPG1-mediated cell death during INF1-induced PTI (Bos *et al.*, 2010; Bos *et al.*, 2006; Gilroy *et al.*, 2011). The manipulation continues at initiation of the necrotrophic phase, with a reversal in the role of the virulence factors. *AVR3a* is downregulated and *INF1* is upregulated in order to activate CMPG1-mediated cell death, since necrosis of the host tissue is beneficial to the pathogen at this stage (Bos *et al.*, 2010). However, the host may prevent this manipulation during the biotrophic phase by activating ETI subsequent to recognition of AVR3a by R3a, which results in a CMPG1-independent HR response (Gilroy *et al.*, 2011).

Since correctly timed cell death is critical in a hemi-biotrophic pathosystem such as with *Phytophthora*, effectors are not the only tool utilized for manipulation of PCD. *P. sojae* produces a necrosis-inducing protein (Nip1) which may act as a toxin that enhances cell death in the late stages of infection. Expression of this gene was found to coincide with the switch from biotrophy to necrotrophy (Qutob *et al.*, 2002). Mechanisms in the plant that mediate timely cell death early in infection may restrict the pathogen, whereas cell death occurring during the necrotrophic phase only aids the pathogen in its necrotrophy.

Though HR is traditionally associated with *R*-gene mediated responses, it is involved in broad-spectrum resistance as well. Chen *et al.* (2011) conducted a study to investigate the difference between defence responses in a susceptible potato cultivar with a transgenic broad-spectrum resistance gene *RB* which confers partial resistance, and a fully resistant cultivar in possession of an *R* gene. A HR was observed in the transgenic and resistant interaction, but not in the wild type susceptible plants. Interestingly, at the time the

necrotrophic phase would have set in, the tobacco HR marker gene *Harpin-induced gene 1* (*Hin1*) (Gopalan *et al.*, 1996) increased in the resistant cultivar, whereas it decreased in the *RB* transgenic (Chen and Halterman, 2011). In two microarray profiling studies, *Hin1* was observed to be upregulated at approximately three days post inoculation (dpi) in partially resistant potato plants inoculated with *P. infestans* (Shibata *et al.*, 2010; Tian *et al.*, 2006; Wang *et al.*, 2005). Possibly the timing and extent of the HR may explain the difference between fully and partially resistant hosts.

There is evidence that a timely HR alone is not sufficient for resistance against *Phytophthora* by itself, and that the oxidative burst may also play a role in the efficacy of the HR. The *A. thaliana* mutant, resistance to *Phytophthora* 1 (*rph1*), displays susceptibility to *P. brassicae* (as opposed to the resistant wild type). It also exhibits a reduced oxidative burst, along with an extended cell death phenotype, and a compromised ability to initiate defence gene expression. *RPH1* is a conserved chloroplast gene that positively regulates defence gene expression and the oxidative burst, which is required to limit cell death during HR. Interestingly, *rph1* plants displayed no enhanced susceptibility toward *Pseudomonas syringae*, *Botrytis cinerea*, or the oomycete *Hyaloperonospora arabidopsis*. However, silencing the potato ortholog of *RPH1* resulted in susceptibility toward *P. infestans.* This gene therefore seems to be specifically involved in responses to *Phytophthora* spp. and may act as an effector target. It is hypothesized that it mediates resistance through the activation of ROS subsequent to recognition when the effector attempts to manipulate it (Belhaj *et al.*, 2009).

The importance of the oxidative burst is evident in the differential regulation of numerous genes related to ROS production and detoxification during infection of potato with *P. infestans*. These genes encode catalase, peroxidase, polyphenol oxidase, superoxide dismutase, NADH oxidase and lipoxygenase, and are found to be differentially regulated in both susceptible and resistant interactions (Beyer *et al.*, 2001; Ros *et al.*, 2004; Tian *et al.*, 2006). Peroxidase especially is responsive to infection in multiple studies. A peroxidase was found to be downregulated in compatible interactions of potato, soybean and papaya in response to challenge with *P. infestans, P. sojae* and *P. palmivora* respectively, and therefore it may be a cross-species target for manipulation by *Phytophthora* effectors (Moy *et al.*, 2004; Porter *et al.*, 2009; Restrepo *et al.*, 2005). Additionally, strong upregulation of peroxidase is present in a resistant pepper cultivar in response to *P. capsici* challenge (Silvar *et al.*, 2008), as well as in resistant taro upon challenge with *P. colocasiae* (Sharma *et al.*, 2009). Therefore the regulation of certain peroxidases could prove to be a key defence mechanism against *Phytophthora*.

The perception of *Phytophthora*-specific PAMPs is proven to result in ROS production. NPP1 and Pep-13, as well as *β*-glucans secreted by *P. sojae* are able to trigger the production of H_2O_2 (Fellbrich *et al.*, 2002; Mithöfer *et al.*, 2001). In *A. thaliana* resistance to *P. parasitica* is mediated by an NADP oxidase-mediated oxidative burst, triggered by CBEL (Larroque *et al.*, 2013). Superoxide anions are released in phosphite-treated *A. thaliana* upon challenge with *P. palmivora*, and in *N. benthamiana* after challenge with *P. nicotianae* and *P. palmivora* (Daniel and Guest, 2006). In tobacco, superoxide was found to be required for the HR, as well as production of the phytoalexin capsidiol (Perrone *et al.*, 2003). *P. sojae* induces a biphasic H_2O_2 burst subsequent to inoculation of soybean. The oxidative burst may play a key role in resistance in this pathosystem, since it is stronger in the partially resistant cultivar as opposed to the susceptible cultivar, and reductants that scavenge ROS enhance susceptibility to the pathogen (Chen *et al.*, 2008). However, a biphasic induction of ethylene and NADPH oxidase-mediated ROS is correlated with increased susceptibility in tobacco inoculated with *P. parasitica* (Wi et al., 2012). In terms of ROS, more is not always better, since it has been shown that a higher degree of lipid peroxidation and higher levels of H_2O_2 is present in susceptible pepper cultivars than in a resistant cultivar upon challenge with *P. capsici*. Therefore, it is hypothesized that protection against oxidative species may also be important for resistance (Koį *et al.*, 2011).

1.5.2.4 Hormone signalling and downstream transcriptional regulation

The roles of SA, JA and ET are fairly well characterized because of their prominent role in plant defence. Other plant hormones such as abscisic acid (ABA), gibberellins (GA), cytokinin (CK), auxin and brassinosteroids (BL) have an effect on plant immunity and the signalling responses to pathogens, but their role in plant defence has not been studied as intensively as that of SA, JA and ET (Robert-Seilaniantz *et al.*, 2011; Robert-Seilaniantz *et al.*, 2007). SA, JA and ET are produced in response to both PTI and ETI. Hormone signalling during ETI is faster, less transient and stronger than during PTI. The current thought is that hormones act synergistically during PTI, but act in a compensatory fashion in ETI. By compensating for each other during ETI, they may act as a "back-up" for another hormone pathway that gets repressed through pathogen manipulation (Tsuda and Katagiri, 2010; Tsuda *et al.*, 2009). Pathogens are able to interfere with hormone signalling through manipulating host hormones or through production of their own hormones, and are even able to mimic plant hormones (Dodds and Rathjen, 2010; Pieterse *et al.*, 2009; Tsuda and Katagiri, 2010).

There is much cross-talk between hormone signalling pathways and though the traditional point of view is that SA and JA pathways act antagonistically, there are instances where synergistic relationships have been observed [reviewed in Pieterse *et al.* (2009) and Robert-Seilaniantz *et al.* (2011)]. It is generally accepted that SA signalling is useful for defence against biotrophs, whereas JA and ET defence provides resistance against necrotrophic pathogens (Glazebrook, 2005). However, the quantity and timing of hormone release may produce a unique profile of phytohormones specific for defence against a particular pathogen, hence there are complex regulatory networks involved in hormone signalling (Pieterse *et al.*, 2009).

SA, JA and ET regulate the activation of particular transcription factors (Robert-Seilaniantz *et al.*, 2011). These transcription factors are then responsible for transcriptional activation of other transcription factors, or certain defence genes in the nucleus that are regulated by the phytohormones. Upon pathogen attack, the products of numerous genes are involved in defence, and therefore they are differentially regulated. The products of many of these genes are already present in the cell, and the genes may be up- or downregulated in response to a pathogen. However, a certain class of proteins are virtually undetectable in the absence of attack, and are induced in the presence of a pathogen. These are termed pathogenesis related (PR) proteins (Van Loon *et al.*, 2006). Some of these proteins have proven *in vitro* and *in vivo* anti-microbial activity (Ferreira *et al.*, 2007). There are currently seventeen *PR* gene families, and these have been extensively reviewed by van Loon *et al.* (2006) and Ferreira (2007). In *A. thaliana*, *PR-1*, *PR-2* and *PR-5* are induced by SA and said to be markers of the pathway, whereas *PR-3* and *PR-4* are induced by JA and ET (Thomma *et al.*, 2011). This is not the case in tobacco, however, where members of the same *PR* family can be regulated by different phytohormones (Niki *et al.*, 1998; Seo *et al.*, 2001; Van Loon *et al.*, 2006).

Despite a wealth of large-scale expression profiling, no clear pattern of defence hormone signalling during *Phytophthora* pathogenesis has emerged. Elucidation of a clear hormone signalling model is confounded by the use of various hosts and tissue types and the diverse *Phytophthora* spp. Another factor to be reckoned with, is the switch from the biotrophic to necrotrophic phase during infection with *Phytophthora,* which requires the host to adjust its defence mechanisms accordingly. The varying lengths of the biotrophic phase present in different pathosystems makes comparison of results from time-course experiments challenging.

Attard *et al.* (2010) conducted a comprehensive study that investigated the roles of SA, JA and ET in *A. thaliana* upon challenge with *P. parasitica*. The requirement of the SA signalling pathway for resistance was investigated through plants impaired in SA biosynthesis and accumulation: *enhanced disease susceptibility 5* (*eds5-1*), *pad4-1*, *eds1-1* and salicylic acid induction deficient *sid2-1* mutants, as well as a *naphthalene hydroxylase G* (*nahG*) transgenic. Mutants of genes influencing the JA/ET pathways [*ethylene insensitive 2* (*ein2-1*), *ethylene receptor 1* (*etr1-3*), *jasmonate resistant 1* (*jar1-1*)] were also evaluated for their contribution to disease resistance. All mutants, as well as the *nahG* transgenic, were more susceptible, with the exception of *sid2-1*, which may be functionally compensated for by *Isochorismate Synthase 2* (*ICS2*). In order to further elucidate the roles of these hormones in the defence response, marker genes for hormone biosynthesis and downstream *PR* gene marker genes were profiled at various time points after inoculation. This revealed a general trend in that SA and JA related genes were transiently upregulated upon penetration of *P. parasitica*, with ET emerging as the dominant signalling hormone during the invasive growth stage of the infection. In contrast, ET and JA mutants (*etr1*, *ein2*, *jar1*) as well as SA mutants [*non-expressor of PR1* (*npr1*), *sid2, nahG* transgenic] of *A. thaliana* were not compromised in their resistance to *P. porri* and so resistance in this pathosystem is mediated through another mechanism (Roetschi *et al.*, 2001).

Smart *et al.* (2003) came to a similar conclusion after investigating *deficient in jasmonate 1* (*def1*), ethylene-insensitive (*never-ripe*) and *nahG* tomato plants. The wild type partial resistance was uninfluenced in plants inoculated with *P. infestans* and therefore it was concluded that partial resistance to *P. infestans* is independant of SA, JA or ET. However, conflicting findings in the same pathosystems make it difficult to draw conclusions. Thaler *et al.* (2004) demonstrated that, contrary to the findings of Smart *et al.* (2003), tomatoes deficient in JA (*def1*) are more susceptible to *P. infestans* than their wild type counterparts. In support of the role of JA in this pathosystem, treatment of tomato plants with the *P. infestans* PAMP, INF1, resulted in the induction of JA and ET responsive genes as well as the accumulation of ET (Kawamura *et al.*, 2009).

Both SA and ET were found to be required for resistance of *N. benthamiana* to *P. infestans.* Young, normally susceptible plants sprayed with acibenzolar-*S*-methyl (ASM), a functional SA analog, displayed a resistant disease phenotype. The role of SA and ET was investigated through the use of virus induced gene silencing (VIGS) to silence *NbICS1* and *NbEIN2* (SA and ET biosynthetic genes, respectively). These genes were required for resistance in mature plants and for INF1 and NPP1.1-induced ROS production. Additionally,

a role in resistance was proposed for SA-regulated cell death and ET-mediated phytoalexin production (Shibata *et al.*, 2010).

SA may also be involved in cell death in potato leaves infiltrated with Pep-13. Halim *et al.* (2004) were able to demonstrate that *nahG* potato plants were impaired in Pep-13 induced cell death, H_2O_2 production, as well as accumulation of JA. Silencing of JA biosynthesis genes, as well as *Coranatine-Insensitive 1* (*COI1*), resulted in impaired Pep-13 responsive ROS and HR, but SA was still able to accumulate. This indicates that SA and JA act in the same pathway to induce ROS and HR in response to Pep-13, and that SA accumulation is independent or upstream of JA synthesis (Halim *et al.*, 2009). JA-impaired susceptible plants are not affected with regards to their defence to *P. infestans* (Halim *et al.*, 2009), but the same plants containing *nahG* are even more susceptible than wild type. Spraying *nahG* plants with the SA functional analog 2,6-dichloro-isonicotinic acid (INA) was able to restore their resistance to wild type levels. This indicates that SA may be an important component of defence in potato against *P. infestans* (Halim *et al.*, 2007). However, JA probably plays a role in resistance mechanisms as well, since silencing of *Allene Oxide Synthase 2* (*AOS 2*) in a resistant potato cultivar compromised resistance (Pajerowska-Mukhtar *et al.*, 2008).

SA and JA signalling play a role in susceptibility of soybean to *P. sojae*. Using microarray analysis, Moy *et al.* (2004) profiled *P. sojae* responsive soybean genes in a susceptible cultivar. The SA pathway was seen to be activated, with genes such as *PR-1* and transcripts belonging to the phenylpropanoid pathway being upregulated. MeJA on the other hand seemed to be suppressed since lipoxygenase (*LOX*), proteinase inhibitors, and terpenoid metabolism being downregulated. The authors hypothesize that the plant may commit to the SA pathway in order to combat the pathogen in the rapid biotrophic stage (less than 24 hpi), and may not be able to revert to the JA pathway in order to combat it appropriately in its role as a necrotroph. The JA pathway may also be suppressed in a compatible interaction as part of the manipulation by the pathogen (Restrepo *et al.*, 2005). Conversely, Chen *et al.* (2011) hypothesize that suppression of *PR-1*, *PR-2* and *PR-5*, which are induced by SA, may be partially responsible for the spread of the pathogen beyond the site of HR in compatible and partially resistant interactions. Furthermore, induced resistance to *P. sojae* is mediated by SA and ET in young soybean seedlings (Sugano *et al.*, 2013). SA may be important for keeping the pathogen confined and preventing establishment.

Ethylene may play a role in vertical resistance of soybean to *P. sojae*, since resistance of an *etr1-1* mutant was compromised (Hoffman *et al.*, 1999), and the upregulation of *ERF1* at 8 and 24 hpi was noted in a resistant cultivar relative to the levels in the compatible interaction

(Narayanan *et al.*, 2009). Ethephon (ethylene) treated *Capsicum annuum* seedlings possessed enhanced resistance against *P. capsici*, whereas treatment with JA and SA had no effect (Núñez-Pastrana *et al.*, 2011). Additionally, a biphasic ethylene burst was noted in *Fagus sylvatica* (beech trees) in response to infection with *P. citricola* (Portz *et al.*, 2011).

Hormones other than SA, JA and ET may also be involved in resistance. Soil drenches and foliar sprays of DL-3-aminobutyric acid (BABA) suppress disease caused by *P. capsici* on squash (Kone *et al.*, 2009). A foliar spray of BABA on potato, is able to confer protection against *P. infestans* that may last till harvest and even increases levels of antimicrobials such as *β*-1,3-glucanase and phytoalexin in the tubers (Andreu *et al.*, 2006). However, it has been shown that SA is required for BABA mediated resistance, since *nahG* potato plants were unable to activate BABA-induced defences unless they were sprayed with INA, which restored the SA deficient phenotype (Eschen-Lippold *et al.*, 2010).

Defence hormones are able to switch on certain defence-related genes, and these genes may be considered marker genes for the hormone signalling pathways. This often happens through WRKY transcription factors that have a DNA binding domain and are responsible for the transcriptional activation of many *PR* genes (Dong *et al.*, 2003; Ishihama and Yoshioka, 2012; Jayaraj *et al.*, 2004; Pandey and Somssich, 2009). Inoculation with *Phytophthora* results in upregulation of WRKY transcription factors in compatible, as well as incompatible interactions (Beyer *et al.*, 2001; Tian *et al.*, 2006; Wang *et al.*, 2005). Strong upregulation of a potato homolog, *StWRKY1*, was noted in a compatible interaction with *P. infestans* (Dellagi *et al.*, 2000). Numerous *PR* genes are regulated upon challenge with *Phytophthora,* and these are recorded in Table 1.2.

The PR-1 protein family is a marker gene of systemic acquired resistance (SAR), and can be induced to very high levels upon infection, but the exact biological role in defence has not been elucidated for this family yet (Jayaraj *et al.*, 2004; Van Loon *et al.*, 2006). It has been noted previously that PR-1 may be important for defence against oomycetes, and it is known to exhibit antimicrobial properties against *P. infestans* zoospore germination *in vivo* and *in vitro* (Niderman *et al.*, 1995; Van Loon *et al.*, 2006). *PR-1* was found to co-regulated with a WRKY transcription factor in a resistant potato-*P. infestans* interaction (Wang *et al.*, 2005). In four-week old resistant potatoes, non-inoculated plants showed accumulation of *PR-1* relative to non-inoculated susceptible plants, and this may prove to be a resistance mechanism (Ros *et al.*, 2005). Differential regulation of *PR-1* in response to *Phytophthora* spp. has been noted in several other studies as well (Table 1.2). Generally speaking, *PR-1* is

most significantly upregulated in incompatible interactions but there is usually a measure of differential expression in the compatible hosts as well.

The PR-2 protein family consists of *β*-1,3-glucanases (Van Loon *et al.*, 2006), which hydrolyze the *β*-1,3-glucans found in oomycete cell walls, which in turn elicit a PTI response (Boller, 1995; Bowles, 1990). PR-2 activity increases during chitosan-mediated protection of *N. benthamiana* against *P. nicotianae* (Falcón-Rodríguez *et al.*, 2011). *PR-2* is often upregulated in both compatible and incompatible interactions, but it may not be involved in resistance. Porter *et al.* (2009) hypothesize that *P. palmivora* possesses an endoglucanase inhibitor that negates the action of *β*-1,3-glucanases from papaya. Probably the role of PR-2 in defence against *Phytophthora* extends as far as releasing of *β*-1,3-glucans (PAMPs) to trigger PTI.

β-1,3-glucanases and chitinases (PR-3, PR-8 and PR-11) have been reported to act synergistically (Boller, 1993; Ferreira *et al.*, 2007; Hardham *et al.*, 1994; Jach *et al.*, 1995; Stintzi *et al.*, 1993; Van Loon *et al.*, 2006). A double role in plant defence has been proposed for chitinases (Collinge *et al.*, 1993; Kasprzewska, 2003), although this may well extend to *β*-1,3-glucanases as well (Ferreira *et al.*, 2007). The double role involves the action of apoplastic chitinases in breaking down the pathogen cell wall, which in turn releases PAMPs which trigger defence responses. Vacuolar chitinases are then involved in directly inhibiting fungal growth by breaking down newly made chitin chains. Chitinases are often differentially regulated in both compatible and incompatible responses to *Phytophthora* spp. (Table 1.2), but there is not a big overlap between studies with both chitinase and *β*-1,3-glucanase, so possibly the synergism of these two proteins is not so important in oomycete defence. Even so, despite the absence of major amounts of chitin in oomycete cell walls, *StWRKY1* and a class I endochitinase were co-regulated in potato inoculated with *P. infestans*, where they were strongly upregulated in an incompatible interaction, and weakly in the compatible (Dellagi *et al.*, 2000). Resistant taro was also found to express chitinase more highly in response to *P. colocasiae* infection than the susceptible cultivar (Sharma *et al.*, 2009).

PR-4 proteins have chitin-binding properties, which may inhibit fungal growth by binding to cell wall chitin (Ferreira *et al.*, 2007). Some of the proteins in this family may be referred to as “hevein-like” (Ferreira *et al.*, 2007; Van Loon *et al.*, 2006). PR-4 is not detected often as a role-player in defence against *Phytophthora*. There is one study in soybean, however, where *PR-4* transcripts were regulated in a biphasic manner. Expression of *PR-4* was downregulated 1-3 hpi in a susceptible interaction following inoculation with *P. sojae*, then upregulated from 6-12 hpi, and repressed again until 24 hpi. This is in contrast to the

resistant cultivar, which displayed a continuous increase in *PR-4* over the same time frame (Chen *et al.*, 2008). Therefore suppression of PR-4 may be a mechanism involved in susceptibility toward *P. sojae*.

The PR-5 family consists of thaumatin-like proteins and osmotin (Ferreira *et al.*, 2007). They are able to form trans-membrane pores in fungi that produce osmotic stress through the leakage of the cells (Kitajima and Sato, 1999; Roberts and Selitrennikoff, 1990). PR-5 proteins have enhanced activity and are thought to act synergistically with a cell-wall degrading counterpart such as *β*-1,3-glucanase or a chitinase (Lorito *et al.*, 1996). This may be true in *Phytophthora* interactions as well since *PR-5* seems to be expressed along with *PR-2* or *PR-3* in most expression profiling studies (Table 1.2).

The PR-6 protein family consists of proteinase inhibitors, which are generally JA responsive. Interestingly, these are sometimes suppressed upon infection with *Phytophthora*. In a potato microarray study, *PR-6* was down-regulated in both resistant and susceptible cultivars (Ros *et al.*, 2004). Down-regulation of *PR-6* was also the case in a compatible soybean-*P. sojae* interaction (Moy *et al.*, 2004). Tian *et al.* (2006) found that *PR-6* was induced in a potato cultivar with horizontal resistance, but maximal induction was found at 48 hpi, the time point at which the necrotrophic growth phase of *P. infestans* sets in. The HR-inducing elicitin, INF1, induced *PR-6* in tomato leaves (Kawamura *et al.*, 2009). The suppression of *PR-6* in certain interactions, in conjunction with the induction of transcripts in necrosis-related scenarios, make it possible to speculate that proteinase inhibitors are suppressed by *Phytophthora* during the biotrophic phase and that expression of these may either enhance or combat necrosis during infection with *Phytophthora*. However, not enough information is available concerning the regulation of *PR-6* to form a sound hypothesis yet.

An SA-related mechanism of defence employed by the plant is the secretion of various proteases into the apoplast where *P. infestans* is located during its biotrophic phase. These may contribute to defence through “executing defence responses, signalling, and pathogen perception” (van der Hoorn and Jones, 2004). In tomato, serine proteases P69B and P69C respond to pathogens and SA, and are classified as PR-7 proteins (Tian *et al.*, 2004; Van Loon *et al.*, 2006). Additionally, tomato contains papain-like cysteine proteases which are also recognized as PR proteins, namely: *Phytophthora*-inhibited protease (PIP1), RCR3 and C14 (Kaschani *et al.*, 2010; Rooney *et al.*, 2005; Tian *et al.*, 2007). Protease inhibitors from *P. infestans* may target and inhibit these proteases, acting as effectors that suppress host defences during the biotrophic phase of infection. For example, Kazal-like serine protease inhibitors, EPI1 and EPI10, interact with and inhibit P69B. EPI1 and EPI10 from the

pathogen and P69B from tomato are co-regulated during infection and the fact that these two different effectors both target P69B suggests that it may play an important role in host defences (Tian *et al.*, 2005; Tian *et al.*, 2004). Cystatin-like protease inhibitors have been shown to inhibit the papain-like cysteine proteases. PIP1 is inhibited by EPICB2, whereas RCR3 and C14 are both inhibited by EPIC1 and EPIC2B (Kaschani *et al.*, 2010; Song *et al.*, 2009; Tian *et al.*, 2007). C14 is present in tomato and potato, and is under diversifying selection in the natural host (wild potato species), which indicates that it is likely to be an effector target (Kaschani *et al.*, 2010). Silencing of C14 increases susceptibility, and it is expressed by potato during the early stages of a resistant interaction. It is expressed in cultivars with field resistance and vertical resistance in the form of *R* genes, indicating that a common pathway leads to expression (Avrova *et al.*, 1999; Kaschani *et al.*, 2010). Due to the similarity of C14 to animal cathepsin B, it is speculated that this protein may be involved in the onset of PCD during the HR (Avrova *et al.*, 1999). Additionally, it is questioned whether EPI1 may play a role in inhibiting plant caspases (so-called saspases) and preventing PCD (Tian *et al.*, 2004).

PR-10 proteins are not characterized well in terms of their biological role in defence, but have diverse putative functions such as ribonuclease activity (Liu and Ekramoddoullah, 2006), cytokinin binding properties (Fujimoto *et al.*, 1998), or an ability to carry steroids (Markovic-Housley *et al.*, 2003). *PR-10* was upregulated in European beech subsequent to challenge with *P. citricola* (Schlink, 2009), as well as in pepper cells during mycelium-elicited defence (Nakazawa-Ueji *et al.*, 2010). It was consistently upregulated in resistant soybean as opposed to inconsistent and lower levels of induction in the susceptible cultivar, in response to *P. capsici* (Chen *et al.*, 2008). Interestingly, overexpression of an ethylene-induced *PR-10* in a susceptible pepper cultivar confers resistance to *P. capsici* (Núñez-Pastrana *et al.*, 2011).

StPRp27, characterized as a PR-17 protein with unknown function (Van Loon *et al.*, 2006), is responsible for broad-spectrum resistance in potato, and may be important for resistance against *P. infestans.* Transient expression of *StPRp27* in *N. benthamiana* conferred enhanced resistance, and overexpression in potato slowed progression of the disease (Shi *et al.*, 2011). This gene is related to the JA pathway, since it was induced by JA (Tian *et al.*, 2003). *PRp27* is reported as upregulated in two susceptible interactions (Ros *et al.*, 2004, 2005) and one horizontally resistant interaction (Tian *et al.*, 2006).

Table 1.2 *PR* families responsive to *Phytophthora* spp. in various hosts

***PR* family**	**Signalling pathway**	**References**
PR1	SA	(Ali *et al.*, 2012; Attard *et al.*, 2010; Bengtsson *et al.*, 2013; Birch *et al.*, 1999; Gyetvai *et al.*, 2012; Lim *et al.*, 2013; Moushib *et al.*, 2013; Moy *et al.*, 2004; Núñez-Pastrana *et al.*, 2011; Ros *et al.*, 2004, 2005; Schlink, 2009; Silvar *et al.*, 2008; Tian *et al.*, 2006; Wang *et al.*, 2005)
PR2	SA	(Beyer *et al.*, 2001; Falcón-Rodríguez *et al.*, 2011; Gyetvai *et al.*, 2012; Lim *et al.*, 2013; Moushib *et al.*, 2013; Porter *et al.*, 2009; Restrepo *et al.*, 2005; Ros *et al.*, 2004, 2005; Schlink, 2009; Silvar *et al.*, 2008)
PR3	JA	(Attard *et al.*, 2010; Beyer *et al.*, 2001; Birch *et al.*, 1999; Dellagi *et al.*, 2000; Gyetvai *et al.*, 2012; Lim *et al.*, 2013; Moy *et al.*, 2004; Orłowska *et al.*, 2012; Restrepo *et al.*, 2005; Ronning *et al.*, 2003; Ros *et al.*, 2004; Schlink, 2009; Sharma *et al.*, 2009; Tian *et al.*, 2006)
PR4	JA	(Attard *et al.*, 2010; Chen *et al.*, 2008; Lim *et al.*, 2013; Wang *et al.*, 2005)
PR5	SA	(Gyetvai *et al.*, 2012; Lim *et al.*, 2013; Orłowska *et al.*, 2012; Restrepo *et al.*, 2005; Ros *et al.*, 2004, 2005; Schlink, 2009; Tian *et al.*, 2006; Trognitz *et al.*, 2002; Wang *et al.*, 2005)
PR6	JA	(Kawamura *et al.*, 2009; Moy *et al.*, 2004; Nakazawa-Ueji *et al.*, 2010; Ros *et al.*, 2004; Schlink, 2009; Tian *et al.*, 2006)
PR7	SA	(Tian *et al.*, 2004; Tian *et al.*, 2007; Tian *et al.*, 2006)
PR10	ET	(Chen *et al.*, 2008; Nakazawa-Ueji *et al.*, 2010; Núñez-Pastrana *et al.*, 2011; Schlink, 2009)
PR12 (PDF1.2)	JA	(Attard *et al.*, 2010; Eshraghi *et al.*, 2013)
PR17	JA	(Gyetvai *et al.*, 2012; Ros *et al.*, 2004, 2005; Shi *et al.*, 2011; Tian *et al.*, 2006; Tian *et al.*, 2003)

The presence of *PR* genes linked to all the signalling pathways may be attributed to the hemibiotrophic nature of the pathogen (Schlink, 2009). Currently, there is no clear model of hormone signalling in response to *Phytophthora*. There has emerged sufficient evidence though, to hypothesize that *Phytophthora* spp. are able to manipulate their hosts during compatible interactions, so that conditions and defence signalling in the host suits the pathogen's needs at a particular time. Therefore, it is often not the defence responses in the host as such that provide protection, but rather the timing and extent or defence regulation, and the host's ability to recognize manipulation of host targets by the pathogen.

1.5.2.5 Phenylpropanoid pathway and phytoalexins

The phenylpropanoid pathway plays a key role in production of secondary metabolites in plants, and compounds produced by this pathway play an important role in defence. The enzyme L-phenylalanine ammonia lyase (PAL) is the entry point enzyme in the phenylpropanoid pathway, and may be responsible for flux control (Blount *et al.*, 2000; Dixon *et al.*, 2002). Along with PAL, cinnamate 4-hydroxylase (C4H) and 4-coumarate:coenzyme A ligase (4CL) form the common pathway for production of hydroxycinnamic acid (Naoumkina *et al.*, 2010). From this point the pathway diverges for the production of various compounds such as flavonoids, coumarins, isoflavonoids, stilbenes and monolignols (Vogt, 2010).

One of the main branches of the phenylpropanoid pathway involved in defence is that of monolignol production, which includes lignins and lignans. Challenge with a pathogen may induce lignification of the cell wall through the oxidative polymerization of monolignols by laccases or peroxidases (Gabaldón *et al.*, 2006; Naoumkina *et al.*, 2010; Sato and Whetten, 2006). Peroxidases are an important class of inducible defence proteins and are classified as PR-9 proteins (Van Loon *et al.*, 2006). They are not only involved in lignin formation, but may also influence defence through formation of suberin, cross-linking of cell wall components and phytoalexin synthesis (Almagro *et al.*, 2009).

Phytoalexins and phytoanticipans are antimicrobial compounds synthesized upon infection, or present before infection, respectively (Dixon, 2001; VanEtten *et al.*, 1994). Many phytoalexins belong to one of the classes of phenolic or phenylpropanoid compounds, such as glyceollin from soybean. However, other phytoalexins may belong to the terpenoids, such as the sesquiterpene capsidiol (found in the Solanaceae) or the sulphur and nitrogen containing indole compound, camalexin, found in the Brassicaceae (including *A. thaliana*) (Ahuja *et al.*, 2011; Dixon, 2001). Camalexin is well-studied, since it is present in *A. thaliana*. Camalexin synthesis is mediated through SA, JA and ET, but can also be regulated without the presence of these hormones. The manner in which this camalexin synthesis is regulated is dependent on the specific pathogen that *A. thaliana* is challenged with (Ahuja *et al.*, 2011).

PAL, as well as other enzymes in the phenylpropanoid pathway are implicated in resistance mechanisms to *P. infestans.* The chemically induced resistance, produced by BABA or fosetyl-aliminium, against *P. infestans* in potato produces increased levels of phenols and phytoalexin in post-harvest tubers, which also have a measure of protection against the late blight pathogen (Andreu *et al.*, 2006). *PAL*, chalcone isomerase (*CHI*), chalcone synthase (*CHS*), chalcone reductase (*CHR*) and peroxidase (*PRX*) are differentially regulated in various studies profiling gene expression of potato upon challenge with *P. infestans* (Beyer

et al., 2001; Ros et al., 2004, 2005; Tian et al., 2006; Wang et al., 2005) and linked to QTL for resistance (Trognitz *et al.*, 2002). The induction of genes encoding these enzymes, as well as genes encoding a certain cytochrome P450 enzyme and γ-glutamylcysteine synthase, may be present in order to increase production of phytoalexins (Beyer *et al.*, 2001; Shibata *et al.*, 2010; Tian *et al.*, 2006; Trognitz *et al.*, 2002). Additionally, polyphenol oxidases (PPOs), which are able to oxidase phenols to toxic quinones (Li and Steffens, 2002), were differentially regulated in three studies (Ros *et al.*, 2004, 2005; Tian *et al.*, 2006).

PPOs seem to play an important role in resistance against *Phytophthora.* The activity of PPO and PRX, as well as the accumulation of phenols, lignin and callose, is positively correlated to *P. infestans* resistance of a wild relative of potato, *S. tarijense* (Korgan *et al.*, 2011). Resistance to blight caused by *P. colocasiae* in *Colocasia esculenta* (taro), is correlated with the accumulation of phenolics and PPO during infection. The ability of the PPOs which are present in the chloroplast to oxidise pre-formed phenols from the vacuole when subcellular compartments are disrupted, may be a resistance mechanism (Sahoo *et al.*, 2009). *PRX* may also play a role in *P. colocasiae*-taro interactions, since it is upregulated in a resistant cultivar relative to the compatible interaction (Sharma *et al.*, 2009).

In pepper plants, the phenylpropanoid pathway and related enzymes also play a central role in defence. *PRX* and sesquiterpene synthase levels were profiled in three pepper cultivars subsequent to infection. Expression levels of these two genes were much higher in the resistant cultivar, though they were upregulated in the susceptible cultivar as well (Silvar *et al.*, 2008). Additionally, *Fusarium oxysporum*-primed pepper plants possess resistance against *P. capsici*. Increased transcript levels of *PRX* and sesquiterpene synthase in leaves, stem and root tissue may play a role in this resistance. Sesquiterpene synthase was upregulated the most dramatically in root tissue (Silvar *et al.*, 2009), which is probably to be expected, considering that *P. capsici* is normally a root pathogen. Koį *et al.* (2011) evaluated three pepper cultivars with varying resistance levels for their response to *P. capsici*. Interestingly, PAL activity was highest in the non-infected leaves of the most resistant cultivar, so it can be inferred that resistance may be mediated by PAL that is present prior to inoculation, not only due to induction after challenge. PAL activity was higher in resistant plants than susceptible, and it is hypothesized that suppression of PAL may be a contributing factor to infection in the compatible interaction.

Phenolics are found to be involved in the model plant *A. thaliana*, where one of the ways that potassium phosphonate treatment prevents infection by *P. palmivora*, is through phenolic

compound accumulation around infected root cells (Daniel and Guest, 2006). In non-model interactions, the phenylpropanoid pathway is also found to play an important role. PAL, CHS and P450 trans-cinnamic 4-monooxygenase are necessary for race-specific resistance to *P. clandestine* in clover. Subsequent to inoculation, incompatible interactions show a rapid increase in mRNA transcripts encoding these enzymes, relative to no differential expression in the compatible interaction (Ma *et al.*, 2010).

One of the most prominent components of plant defence against *Phytophthora* is phytoalexin production. Evidence for this is found in the model systems of tobacco and *A. thaliana*, as well as the well studied *P. sojae* interaction with soybean. Silencing of *N. benthamiana* 5-*Epi*-aristolochene dihydroxylase (*NbEAH*) and 5-*Epi*-aristolochene synthase (*NbEAS*), required for the production of capsidiol in *N. benthamiana*, resulted in reduced resistance to *P. infestans* (Shibata *et al.*, 2010). Challenge of cell suspension cultures of *N. benthamiana* with *P. nicotianae* resulted in superoxide-dependant production of capsidiol in incompatible interactions. The non-host interaction with *P. palmivora* yielded the same results, except that capsidiol production was even faster following inoculation (Perrone *et al.*, 2003). A decrease in infection of *N. benthamiana* by *P. nicotianae* was noted subsequent to spraying with chitosan, and this protection was correlated with increased PAL activity (Falcón-Rodríguez *et al.*, 2011), which may be involved in phytoalexin production.

The production of the isoflavonoid phytoalexin, glyceollin, is important for resistance of soybean to *P. sojae*. Silencing of *CHR* or isoflavone synthase, which are involved in production of 5-deoxyflavonoids (specifically glyceollin), resulted in loss of resistance and cell death induced by the pathogen and PAMPs. This suggested that resistance and PCD is mediated by isoflavones (Graham *et al.*, 2007; Subramanian *et al.*, 2005). Additionally, susceptibility mediated by abscisic acid (ABA) in soybean is correlated with a decrease in PAL activity and transcript expression, as well as a large decrease in glyceollin (Ward *et al.*, 1989). The role of glyceollin-mediated resistance was also investigated by Mithofer *et al.* (2001) and it was shown that the Ca^{2+} influx induced by *β*-glucan is required for the phytoalexin's production (Mithöfer *et al.*, 2001), though H_2O_2 is not (Mithöfer *et al.*, 1997). Support for the central role of glyceollin in soybean defence against *P. sojae* is found in a microarray study by Moy *et al.* (2004). Other than an upregulated *PPO* and *PRX*, phenylpropanoid related genes encoding phytoalexin production were found to be strongly upregulated. These included *CHS* and *CHR*, isoflavone synthase and reductase, caffeoyl-CoA O-methyltransferase, 2'-hydroxydihydrodaidzein reductase, and *PAL*. In potato, preformed flavonoids were are shown to be involved in resistance to *P. infestans* (Henriquez *et al.*, 2012).

Roetschi *et al.* (2001) found the *A. thaliana pad2* mutant to be hypersusceptible to *P. porri*. This mutant blocks production of camalexin partially, as opposed to complete blockage in a *pad3* mutant (Glazebrook and Ausubel, 1994). However, since resistance in the *pad3* mutant was not affected, the authors hypothesized that resistance is not due to a requirement for camalexin. Surprisingly, a follow up study proved that camalexin is indeed required for resistance, but in conjunction with the phytoanticipin indole glucosinolate (iGS). A series of mutant studies revealed that the hypersusceptibility of the *pad2* mutant is as a result of a loss of iGS in conjunction with reduced camalexin levels. The role of both iGS and camalexin is supported by *in vitro* inhibition of *P. brassicae* by these compounds, as well as synchronized increases in transcript levels for enzymes involved in their synthesis. Therefore, the current hypothesis is that the phytoanticipin iGS "buys time" for the activation of defence, with newly produced camalexin exerting its antimicrobial activity later, and therefore both are necessary for resistance (Schlaeppi *et al.*, 2010). Recently, the role of iGS and camalexin in *Phytophthora* resistance in *A. thaliana* was confirmed when *pad2*, *pad3*, and *pad4* mutants were proved susceptible to *P. capsici* (Wang et al., 2013).

1.5.2.6 Cytoplasmic aggregation responses

A very rapid host response to appressorium formation by *Phytophthora,* is the aggregration of cytoplasmic components beneath the appressorium or next to hyphae. Actin microfilaments are rearranged so as to be concentrated at the site of penetration and organelles such as endoplasmic reticulum (ER), Golgi bodies and peroxisomes also become localized near the site of penetration (Daniel and Guest, 2006; Gross *et al.*, 1993; Takemoto *et al.*, 2003). Because of this aggregration, materials such as callose and phenolics that are involved in wall appositions are secreted at the site where they are needed (Fellbrich *et al.*, 2002; Soylu and Soylu, 2003; Soylu, 2004). Cell wall appositions are important for formation of physical and chemical barriers that prevent penetration by the pathogen. This is evident in Port-Orford-Cedar root-and stem cells, which respond to challenge with *P. lateralis* by forming cell wall appositions, thickening cell walls and accumulating osmophillic granules and crystals around the hyphae and in cell walls (Oh and Hansen, 2007). Various compounds are used to defend against invading hyphae. Cell wall appositions contain a variety of chemicals such as callose, phenolics, silicon, H_2O_2, PRX and enzyme inhibitors (Aist, 1976; Schmelzer, 2002; Zeyen *et al.*, 2002). PR proteins, phytoalexins and toxins are secreted in addition to the aggregation and apposition response, forming the basal resistance which is capable of preventing any further disease development in non-host interactions (Hardham, 2007). These responses to *Phytophthora* infection are reviewed in detail by Hardham and Shan (2009).

1.6. Host responses to *P. cinnamomi*

1.6.1 *A. thaliana*

Screening numerous *A. thaliana* ecotypes against *P. cinnamomi* showed that the ecotypes exhibit a range of varying degrees of susceptibility. However, *P. cinnamomi* was able to sporulate on the leaves and roots of all the ecotypes. H_2O_2 production, cell death, and restricted lesions were observed to be correlated with resistance. The least susceptible ecotypes were found to possess increased tolerance at older ages, whereas the most susceptible did not vary in resistance at different ages (Robinson and Cahill, 2003). This is similar to a finding in potato where age-related resistance was present in resistant cultivars (Ros *et al.*, 2004).

Susceptible *A. thaliana* seedlings (accession Ler), are primed for defence to *P. cinnamomi* by phosphite to such an extent that lesion size decreases drastically, and defence responses become similar to those observed in the non-host Col-0. Prior to inoculation, phosphite was able to induce transcription of SA-responsive genes *PR-1* and *PR-5*, as well as JA marker genes thionin (*THI2.1*) and plant defensin (*PDF1.2*). It also boosted H_2O_2 production at the site of penetration by the pathogen. Callose deposition was augmented in phosphite primed plants, and timing of the callose response was six hours earlier than non-phosphite treated plants. Therefore a phosphite-induced change in timing and magnitude of certain defence mechanisms was able to convert a compatible interaction to incompatible (Eshraghi *et al.*, 2011).

Other defence mechanisms than those profiled by Eshraghi *et al.* (2011) may be required for successful defence against *P. cinnamomi* in *A. thaliana*. Rookes *et al.* (2008) compared the role of various defence pathways and genes in leaves and roots. *P. cinnamomi* is able to reproduce asexually on the roots of Col-0, but not in the leaves. In the leaves, induced expression of *PDF1.2, PR-1* as well as glutathione-S transferase 1 (*GST1*), an oxidative burst marker gene, was present. *PDF1.2* and *PR-1* induction was not detected in the roots, but *GST1* was, though it was induced to a lesser degree than in the leaves. Thirteen defence mutant/overexpression lines of Col-0 were tested for increased susceptibility, but there was no difference in disease phenotype compared to their wild type counterparts. The JA, SA and ET signalling mutants were therefore not impaired in resistance, indicating that there are other resistance mechanisms in play in this non-host interaction. Another study on Col-0 confirmed that resistance is not impaired in JA/ET mutants, but rather that ABA signalling could be a partial resistance mechanism in this ecotype (Eshraghi *et al.*, 2013).

1.6.2 *Persea americana* (Avocado)

Using proteomics, sixteen proteins were found to be more abundant in an infected tolerant avocado cultivar's roots, as opposed to the non-inoculated control. Two *GST*s, which may be involved in ROS, were identified, but the majority of the identified proteins are involved in monolignol and isoflavonoid biosynthesis which form part of the phenylpropanoid pathway (Acosta-Muñiz *et al.*, 2012). Therefore, phytoalexin production and cell wall strengthening may play an important role in the tolerance displayed by this cultivar.

Garcia-Pineda *et al.* (2010) investigated the role of certain defence mechanisms in the roots of a susceptible avocado rootstock. Inoculation with *P. cinnamomi* resulted in an oxidative burst involving H_2O_2, as well as a sharp increase in nitric oxide (NO). Both of these are involved in mediating HR. Furthermore, PRX activity was found to increase, and since this did not lead to an increase in lignin, it may contribute to H_2O_2 accumulation. There was also an observed decrease in epicatechin, a flavonoid potentially involved in scavenging ROS. Cumulatively, this data led the authors to speculate that an increase in ROS, as well as decreased ability to scavenge these molecules, may contribute to susceptibility by enhancing HR-related cell death, and thereby creating a favourable environment for *P. cinnamomi* to colonize during its necrotrophic phase of infection (which is by far the largest part of its lifestyle). This is in accordance with Koj *et al.* (2010), who mentioned that protection against ROS is required for resistance in pepper plants infected by *P. capsici*. Therefore correct regulation of ROS is an important component of host defences.

ROS related defence mechanisms were also detected in an EST sequencing study involving a tolerant avocado rootstock challenged with *P. cinnamomi* mycelia (Mahomed and Van den Berg, 2011). Cytochrome P450-like TBP, which plays a role in ROS and HR, was induced along with a metallothionein, which acts as a ROS scavenger. Genes potentially involved in actin polarization, *mlo* and *profilin*-like, were detected, and may be involved in the cytoskeletal rearrangements that occur upon infection with *Phytophthora*. *PsemI*, a PR-10 protein, as well as thaumatin (PR-5) were detected. Furthermore, two genes involved in the core part of the phenylpropanoid pathway, namely *4-CL* and *C4H* were detected, as well as an oxysterol-binding protein and an aquaporin (Mahomed and Van den Berg, 2011). It is interesting to note the upregulation of aquaporin in this study. An aquaporin was downregulated in both soybean, potato and papaya following inoculation with *P. sojae, P. infestans* and *P. palmivora* respectively (Moy *et al.*, 2004; Porter *et al.*, 2009; Restrepo *et al.*, 2005). Porter *et al.* (2009) hypothesize that this aquaporin may be a cross-species effector target of *Phytophthora*, since it is suppressed in three different compatible pathosystems. The upregulation of aquaporin in this study, may therefore contribute to the tolerance

displayed. The regulation of oxysterol-binding protein was previously noted in a suppression subtractive hybridization (SSH) study by Avrova *et al.* (2004), and they speculate that this may regulate the availability of oxysterols to *Phytophthora*, which cannot make its own sterols, and therefore utilizes plant sterols through the secretion of sterol-binding elicitins (Hendrix, 1970).

1.6.3 *Eucalyptus* spp.

In another tree host of *P. cinnamomi*, *Euclayptus,* several studies have been conducted, but the majority with regards to molecular defence were conducted approximately two decades ago. Reduction of two types of cytokinins was observed in the susceptible *E. marginata* as little as three days after inoculation with *P. cinnamomi*. Field resistant *E. calophylla* did not possess any changes in cytokinin levels. It is hypothesized that the changes in cytokinins may be related to disturbances in ABA balance, thereby explaining the water-stress of the trees early on in infection despite little root damage detected at that stage (Cahill *et al.*, 1986). It is now known that ABA may mediate susceptibility in plants, and also that cytokinins are related to the SA signalling pathway (Asselbergh *et al.*, 2007; Robert-Seilaniantz *et al.*, 2011; Ton and Mauch-Mani, 2004).

The activation of the phenylpropanoid pathway is a clearly recurring theme in the response of plants to challenge with various *Phytophthora*s. There is support for this in *P. cinnamomi* infected *Eucalyptus* roots as well. Field resistant *E. calophylla* exhibited an increase in lignin, PAL activity and soluble phenolics, whereas *E. marginata* displayed hardly any change in the levels of these compounds. By blocking PAL with an inhibitor, a disease phenotype similar to that of the susceptible *E. marginata* was observed (Cahill and McComb, 1992). The role of phenolics was confirmed in a follow up study, where selected *E. marginata* seedlings displaying resistance traits were found to have similar defence mechanisms: PAL activity, lignin and phenolic compounds increased upon inoculation (Cahill *et al.*, 1993).

1.6.4 Other *P. cinnamomi* hosts

Lupinus angustifolius (blue lupin), a susceptible host of *P. cinnamomi* produced an isoflavonoid and saponin upon inoculation. Application of phosphite induced resistance, and though the same metabolites were produced, the concentrations varied (Gunning *et al.*, 2013). In maize, a resistant monocot, two phytoalexin terpenoids, as well as JA and ET biosynthesis were up-regulated upon inoculation and are thereby implicated in resistance (Allardyce *et al.*, 2013).

Phenolic secondary metabolites were found to be important in chestnut infected by *P. cinnamomi*, where a large increase in phenol content was observed in leaves of resistant trees after inoculation, and there was no change in the phenol content of susceptible trees (Dinis *et al.*, 2011). In oak trees, *P. cinnamomi* inoculation results in decreased water content, net photosynthesis and stomatal conductance, but treatment with the elicitins cryptogein or capsicein negate these effects and induce resistance to the pathogen (Medeira *et al.*, 2012; Sghaier-Hammami *et al.*, 2013).

1.7 Summary and recent advances

The specific responses of plants to various *Phytophthora* spp. have been examined in this review. These defence mechanisms correlate well with what is known regarding plant defence already. The most critical components of defence in these interactions seem to be correct timing of the oxidative burst and HR-related cell death, as well as the production of phenolic compounds. Phytoalexins especially seem to play an important role in resistance against *Phytophthora,* something which was also noted over a decade ago by Erwin and Ribeiro (1996). No specific role has been elucidated for the defence hormones JA, ET and SA yet. A general model depicting the elements of *Phytophthora*-host interactions has been compiled (Figure 1.2). Though it is useful to find common responses between pathosystems, each pathosystem needs to be characterized individually, in order to find the defence responses that can be selected for or manipulated to improve yield of the crop. For example, the *Phytophtora-Eucalyptus* pathosystem seems to involve the regulation of phenolic compounds, which is something generally noted in the other *Phytophthora* hosts as well. However, something that seems to be more specific to this system is the early water stress, related to ABA disturbance during infection.

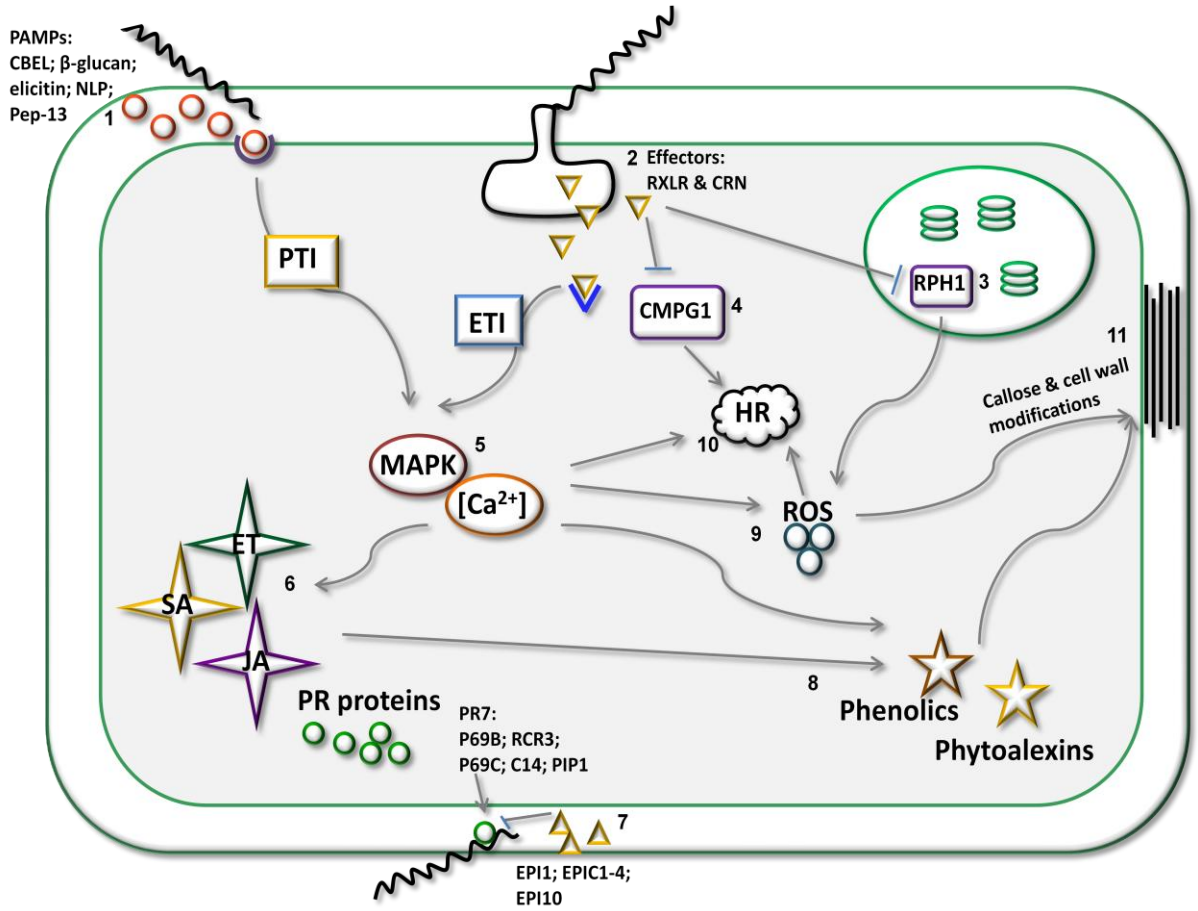

Figure 1.2 Model of defence responses involved in various *Phytophthora*-plant interactions. [1]Various *Phytophthora* PAMPs interact with PRR's in the plasma membrane (Hein *et al.*, 2009) to trigger PTI, while [2]effectors are secreted from the haustorium into the cell (Haas *et al.*, 2009) to manipulate host targets such as RPH1[3] in the chloroplast (Belhaj *et al.*, 2009) and CMPG1, a positive regulator of PCD[4] (Gilroy *et al.*, 2011). When effectors are detected by host *R* proteins, ETI results. Both PTI and ETI trigger [5]MAPK and [Ca^{2+}] signalling cascades (Daxberger *et al.*, 2007; Mithöfer *et al.*, 2001) and downstream defence responses through [6]hormone signalling and PR protein production (Attard *et al.*, 2010; Halim *et al.*, 2007; Moy *et al.*, 2004; Ros *et al.*, 2004). [7]PR-7 proteins are inhibited by apoplastic effectors from *Phytophthora* (Tian *et al.*, 2007). ETI and PTI also result in [8]activation of the phenylpropanoid pathway (Cahill *et al.*, 1993; Schlaeppi *et al.*, 2010), [9]ROS (Chen *et al.*, 2008) and [10]HR (Vleeshouwers *et al.*, 2000). [11]Callose and cell wall modifications are formed through deposition of monolignols and cross-linking of cell wall components (Hardham and Shan, 2009).

Study of the host responses to *Phytophthora* spp. has advanced greatly in the last decade, with microarray profiling playing a large role in this progress. However, there are only a handful of publications describing molecular defence mechanisms for most *Phytophthora* pathosystems, despite the economic impact of many of these species. Certain defence responses are repeatedly shown to be important, but consistent study in a model *Phytophthora*-host pathosystem is required to truly elucidate the finer mechanisms of the

interaction. *A. thaliana* would be particularly useful to elucidate the role of defence hormones and PR proteins in response to *Phytophthora*, since there is currently no clear model or hypothesis concerning this, and much is known about these defences in *A. thaliana*. Importantly, a consensus is required in the research community regarding which *Phytophthora* spp. should form a model pathosystem with *A. thaliana*. Several species have been studied in conjunction with this model plant, but there has been little continuity thus far.

Though *A. thaliana* is exceedingly useful to form basic hypotheses, certain mechanisms pertaining to defence remain specific to the host-pathogen combination. The lowered costs of NGS and the high-throughput data that can be obtained from it for model and non-model systems alike, is likely to accelerate what is known of molecular defence mechanisms in various *Phytophthora*-plant interactions. Already, the host responses of potato and raspberry upon challenge with *Phytophthora* spp. have been profiled using RNA-seq (Gao *et al.*, 2013; Ward and Weber, 2012). Integration of high throughput expression profiling data with proteomics and metabolomics will be essential in order to validate the hypotheses formed from RNA-level analyses.

During investigation of defence, the study of incompatible pathosystems may provide an idea of mechanisms that prevent successful infection. Even so, they do not provide information with regards to what actually causes disease establishment. Valuable information can be gleaned from the study of a compatible interaction. Various attempts at curbing pathogen invasion by the host are present, and thereby potential defence mechanisms can be identified. These mechanisms have the potential to be effective, but are normally just “too little, too late” in the susceptible host. Tao *et al.* (2003) did large-scale expression profiling to determine the differences between compatible and incompatible interactions. They found that the responses are very similar, but that the amplitude of the compatible interaction’s responses is low compared to the incompatible interaction in the early stages of infection. After a delay, the compatible interaction shows a marked increase in response amplitude. In a review comparing differences between compatible and incompatible interactions in the Solanaceae, Desender *et al.* (2007) also concludes that host defence responses in compatible systems occur at lower levels and later than in the incompatible counterparts. This widely accepted point of view has been confirmed in a *Phytophthora* pathosystem as well (Gao *et al.*, 2013).

The overlap between compatible and incompatible pathosystems and usefulness of studying a compatible interaction is further highlighted in a study involving defence responses of *A. thaliana* and the oomycete *H. arabidopsis*. More than 97% of the upregulated and

downregulated genes were regulated in the same direction in both compatible and incompatible interactions (Huibers *et al.*, 2009). Most importantly, a set of 'compatible specific' genes were regulated only in the susceptible interaction. These genes could be manipulation targets of pathogen effectors. In compatible pathosystems, downregulated genes could be an indication of suppression by the pathogen (Porter *et al.*, 2009; Restrepo *et al.*, 2005).

Rapid advances have been made recently with regards to understanding the way *Phytophthora* effectors interact with host targets. A host protein, BSU-LIKE PROTEIN 1 (BLS1) is a putative phosphatase and mediates the recognition of *P. infestans* effector AVR2 and the *Solanum demissum* resistance protein R2 (Saunders *et al.*, 2012). Subcellular localization is important subsequent to effector recognition. After recognizing AVR3a, potato resistance protein R3a relocalizes from the cytoplasm to endosomes and triggers HR (Engelhardt *et al.*, 2012). Another *P. infestans* effector, Pi03192, interacts with two NAC transcription factors at the endoplasmic reticulum membrane, to prevent their re-localization to the nucleus during PTI (McLellan *et al.*, 2013). There is evidence that *Phytophthora* may modulate auxin levels, since *P. parasitica* Penetration Specific Effector 1 (PSE1) disturbs developmental processes and results in lowered auxin levels at root tips (Evangelisti *et al.*, 2013). Additionally, some 21-nucleotide small RNA's found in *Phytophthora* genomes are located in CRN effector genes and are predicted to target some amino acid/auxin permeases (Fahlgren *et al.*, 2013). Two *P. sojae* effectors also manipulate the MAPK signalling pathway. Effector Avh331 suppressed MAPK-based defences in *A. thaliana* in response to *P. capsici* elicitors (Cheng *et al.*, 2012), and effector Avh241 requires two MAPK proteins in order to induce cell death (Yu *et al.*, 2012). A high-throughput pipeline using effectors to find interacting host proteins and determine subcellular localization of effectors has been proposed (Pais *et al.*, 2013).

Other than finding possible effector targets, another outcome of studying a compatible interaction is information regarding indirect effects of infection in the host. This includes changes in photosynthesis, respiratory metabolism, water stress and growth processes (Agrios, 2005). Knowledge of the disruption of these components of plant physiology assists in understanding factors involved in the disease phenotype caused by a particular pathogen.

The interaction between *Eucalyptus* and *Phytophthora* has not received much attention in terms of gene expression profiling upon infection. Yet, *Phytophthora* root rot is an important disease of eucalypts. This study aimed to elucidate the mechanisms involved in attempted defence by a susceptible *Eucalyptus* host upon challenge with *Phytophthora cinnamomi*.

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Chapter 2

Establishment of a pathosystem between *Eucalyptus* and *Phytophthora*

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I conducted all the laboratory work and analysis contained in this chapter (except for technical assistance acknowledged at the end of the chapter) and was responsible for writing the chapter. Noëlani van den Berg gave valuable insights on different *Phytophthora* inoculation methods and provided field trip opportunities to find *Phytophthora* isolates. Alexander A. Myburg provided practical insights in critical decisions during the course of the project. Sanushka Naidoo was the main supervisor for the project. Noëlani van den Berg and Sanushka Naidoo critically reviewed the chapter.

2.1 Abstract

Certain commercially cultivated cold-tolerant eucalypts in South Africa are susceptible to Phytophthora root rot (PRR) and the study of genetic mechanisms responding to infection could provide insight into resistance factors. In order to profile *Eucalyptus* host responses upon challenge with *Phytophthora*, an effective compatible pathosystem is required. In particular, the pathosystem should provide enough tissue that is suitable for large-scale RNA-extractions, and progression of the disease needs to be consistent and predictable. With this end in mind, this study aimed to establish a pathosystem with a susceptible host and virulent *Phytopthora* spp. During the search for a virulent *P. cinnamomi* isolate from infected trees in the field, several *Phytophthora* isolates were found on some young *Eucalyptus* trees in the Tzaneen area, Limpopo, South Africa. The regions encoding the internal transcribed spacer (ITS), β-tubulin (β-*tub*), translation elongation factor 1 alpha (*EF*-1α), NADH dehydrogenase subunit 1 (*nadh*1) and cytochrome c oxidase subunit 1 (*cox*I) of these *Phytophthora* isolates were sequenced and analyzed using parsimony, maximum likelihood, and Bayesian inference based phylogenetic methods for species identification. Identification at species level was not possible, but the isolates were confirmed to be closely related to the *P. parsiana* species complex which belongs to clade 9. Three isolates were selected for virulence screening on *E. nitens*, and two of these were used to compare virulence on a tolerant (*E. grandis*) and susceptible (*E. nitens*) host at standard (25°C) and elevated (30°C) temperatures. Though the elevated temperature enabled increased virulence of these isolates, comparison with *P. cinnamomi* proved that *P. cinnamomi* remains one of the most virulent *Phytophthora's* in South Africa. As a result, *P. cinnamomi* was chosen as a virulent pathogen for inoculation on the susceptible *E. nitens*. Using stem inoculations, the progression of lesion development over a short time-course was documented, and confocal microscopy confirmed the presence of hyphae within host stem tissue as early as 48 hpi. *E. nitens* stem tissue harvested four days after inoculation with *P. cinnamomi* hyphae should be suitable material for RNA-seq profiling of host responses in order to identify possible host defence mechanisms and causes of susceptibility.

2.2 Introduction

Phytophthora cinnamomi has affected native vegetation in the south-western parts of Australia for several decades (Cahill *et al.*, 2008; Podger *et al.*, 1965). Numerous scientific studies have stemmed from this epidemic, including extensive research involving the native genus, *Eucalyptus* (Shearer and Smith, 2000). Susceptible eucalypts display die-back as well as root- and collar rot when infected with *Phytophthora*, and in South Africa this disease threatens certain commercially cultivated species (Linde *et al.*, 1994a; Linde *et al.*, 1994b; Maseko *et al.*, 2007). A pathosystem is classically defined as a "disease triangle," which is composed of a virulent pathogen, a susceptible host and favourable environmental conditions (Agrios, 2005; Robinson, 1976). Accordingly, South African-based research on host responses of *Eucalyptus* to *Phytophthora* requires a reliable pathosystem composed of a virulent *Phytophthora* isolate and a susceptible *Eucalyptus* host that responds predictably to infection under certain conditions. For research of Phytophthora root rot (PRR) in the South African context, the *Eucalyptus* species utilized should be easily accessible and routinely used by forestry companies to ensure relevance and availability of the material for ongoing work. However, the commonly studied susceptible *Eucalyptus* species, such as *E. marginata* (Shearer and Smith, 2000), are not routinely cultivated for South African forestry. Additionally, the *Phytophthora* species chosen should be present locally to avoid complications with quarantine, and the isolate chosen should be highly virulent for optimal results.

A handful of studies, mostly focused on the diversity and distribution of *Phytophthora*, have been conducted on the *Eucalyptus-Phytophthora* interaction in South Africa. Maseko *et al.* (2007) and Linde *et al.* (1994b) investigated the presence of *Phytophthora* spp. in diseased *Eucalyptus* plantations in different areas of the country. *P. nicotianae*, *P. cinnamomi* and *P. boehmeriae* isolates were collected and *P. frigida* and *P. alticola* were newly described (Maseko *et al.*, 2007). *P. nicotianae* is highly prevalent in South African plantations (Maseko *et al.*, 2001), but *P. cinnamomi* remains one of the most virulent species on *Eucalyptus* (Linde *et al.*, 1994b; Maseko *et al.*, 2007). Isolates from the study by Maseko *et al.* (2007) were deposited in the Forestry and Agricultural Biotechnology Institute (FABI) culture collection based at the University of Pretoria, and are therefore accessible to South African researchers. However, this was a decade ago and there is reason to believe that cultures could be less pathogenic when stored *in vitro* over a long time (Linde *et al.*, 1999). These older isolates could be "revived" by inoculating a host and using a re-isolate for subsequent infection trials. However, the ideal is to obtain a more recent isolate from infected trees in the field.

PRR in South African plantations is avoided by planting resistant clones such as *E. grandis* or hybrids of resistant species (Wingfield and Knox-Davies, 1980). However, certain high-altitude

areas are prone to frost and cold spells in winter, and require planting of cold-tolerant eucalypts (Gardner and Swain, 1996; Swain and Gardner, 2003). These include species such as *E. nitens, E. smithii, E. dunnii* (subgenus *Symphyomyrtus*) and *E. fastigata, E. fraxinoides, E. elata* (subgenus *Monocalyptus*) (Pryor and Johnson, 1971). In a comparison of these subgenera, Brown (1977) concluded that susceptible *Monocalyptus* species were highly sensitive to *P. cinnamomi*, whereas *Symphyomyrtus* species were less sensitive to *P. cinnamomi* and are therefore generally regarded as more resistant (Shearer and Smith, 2000). This is reflected in South African *Eucalyptus* plantations, since cultivation of cold-tolerant species belonging to *Monocalyptus* has been discontinued due to severe PRR problems (Clarke and Jones, 1998; Linde *et al.*, 1994a). However, PRR prevails on trees belonging to the *Symphyomyrtes*, since various *Phytophthora* spp., including *P. cinnamomi*, have been isolated from *E. smithii*, *E. nitens, E. dunnii* and other species belonging to this subgenus (Linde *et al.*, 1994b; Maseko, 2010; Maseko *et al.*, 2007). Strangely, *E. fastigata*, *E. fraxinoides* and *E. smithii* are all regarded as resistant in their native environment and susceptibility to *P. cinnamomi* within South African plantations is unexplained (Cahill *et al.*, 2008). PRR continues to be a problem in localized areas of South African *Eucalyptus* plantations (Tree Pathology Co-operative Program, 2002), and is particularly problematic in certain *E. nitens* stands (J. Roux, personal communication).

Phytophthora has been divided into ten clades based on sequence variation across seven loci (Blair *et al.*, 2008). The internal transcribed spacer (ITS) (Cooke *et al.*, 2000b), β-tubulin (β-*tub*) and translation elongation factor 1 alpha (*EF*-1α) (Kroon *et al.*, 2004) are commonly used genomic regions for phylogenetic analysis. Primers have also been designed to target mitochondrial gene regions such as NADH dehydrogenase subunit 1 (*nadh*1) (Kroon *et al.*, 2004) and cytochrome c oxidase subunit 1 (*cox*I) (Martin and Tooley, 2003). In the last decade, the number of described *Phytophthora* spp. have more than doubled, with 117 species formally described at present (Brasier, 2009; Érsek and Ribeiro, 2010; Martin *et al.*, 2012). Particularly within clade 9, there has been dramatic expansion of newly described *Phytophthora* spp. This has resulted in some difficulty with delineating certain species, such as *P. parsiana* isolates, which are now considered a species complex (Kroon *et al.*, 2012).

Numerous *Phytophthora* spp. belonging to various clades are virulent to *Eucalyptus.* These include: *P. alticola, P. boehmeriae*, *P. cactorum, P. captiosa, P. cinnamomi*, *P. citrophthora, P. cambivora, P. citricola, P. cryptogea, P. drechsleri, P. fallax, P. elongata, P. frigida, P. heveae, P. megasperma, P. multivora, P. nicotianae* and *P. palmivora* (Dick *et al.*, 2006; Erwin and Ribeiro, 1996; Maseko *et al.*, 2007; Rea *et al.*, 2010; Scott *et al.*, 2009; Shearer and Smith, 2000).

Eucalypts can be infected with *Phytophthora* by means of stem inoculation using mycelial plugs (Tippett *et al.*, 1983), or by zoospore-mediated root inoculation (Burgess *et al.*, 1998; O'gara *et al.*, 1996), which best mimics natural infection. However, zoospore inoculation is associated with several disadvantages when used for large trials which involve resistance screening or harvesting of soil-free material for RNA extractions. This method requires an established hydroponics or aeroponics system, and zoospores are difficult to obtain for certain species, such as *P. cinnamomi*, despite detailed protocols (Byrt and Grant, 1979; Chen and Zentmyer, 1970). Also, the amount of zoospores that will be obtained for a trial is often unpredictable and unreliable due to the complex conditions required for sporangial production (Erwin and Ribeiro, 1996). Production of zoospores for large time-course trials (approximately 200 trees) is challenging. If roots are dipped, impractically large volumes of zoospore suspension is required for proper infection. If roots are inoculated individually, time constraints prove problematic, since numerous roots per tree would need to be inoculated to obtain enough tissue, and only a limited timeframe is available to apply the inoculum before the released zoospores shed their flagella and die.

Though stem inoculation does not mimic natural infection mechanisms, this method overcomes several of the drawbacks of root inoculations. Stem inoculations are easily scored by measuring beneath-bark necrotic lesions. Several studies have utilized this method to assess virulence, screen for host resistance, and prove pathogenicity of an isolate on a given host (Dudzinski *et al.*, 1993; Hajebrahimi and Banihashemi, 2011; Linde *et al.*, 1994b; Linde *et al.*, 1999; Maseko *et al.*, 2007; Scott *et al.*, 2012; Stukely and Crane, 1994; Tippett *et al.*, 1985). Stem inoculations do not necessarily reflect the degree of resistance or susceptibility of a host, since the lesion lengths do not correlate precisely with the degree of root rot symptoms (Shearer and Smith, 2000; Tippett *et al.*, 1985). However, this inoculation technique is standardized and it provides a relative and consistent estimate of pathogenicity between treatments within the same trial. Moreover, rankings of host susceptibility derived from stem and root inoculation data are similar (Dudzinski *et al.*, 1993; O'gara *et al.*, 1996; Stukely and Crane, 1994; Tippett *et al.*, 1985). Stem tissue can provide large amounts of soil-free, high quality RNA for the purpose of investigating host responses (Gambino *et al.*, 2008). Though hyphae are slower in penetrating the host and evoking host responses than zoospores (Cahill *et al.*, 1989), the time-points at which stem tissue is harvested can be adjusted accordingly.

This study aimed to establish a compatible pathosystem between a *Eucalyptus* species and a virulent *Phytophthora* isolate. The purpose of this system was to ensure a reliable and repeatable method of obtaining infected material for profiling of the host's disease-responsive transcriptome.

2.3 Materials and Methods

2.3.1 Biological materials

2.3.1.1 *Phytophthora* isolates

Phytophthora isolates were obtained from diseased root and collar tissue of (approximately) one year-old *Eucalyptus* spp. growing on the edge of the Tzaneen dam at 23°45′45.1″S; 30°7′40.5″E (possibly offspring from the adjacent *E. grandis* hybrid plantation). Isolates with *Phytophthora*-like culture morphology were identified following isolation on *Phytophthora* specific media (see section 2.3.2 below) and named field isolate (FI) 1, FI 2, FI 3, etc. Three of these isolates were deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI, University of Pretoria, South Africa) under the numbers CMW37795, CMW37796 and CMW37797. The *P. cinnamomi* isolate CMW26310 was isolated in 2002 from diseased *E. dunnii* in Lothair, Kwa-Zulu Natal, South Africa (Maseko, 2010).

2.3.1.2 Plant material

E. nitens seedlings were obtained from Sappi Forest Research, Shaw Research Centre, KwaZulu-Natal and the *E. grandis* clone, Tag5, was obtained from Mondi Forestry, Kwambonambi Nursery, KwaZulu-Natal. All trees were grown in pine-bark seedling mix until stems were thicker than 0.5 cm for stem inoculations (approximately 1 year old). The trees were kept in a greenhouse with temperature ranging between 20°C minimum and 28°C maximum (unless otherwise specified), and a 12-hour day artificial lighting regime supplemented natural sunlight. Trees were watered daily and received fertilizer shortly after they were transplanted approximately six months before inoculation.

2.3.2 Isolation of *Phytophthora* spp. from plant material and routine culturing

Isolations were performed by excising lesion margins on the roots, root collar or stem and placing these on modified *Phytophthora*-selective NARPH medium (Hüberli *et al.*, 2000) [½ strength potato dextrose agar (PDA; Merck, Darmstadt, Germany) amended with 50 µg/ml nystatin (Sigma-Aldrich®, St. Louis, Missouri), 200 µg/ml ampicillin (Sigma-Aldrich®), 10 µg/ml rifampicin (Melford Laboratories, Suffolk, UK), 25 µg/ml pentachloronitrobenzene (PCNB; Sigma-Aldrich®), and 50 µg/ml hymexazol (Tachigaren; Sankyo Company, Tokyo, Japan)]. Hyphae grown on the selective medium were subcultured onto water agar (WA; 17 g/L agar). Pure cultures were obtained by transferring hyphal tips from WA to clarified V8 agar [cV8A; modified from Erwin and Ribeiro (1996): 200 ml/L V8 juice (Campbell Soup Company, Camden, New Jersey), 20 g/L $CaCO_3$ (Merck), 20 g/L agar)]. Pure cultures were routinely maintained and grown for inoculations on cV8A. For long-term storage, cV8A blocks containing hyphae were placed in air-tight glass bottles containing sterilized grass (*Pennisetum clandestinum*) and sterile distilled water (Boesewinkel, 1976).

2.3.3 *Phytophthora* DNA extractions

Aerial hyphae were ground in 50 µl Prepman® Ultra (Applied Biosystems, Foster City, CA) and heated to 95°C for 10 minutes. This was centrifuged for 10 minutes at 14500 x g in a MiniSpin® centrifuge (Eppendorf, Hamburg, Germany) and the supernatant (containing the crude DNA extract) was removed and stored at 4°C.

2.3.4 Template amplification from *Phytophthora* DNA

The ITS region of all isolates (Cooke *et al.*, 2000a) and the β-*tub*, *EF*-1α, *nadh*1 (Kroon *et al.*, 2004) and *cox*I (Martin and Tooley, 2003) regions of CMW37795, CMW37796 and CMW37797 were amplified. Polymerase chain reactions (PCR) were prepared as follows: 1 µl crude DNA template, 1X S-T ExSel buffer (Southern Cross Biotechnologies, Cape Town, South Africa), 0.2 mM dNTPs (Fermentas, Ontario, Canada), 0.25 µM forward and reverse primer (see Table 2.1 for details), 0.8 U ExSel High Fidelity Taq (Southern Cross Biotechnologies), made up to 20 µl with double distilled Sabax water (Adcock-Ingram, Johannesburg, South Africa). PCR cycles were composed of: an initial denaturing cycle of 94°C for 2 minutes and 35 cycles of 94°C for 30 s, annealing for 30 s (except *cox*I, which had an annealing time of 1 minute), and a 30 s extension at 72°C. A final extension at 72°C for 10 minutes, and a 4°C hold was applied.

Amplicons were resolved using electrophoresis on ethidium bromide-stained 1 % (w/v) agarose gels, visualized under UV light in a Molecular Imager® Gel Doc™ XR$^+$ Imaging System (Bio-Rad, Hercules, California) and viewed in Image Lab v2.0.1 (Bio-Rad). A standard molecular marker, 100 bp O'GeneRuler (Fermentas) was used on all gels. Where multiple bands were present, 0.8% w/v agarose gels were prepared and the band of desired size was excised on a UV transilluminator. The Nucleospin gel and PCR cleanup kit (Macherey-Nagel, Düren, Germany) was used according to manufacturer's instructions and the purified product was resuspended in 20 µl double distilled water.

2.3.5 Cloning of ITS region

Amplicons of the ITS region of isolates CMW37795, CMW37796, CMW37797, FI5, FI6, FI13, FI17, FI18, FI26 and FI48 were cloned into blue-white selection vector pTZ57R/T using the InsTAclone™ kit (Fermentas), due to multiple peaks when sequencing directly from PCR products. Ligation was performed for one hour at 37°C and left at 4°C overnight. Briefly, transformation was performed as follows: the ligated vector was added to competent DH5α *Escherichia coli* cells and incubated on ice for 10 minutes, followed by a brief heat shock step and subsequent cooling on ice. Cells were incubated in SOC media (Hanahan, 1983) for an hour and then plated onto Luria-Bertani plates supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG; Fermentas), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-

Gal; Fermentas) and carbenicillin (Melford Laboratories). Eight white recombinant colonies per isolate were selected and M13 primer-based (Vieira and Messing, 1982) colony PCR was used to confirm the presence of the ITS insert. Initial denaturation was performed at 95°C for a minute, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C. A final extension was done at 72°C for 10 min. One clone was chosen to represent each isolate and plasmid was extracted using the GeneJet™ plasmid miniprep kit (Fermentas) according to manufacturer's guidelines.

Table 2.1 Primers and annealing temperatures used to amplify the various gene regions of *Phytophthora* isolates

Target DNA	Primer	Primer sequence (5' – 3')	Annealing temperature (°C)	Expected product size (bp)	Reference
ITS	ITS4	TCCTCCGCTTATTGATATGC	55	862-942	White *et al.* (1990) Cooke *et al.* (2000)
	ITS6	GAAGGTGAAGTCGTAACAAGG			
β-*tub*	TUBUF2	CGGTAACAACTGGGCCAAGG	60	989	Kroon *et al.* (2004)
	TUBUR1	CCTGGTACTGCTGGTACTCAG			
EF-1α	ELONGF1	TCACGATCGACATTGCCCTG	60	972	Kroon *et al.* (2004)
	ELONGR1	ACGGCTCGAGGATGACCATG			
*nadh*1	NADHF1	CTGTGGCTTATTTTACTTTAG	53	897	Kroon *et al.* (2004)
	NADHR1	CAGCAGTATACAAAAACCAAC			
*cox*I	FM83 (R)	CTCCAATAAAAAATAACCAAAAATG	56	1200	Martin and Tooley (2003)
	FM84 (F)	TTTAATTTTTAGTGCTTTTGC			
	FM50 (F)	GTTTACTGTTGGTTTAGATG	Internal sequencing primers		
	FM85 (R)	AACTTGACTAATAATACCAAA			
M13	M13F	CACGACGTTGTAAAACGAC	53	>1000	
	M13R	GGAAACAGCTATGACCATG			

2.3.6 Sequencing

Due to its length, the *cox*I fragment was sequenced using primers FM83 and FM84, as well as internal primers FM50 and FM85 (Martin and Tooley, 2003) to ensure sufficient high-quality sequencing coverage. M13 primers were used to amplify clones with the ITS insert for sequencing, using 100 ng purified plasmid as template. Other gene regions were sequenced with the same primers used to amplify them. PCR products to be sequenced were purified using ethanol precipitation and resuspended in 5 µl Sabax water. Cycle sequencing reactions were prepared as follows: 2.5 µl purified PCR product (<100 ng), 0.5 µl 100 mM forward or reverse primer, 2 µl BigDye® Terminator v3.1 ready reaction mix (Applied Biosystems), 2 µl 5X sequencing buffer (Applied Biosystems) and 3 µl ddH_2O. Sequencing was conducted at 96°C

denaturation for 1 min and 25 cycles of 96°C denaturation for 10 s, 50°C for 5 s, 60°C for 4 min. Ethanol precipitation was repeated to obtain a purified product and the sequences were analyzed on an Applied Biosystems® 3100 Genetic Analyzer. CLC Main Workbench v6.6.1 (CLC bio, Aarhus, Denmark) was used to visualize trace data and assemble forward and reverse strands.

2.3.7 Phylogenetic and sequence analysis

2.3.7.1 Dataset compilation

Sequence data for the unknown *Phytophthora* isolates were included in two datasets. One dataset included species from every *Phytophthora* clade and used *Pythium ultimum* as an outgroup (see Table 2.2). Sequences for *Py. ultimum* were obtained from the genome sequence on GenBank (Benson *et al.*, 2011). The other dataset included only clade 9 *Phytophthora* spp., with sequence information for two isolates per species wherever available (see Table 2.3). Additional isolates of *P. parsiana* and *P. hydropathica* were included due to their close relationship to the unknown isolates in question. Sequence information was obtained from GenBank and aligned using Muscle (Edgar, 2004) in Seaview (Gouy *et al.*, 2010).

Three datasets were prepared for multiple gene phylogenies: one containing ITS, β-*tub*, *EF*-1α only and another two datasets containing these regions with either *nadh*1 or *cox*I added. If sequence information for all the gene regions from a species analyzed in one of these combined datasets was not present, that species and/or isolate was excluded from that particular dataset.

2.3.7.2 Maximum parsimony analysis

Parsimony non-informative, as well as missing and ambiguous characters were excluded from the datasets in the Macintosh version of PAUP* 4.0 (Swofford, 2004). Trees were rooted at the *Py. ultimum* or *P. boehmeriae* outgroup. A heuristic search with the tree-bisection-reconnection (TBR) branch-swapping method was performed using a hundred random starting trees. Support values for clades were generated using a thousand bootstrap replicates. Partitioned datasets containing multiple gene regions were subjected to a partition homogeneity test (PHT) to establish congruency of data for the construction of multi-gene phylogenies.

2.3.7.3 Maximum likelihood analysis

A corrected Akaike Information Criterion (AIC) calculation (Akaike, 1973) was performed in JModelTest v.0.1.1 (Posada, 2008) to find the most suited model for each dataset analysed using PhyML (Guindon *et al.*, 2010). A starting tree was found using the BioNJ and the nearest

neighbour interchange (NNI) algorithm was used to improve tree topology. Bootstrap analysis was performed using a thousand replicates. Sequence data for multiple gene analysis was concatenated using SequenceMatrix (Vaidya *et al.*, 2011).

2.3.7.4 Bayesian inference analysis

The most appropriate parameters for Bayesian inference were determined using MrModelTest 2.2 (Nylander, 2004). Analyses were conducted in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Outgroups were defined as *Py. ultimum* for analyses on all *Phytophthora* clades, and *P. boehmeriae* for analysis of clade 9 *Phytophthora* spp. Chains were sampled once every thousand generations out of a total of three million generations. Tracer v1.5 (Rambaut and Drummond, 2005) was used to visualize trace data from MrBayes and subsequently a standard burnin of 10% (300 sampled generations) was defined in MrBayes when summarizing the data.

2.3.7.5 Analysis of ITS variability

All unknown isolates in question were aligned together with the two phylogenetically closest reference sequences from the ITS phylogenetic tree. The alignments were examined for sites that were variable between the two reference sequences. The variation at these sites was compared with respect to the isolate's ITS sequence.

2.3.7.6 Graphical representation of phylogenetic trees

Output tree files were visualized and edited in TreeGraph2 (Stöver and Müller, 2010). Maximum likelihood trees were artificially rooted at the appropriate outgroups. Support values from parsimony, maximum likelihood and Bayesian inference trees were superimposed on the maximum likelihood tree topology. Clades with weak support values (bootstrap values less than 50% and posterior probability less than 0.95) from all three analyses were collapsed for ease of viewing.

Table 2.2 *Phytophthora* spp. and associated Genbank accession numbers for various gene regions used for constructing phylogenetics trees with representatives from all clades.

Species	Isolate[a]	Isolate[b]	Clade	ITS	β-*tub*	*EF*-1α	*coxI*	*nadh1*
P. aquimorbida	40A6		9	FJ666127	GQ294534		GQ294536	GQ294538
P. arecae	P10213	CBS148.88	4	HQ643146	EU080810	EU080811	AY564164	AY563991
P. boehmeriae	P6950		10	HQ261504	EU080162	EU080163	AY564165	AY563992
P. botryosa	P6945	CBS533.92	2a	HQ261507	EU079935	EU079936	AY564166	AY563993
P. cactorum	P0714	[c]P6183	1a	HQ261514	EU080278	EU080279	AY564167	AY563994
P. cambivora	P0592	PC643	7a	HQ261516	EU080551	EU080552	DQ202502	DQ202494
P. captiosa	P10719		9	HQ261522	EU079659	EU079660		
P. chrysanthemi	NBRC 104917		9	AB437135	AB511995	AB511925		
P. cinnamomi	P2159	RADICI B/10A6	7b	AY302164	EU079757	EU079758	AY564169	AY563996
P. citrophthora	P6310	CBS274.33	2a	FJ801913	EU080538	EU080539	AY564171	AY563998
P. cryptogea	P1088	HR1/ss/pp/99	8a	HQ261549	EU080447	EU080448	AY564174	AY564001
P. erythroseptica	P1699	CBS951.87	8a	FJ801784	EU079828	EU079829	AY564176	AY564003
P. fallax	P10725		9	HQ261557	EU080035	EU080036		
P. fragariae var. fragariae	P3821	A2/NS4	7a	FJ802063	EU080652	EU080653	AY564178	AY564005
P. gonapodyides	P3822	P245	6	FJ801392	EU080531	EU080532	AY564181	AY564008
P. heveae	P10167	CBS296.29	5	GU259516	EU080796	EU080797	AY564182	AY564009
P. hydropathica 1D12	ID12		9	EU583797	GQ260068	GQ260060		GQ260064
P. hydropathica 44A9	44A9		9	EU583796	GQ260070	GQ260062		GQ260066
P. hydropathica 5D1	5D1 (type)		9	FJ196760	GQ260069	GQ260061		GQ260065
P. idaei	P6767	IMI313727	1a	HQ261579	EU080130	EU080131	AY564185	AY564012
P. ilicis	P3939	[c]PD91/595	3	HQ261583	EU079860	EU079861	AY129172	AY564013
P. infestans	P10650	Pic99186	1c	HQ261589	EU079626	EU079627	AY564150	AY563977
P. insolita	P6195	IMI288805	9	HQ261591	EU080176	EU080177	AY564188	AY564015
P. inundata	P8478	Isolate 167	6	HQ261594	EU079942	EU079943	EU199101	EU199095
P. iranica	P3882		1b	HQ261598	EU080112	EU080113	AY564189	AY564016
P. irrigata	23J7		9	FJ196758	EU595775	EU595778		EU595786
P. katsurae	P10187	CBS587.05	5	HQ261601	EU080803	EU080804	AY564190	AY564017
P. kernoviae	P10681		10	HQ261603	EU079646	EU079647		
P. lateralis	P3888	CBS168.42	8c	FJ802093	EU079853	EU079854	AY564191	AY564018
P. macrochlamydospora	P10267		9	HQ261606	EU080005	EU080006		
P. megakarya	P8516	[c]IMI337098	4	HQ261609	EU079970	EU079971	AY564193	AY564020
P. megasperma	P3136	[c]IMI133317	6	HQ261613	EU080059	EU080060	AY564194	AY564021
P. morindae	Ph697.P238		10	FJ469147		FJ469148		
P. nicotianae	P6303	P582	1		EU080599	EU080600	AY564196	AY564023
P. palmivora	P0255	CBS236.3	4	HQ261635	EU080339	EU080340	AY564197	AY564024
P. parsiana C25	C25 (type)		9	AY659739	AY659746	AY659753	AY659759	
P. parsiana SCRP237	SCRP237		9	AY659736	AY659743	AY659750	AY659756	
P. parsiana SUC19	SUC19		9	AY659738	AY659745	AY659752	AY659758	
P. parsiana SUC7	SUC7		9	AY659737	AY659744	AY659751	AY659757	
P. parsiana SURf17	SURf17		9	AY659741	AY659748	AY659755	AY659761	
P. parsiana SURf6	SURf6		9	AY659740	AY659747	AY659754	AY659760	
P. phaseoli	P10145	CBS556.88	1c	HQ261642	EU080749	EU080750	AY564159	AY563986
P. polonica	P15005	[c]UASWS0198	9	HQ261646	EU080257	EU080258		DQ399847
P. pseudosyringae	P10437		3	FJ801438	EU079563	EU079564		
P. quininea	P3247	CBS407.48	9	HQ261661	EU079803	EU079804	AY564200	AY564027
P. ramorum	P10328		8c	HQ261662	EU080684	EU080685	AY564208	AY564034
P. sojae	P10301	PD93/51	7b	HQ261676	AY564047	AY564104	AY564162	AY563989
P. vignae	P6497		7b	HQ261724	EU079783	EU079784	HQ261471	AY564032
P. sp *cuyabensis*	P8213		9	FJ801990	EU080665	EU080666		
P. sp *lagoriana*	P8223		9	EF590256	EU080365	EU080366		
P. syringae	P10330	CBS364.52	8b	HQ261716	EU080558	EU080559	AY564203	AY564030
P. tentaculata	P8497	CBS552.96	1b	HQ261717	EU079956	EU079957	AY564204	AY564031
P. tropicalis	P10329	PD97/11132	2b	GU259255	EU080306	EU080307	AY564161	AY563988
Pythium ultimum	BR114			ADOS 01001233.1 5846..6701	ADOS 01001717.1 14056..14992	ADOS 01001574.1 51747..52619	GU138662.1 36817.. 37684	GU138662.1 2790..3621

[a]Indicates the isolates from which ITS, *β-tub* and *EF-1α* sequences were derived
[b]Indicates the isolates from which *nadh1* and *coxI* sequences were derived
[c]Indicates isolates from which only *nadh1* sequence data was derived, *coxI* data for that species was obtained from isolate [a]
Grey cells indicate a lack of sufficient sequence data available for the gene region of that particular isolate for phylogenetic analysis

Table 2.3 *Phytophthora* spp. and associated GenBank accession numbers for various gene regions used for constructing phylogenetics trees with representatives from clade 9.

Species	Isolate	Clade	ITS	β-*tub*	*EF*-1α	*cox*I	*nadh*1
P. aquimorbida	40A6	**9**	FJ666127	GQ294534		GQ294536	GQ294538
P. aquimorbida	40E3	**9**	FJ666128				
P. aquimorbida	40F9	**9**	FJ666129				
P. aquimorbida	44G9	**9**	FJ666130				
P. boehmeriae	P6950	**10**	HQ261504	EU080162	EU080163	AY564165	AY563992
P. captiosa	P10719	**9**	HQ261522	EU079659	EU079660		
P. captiosa	P10721	**9**	FJ801493	EU079666	EU079667		
P. chrysanthemi	GF749	**9**	AB437135	AB511995	AB511925		
P. chrysanthemi	GF753	**9**	AB511826	AB511996	AB511927		
P. constricta	CBS125801	**9**	HQ013225			HQ013207	
P. constricta	VHS16127	**9**	HQ013224			HQ013206	
P. fallax	P10725	**9**	HQ261557	EU080035	EU080036		
P. fallax	P10723	**9**	HQ261558				
P. hydropathica	1D12	**9**	EU583797	GQ260068	GQ260060		GQ260064
P. hydropathica	23J8	**9**	GQ280346				
P. hydropathica	33H1	**9**	FJ196761				
P. hydropathica	44A9	**9**	EU583796	GQ260070	GQ260062		GQ260066
P. hydropathica	44J1	**9**	EU583794		GQ260063		GQ260067
P. hydropathica	5C11	**9**	FJ914882				
P. hydropathica	5D1 (type)	**9**	FJ196760	GQ260069	GQ260061		GQ260065
P. insolita	P6195	**9**	HQ261591	EU080176	EU080177	AY564188	AY564015
P. irrigata	23J7	**9**	FJ196758	EU595775	EU595778		EU595786
P. irrigata	44E4	**9**	EU595772	EU595776	EU595779		EU595787
P. macrochlamydospora	P10267	**9**	HQ261606	EU080005	EU080006		
P. macrochlamydospora	P10266	**9**	FJ801353	EU079999	EU080000		
P. parsiana	C25 (type)	**9**	AY659739	AY659746	AY659753	AY659759	
P. parsiana	SCRP237	**9**	AY659736	AY659743	AY659750	AY659756	
P. parsiana	C19	**9**	AY659738	AY659745	AY659752	AY659758	
P. parsiana	C7	**9**	AY659737	AY659744	AY659751	AY659757	
P. parsiana	Rf17	**9**	AY659741	AY659748	AY659755	AY659761	
P. parsiana	Rf6	**9**	AY659740	AY659747	AY659754	AY659760	
P. polonica	[a]P15005	**9**	HQ261646	EU080257	EU080258		DQ399848
P. polonica	UASWS0198	**9**	DQ396410	DQ399844	DQ399850		DQ399847
P. quininea	P3247	**9**	HQ261661	EU079803	EU079804		
P. quininea	CBS 407.48	**9**	HQ643338	AY564085	AY564141	AY564200	AY564027
P. sp cuyabensis	P8213	**9**	FJ801990	EU080665	EU080666		
P. sp cuyabensis	P8224	**9**	EF590254	EU080372	EU080373		
P. sp lagoriana	P8223	**9**	EF590256	EU080365	EU080366		
P. sp lagoriana	P8220	**9**	FJ802119		EU080359		

[a]For *P. polonica,* the isolate used for *nadh1* sequence data is UASWS0199

Grey cells indicate a lack of sufficient sequence data available for the gene region of that particular isolate for phylogenetic analysis

2.3.8 Pathogenicity trials

2.3.8.1 Inoculations

A 4 mm cork borer was used to remove the periderm or bark and create a fresh wound approximately 20-30 cm from the base of the stem. A mycelial plug from the margin of a three-day-old *Phytophthora* colony was placed on the wound. For mock-inoculation, a sterile cV8 agar plug was used. The displaced periderm or bark was replaced and covered with a moist piece of sterile cheesecloth placed over the wound site, which was held in place with a piece of tin foil wrapped around the stem. Parafilm (Parafilm, Chicago, IL) was used to secure the tin foil and seal in the moisture. After six weeks the bark around the inoculation site was removed and the extent of necrosis or oxidization was measured in both infected plants and the uninfected controls. Dead trees in the 30°C trial were excluded from statistical analyses since lesions were difficult to record on these and thus introduced a bias in the data. In all plant trials, the causal agent of the disease symptoms was confirmed to be the same as the inoculated isolate. This was achieved by re-isolations from the lesion margin (three individual trees of each treatment were sampled, with 10 pieces of stem tissue tested per sample) and subsequent inspection of culture morphology and ITS sequence comparisons.

2.3.8.2 Determination of *Phytophthora* isolates' pathogenicity

E. nitens seedlings at 25°C were inoculated with CMW37795, CMW37796 and CMW37797. Mock inoculations were included as a negative control. The isolate CMW26310 (*P. cinnamomi*) used as a positive control for infection, was passed through a host, *E. nitens*, to revive virulence subsequent to long-term storage. The re-isolate was sequenced to ensure that it is still the same as the original isolate. Between nine and 12 individuals were screened for each treatment in the trial. Symptoms were noted at six weeks following inoculation. In order to confirm results, the trial was repeated.

2.3.8.3 Effect of temperature and host on virulence of *Phytophthora* isolates

To determine the effect of temperature and host species on virulence of the unknown *Phytophthora* spp. isolates, *E. nitens* seedlings and TAG5 *E. grandis* clones were inoculated with CMW37796 and CMW37797 at two temperatures: 25°C and 30°C. Under all conditions a negative mock-inoculated and positive control with *P. cinnamomi* CMW26310 was included for comparative purposes. Ten to 11 plants were used per treatment.

2.3.9 Statistical analysis

The beneath-bark lesion measurements obtained from pathogenicity trials were tested for normality using a Shapiro-Wilk test in GraphPad Prism 6 (Motulsky, 1999). Lesions from mock-inoculated plants were compared to infected plants using the non-parametric Mann-Whitney

test at a confidence level of 95% (GraphPad Prism 6). Graphs were drawn in Microsoft® Office Excel 2007 using the average lesion length for bar height, with error bars calculated using standard error.

2.3.10 Microscopy

2.3.10.1 Preparation of plant material

Approximately 1.5-2 cm of stem tissue surrounding the inoculation site of *P. cinnamomi* infected and mock-inoculated *E. nitens* tissue was harvested in triplicate at 24 hpi, 48 hpi, 96 hpi and 1 wpi. The harvested tissue was fixed in formalin-acetic acid-alcohol [FAA; 100 ml/L formalin (Sigma-Aldrich®), 50 ml/L glacial acetic acid (Bio-Zone chemicals, Johannesburg, South Africa), 500 ml/L 95% ethanol] and stored at room temperature. For confocal microscopy, thin hand sections (longitudinal- and cross-sections) were made of stem tissue away from the immediate vicinity of the inoculation site. These sections were stained in 0.01% Calcufluor white fluorescent brightener 28 (Sigma-Aldrich®) for five minutes and rinsed three times in distilled water. The stained tissue was mounted in water on a glass microscope slide and sealed with petroleum jelly and a cover slip.

2.3.10.2 Visualization

Representative stem tissue samples from each time-point were visualized under a Stemi SV6 stereo microscope (Zeiss, Munchen, Germany) to observe the progression of disease over time in the stems. Images were captured using an AxioCam MRc digital camera (Zeiss) and Axiovision 4.7 software (Zeiss). The presence of hyphae inside the sampled tissue at various time-points was verified through use of a confocal laser scanning microscope (CLSM 510 Meta, Zeiss). To view Calcufluor-stained tissue, a wavelength of 405 nm was used, and a 543 nm wavelength was used to visualize autofluorescence. LSM Image Browser v4.2.0.121 (Zeiss) was used to view images generated from the confocal microscopy.

2.4 Results

2.4.1 Recovery of unknown *Phytophthora* spp.

In an attempt to obtain a highly virulent, recently isolated *P. cinnamomi*, sampling was conducted in the Tzaneen area, where numerous *P. cinnamomi*-infected avocado orchards are present. On the shore of the Tzaneen dam, offspring from an *E. grandis* hybrid plantation appeared to be dying (Figure 2.1 A-B). No trees with early to intermediate wilt or die-back symptoms were observed in the vicinity. Some of the roots of the diseased trees displayed discoloured, water-soaked lesions that are typical of root rot symptoms.

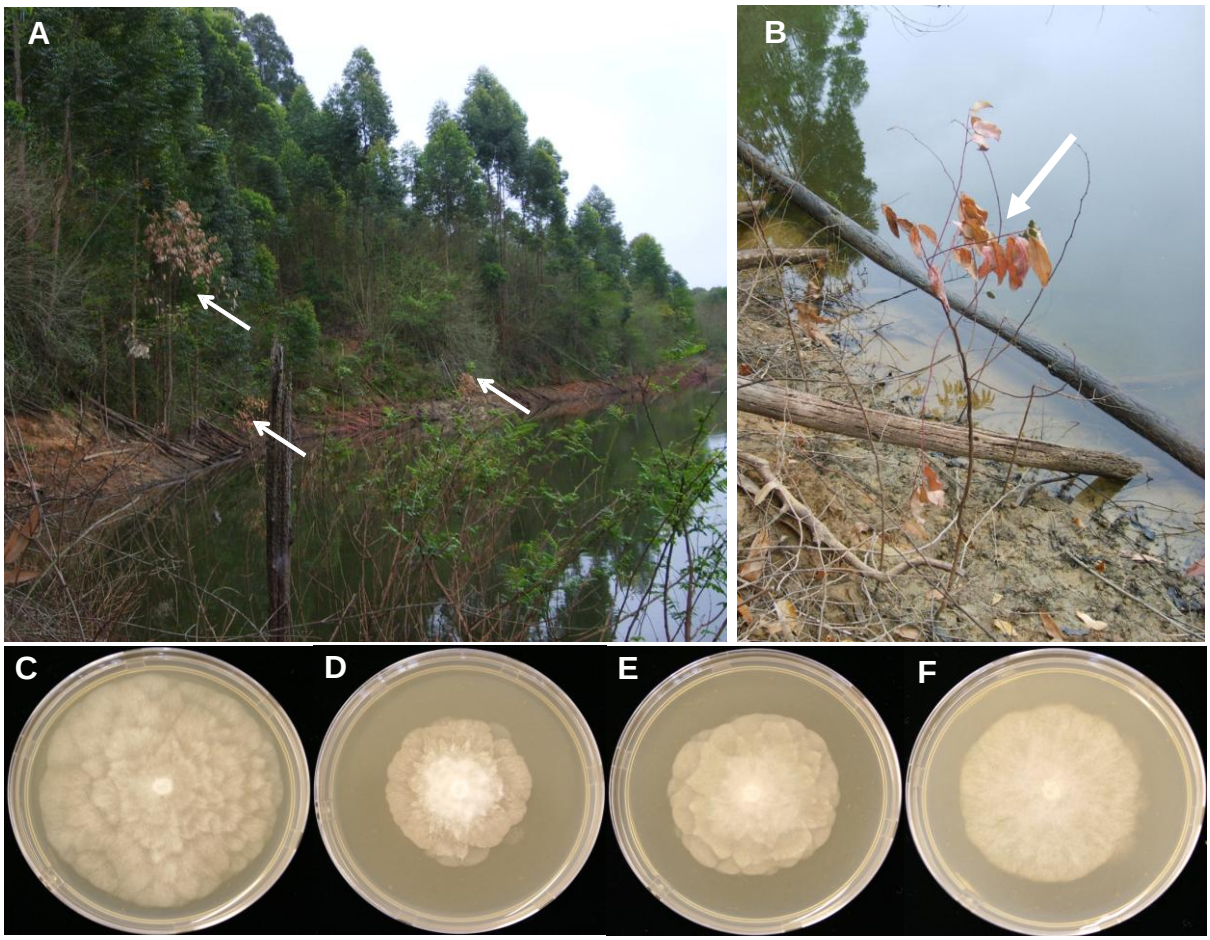

Figure 2.1 (A) White arrows indicate some of the dead *Eucalyptus* trees on the shore of the Tzaneen dam. (B) Close up of one of the dead trees that were sampled (C-E) Culture morphology on cV8 agar of (C) CMW37795 (D) CMW37796 (E) CMW37797 and (F) CMW26310 (*P. cinnamomi*).

Twenty-five isolates with similar culture morphology on ½ PDA were recovered from the diseased root and root collar tissue sampled. Several isolates were lost in long term storage, so only fifteen were used for phylogenetic analysis, with three (CMW37795, CMW37796, CMW37797) being selected for analysis of multiple gene regions and pathogenicity trials. All the isolates form a flower-like pattern on cV8 agar, although there is large variation among

them in terms of growth rate and colony morphology. For example, CMW37795 grew more rapidly than CMW37796 and CMW37797, and the cultures look quite different (Figure 2.1 C-E). CMW37796 produces more aerial hyphae in culture, and grows with a more irregular shape. CMW37795 and CMW37797 have more similar, regular features, but those of CMW37797 are more round and appear smoother. None of the isolates that were recovered had culture morphology similar to *P. cinnamomi* (Figure 2.1 F).

2.4.2 Amplification and sequencing

The ITS region of 17 isolates was successfully amplified, but upon sequencing the PCR products, only FI 7, FI 8, FI 30, FI 36, FI 44, FI 45, FI 46 yielded usable sequence information. ITS sequence data for the other isolates (FI 5, FI 6, FI 13, FI 17, FI 18, FI 26, FI 48, CMW37795, CMW37796 and CMW37797) showed mixed and irregular peaks on the electropherograms obtained after sequencing. Therefore the ITS region of these isolates were cloned in order to obtain clearer sequence data. Possibly the mixed peaks obtained could indicate that the isolates are hybrids. However, only one colony was selected per isolate for sequencing, since a more in-depth investigation into the possibility of hybrids was beyond the scope of the project. A gel depicting the products of a colony PCR to screen for transformed colonies is included in Appendix A.

The amplified β-*tub*, *EF*-1α, *nadh*1 and *cox*I regions of isolates CMW37795, CMW37796 and CMW37797 were successfully obtained according to expected product sizes, following PCR-based amplification (Figure 2.2 A-E). There were no problems with mixed sequencing signal for these gene regions, and when the sequences were submitted to BLASTN analysis on NCBI the results were composed of *Phytophthora* spp. sequence data representing the same gene region as the query sequence. Sequence data for all gene regions of the isolates can be found in Appendix B.

2.4.3 Phylogenetic analysis

Phylogenetic analysis of separate gene regions using parsimony, maximum likelihood and Bayesian inference was performed. A dataset with *Phytophthora* spp. representing all clades within the genus was used to attempt identification of the unknown *Phytophthora* isolates. However, trees resulting from this dataset (Appendix C) do not provide sufficient resolution to identify the isolates in question, since the sequences included are too diverse. To reduce the amount of homoplasy, a dataset containing only sequences from clade 9 *Phytophthora* species (with the clade 10 *P. boehmeriae* as outgroup) was used to perform phylogenetic analyses (Figures 2.3 - 2.8). The models utilized for PhyML and MrBayes are noted in Appendix D. Multigene phylogenies were attempted using PAUP and PhyML, but the results

from the different regions were not congruent enough to provide trustworthy trees, as indicated by very low PHT and high log-likelihood values respectively.

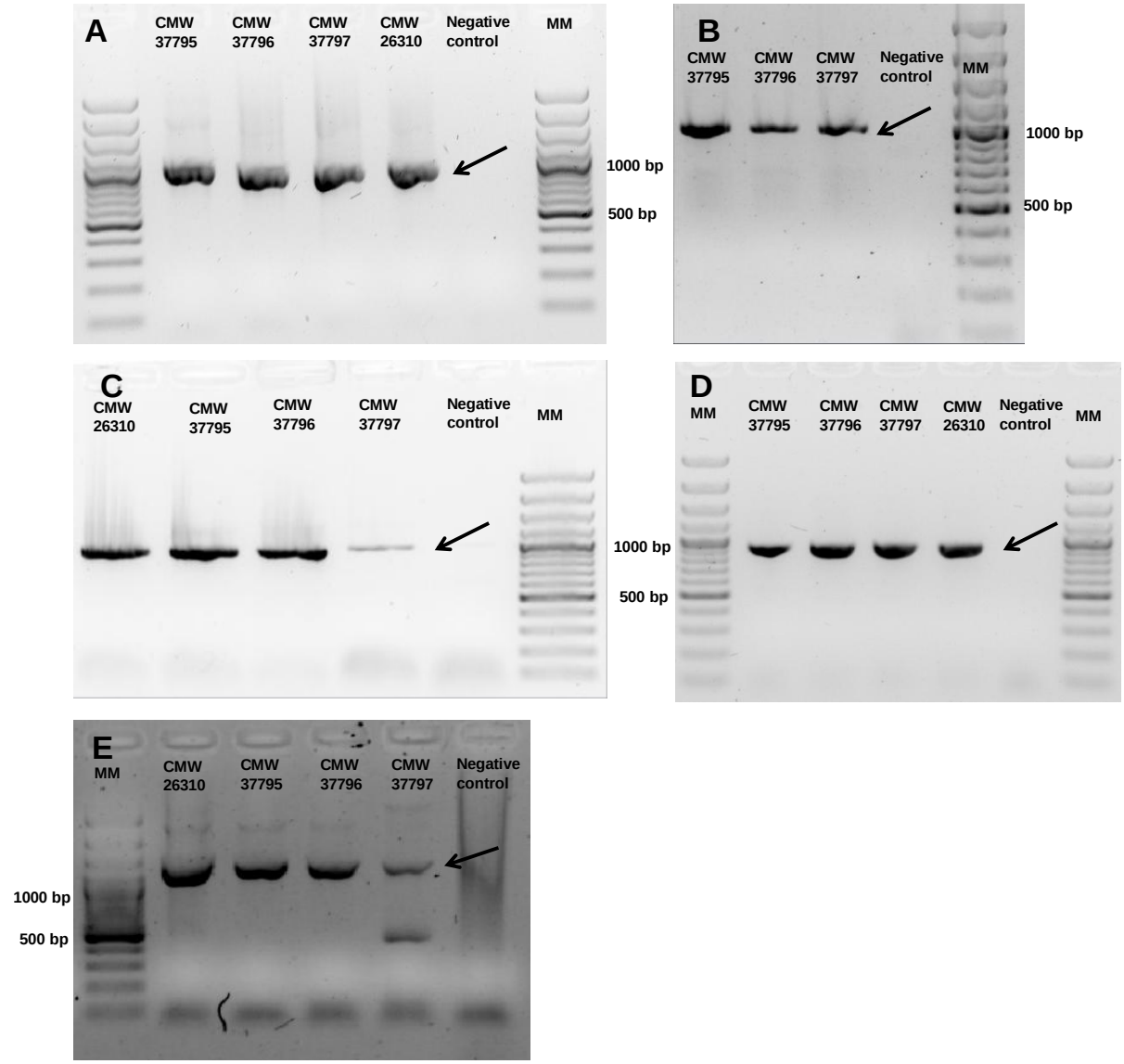

Figure 2.2 Amplification of (A) ITS, (B) β-*tub*, (C) *EF*-1α, (D) *nadh*1 and (E) *cox*I regions from CMW37795, CMW37796 and CMW37797. CMW26310 was used as a positive control, and a no-template negative control was included. Lanes marked MM contain the molecular marker – 100 bp Fermentas O'GeneRuler.

Two ITS trees were constructed, one including all 17 isolates for which ITS was sequenced (Figure 2.3) and one with the three selected isolates, CMW37795, CMW37796 and CMW37797 (Figure 2.4). The latter ITS tree is provided for ease of viewing and comparison with the other gene regions where only these isolates were analysed. From the analysis of the ITS region, it is evident that the various isolates are probably different *Phytophthora* spp. All the isolates fall within a large, strongly supported sub-clade which encompasses *P. aquimorbida*, *P. chrysanthemi*, *P.* sp *cuyabensis*, *P. irrigata*, *P.* sp *lagoariana*, *P. parsiana* and *P.*

hydropathica. Within this sub-clade, another well-supported clade is found. This clade includes *P.* sp *lagoariana*, two isolates belonging to the so-called *P. parsiana* species complex, and the majority of isolates from this study. Only FI 17 and FI 5 do not group within this smaller clade. One finding of note is the distinct grouping of the six *P. parsiana* isolates into three groups, similar to that found in the original species description (Mostowfizadeh-Ghalamfarsa *et al.*, 2008).

Phylogenetic analysis of the β-*tub* region (Figure 2.5) reveals a well-supported subclade containing CMW37795 and CMW37797, which groups with *P.* sp *lagoariana*. CMW37796 groups along with *P. hydropathica* and the *P. parsiana* isolate C25 (the type for this species). This contradicts the ITS tree, which separates CMW37795 and CMW37797 from *P.* sp *lagoariana*. Analysis of the *EF*-1α region (Figure 2.6) does not provide any additional information, since all the *P. lagoariana*, *P. hydropathica* and *P. parsiana* isolates group with the three isolates in question in a well-supported, but unresolved clade. Due to a lack of information for *cox*I (Figure 2.7) with regards to *P. hydropathica* and *P. lagoariana*, the three isolates grouped with *P. parsiana* isolates only, with CMW37797 and CMW37796 forming a subclade. There is a scarcity of information regarding the *nadh*1 region as well (Figure 2.8), since only *P. hydropathica* data, but no *P. parsiana* or *P. lagoariana* sequence data is available for this. However, the *P. hydropathica* isolates form a very strongly supported subclade, with the three isolates in question forming a strongly supported sister clade. This indicates that, based on this region's information, they are not *P. hydropathica*. Similar to the *coxI* region, CMW37797 and CMW37796 form a well-supported subclade. From these analyses, CMW37795, CMW37796 and CMW37797 may be either *P.* sp *lagoariana,* or similar to one of the non-type *P. parsiana* isolates.

Since *P. parsiana* Rf17 and *P.* sp. *lagoariana* grouped the most closely with the unknown isolates in the ITS phylogenetic tree (Figure 2.3), these sequences were used to find variable sites for analysis of ITS variability. The variation between isolates was compared to establish whether the isolates could be a hybrid of the two closest known species. The *P.* sp. *lagoariana* isolate seems to share the most similarity with the unknown isolates. The *P. parsiana* isolate also shares some variable sites with the isolates, but it is scattered throughout the whole region. From the information available, it seems unlikely that the unknown isolates are hybrids of *P. parsiana* Rf17 and *P.* sp. *lagoariana*, since there is no clear inheritance pattern of part of the ITS from any one of the possible parents (Table 2.4). The white spaces in the unknown isolates' sequence further clarify that the isolates are phylogenetically distinct, since these sites are not present in either *P. parsiana* Rf17 or *P.* sp. *lagoariana*.

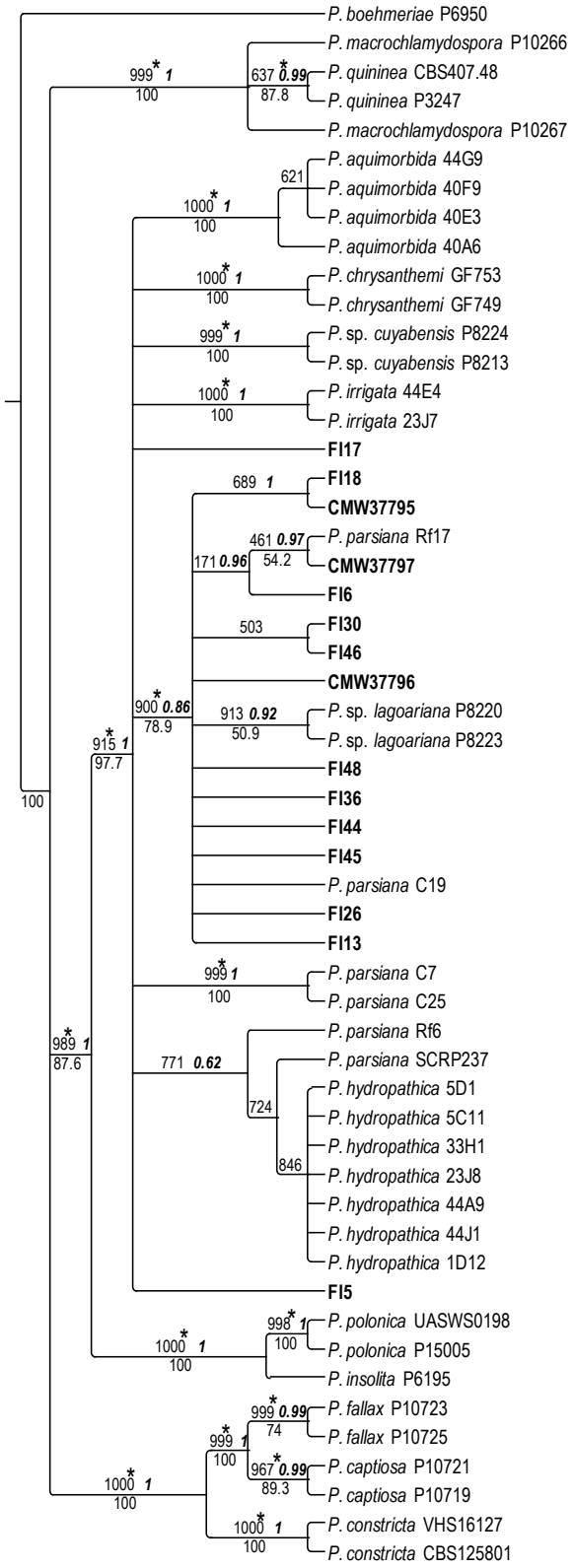

Figure 2.3 Cladogram derived from maximum likelihood analyses of the ITS region of various clade 9 *Phytophthora* spp. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from Bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

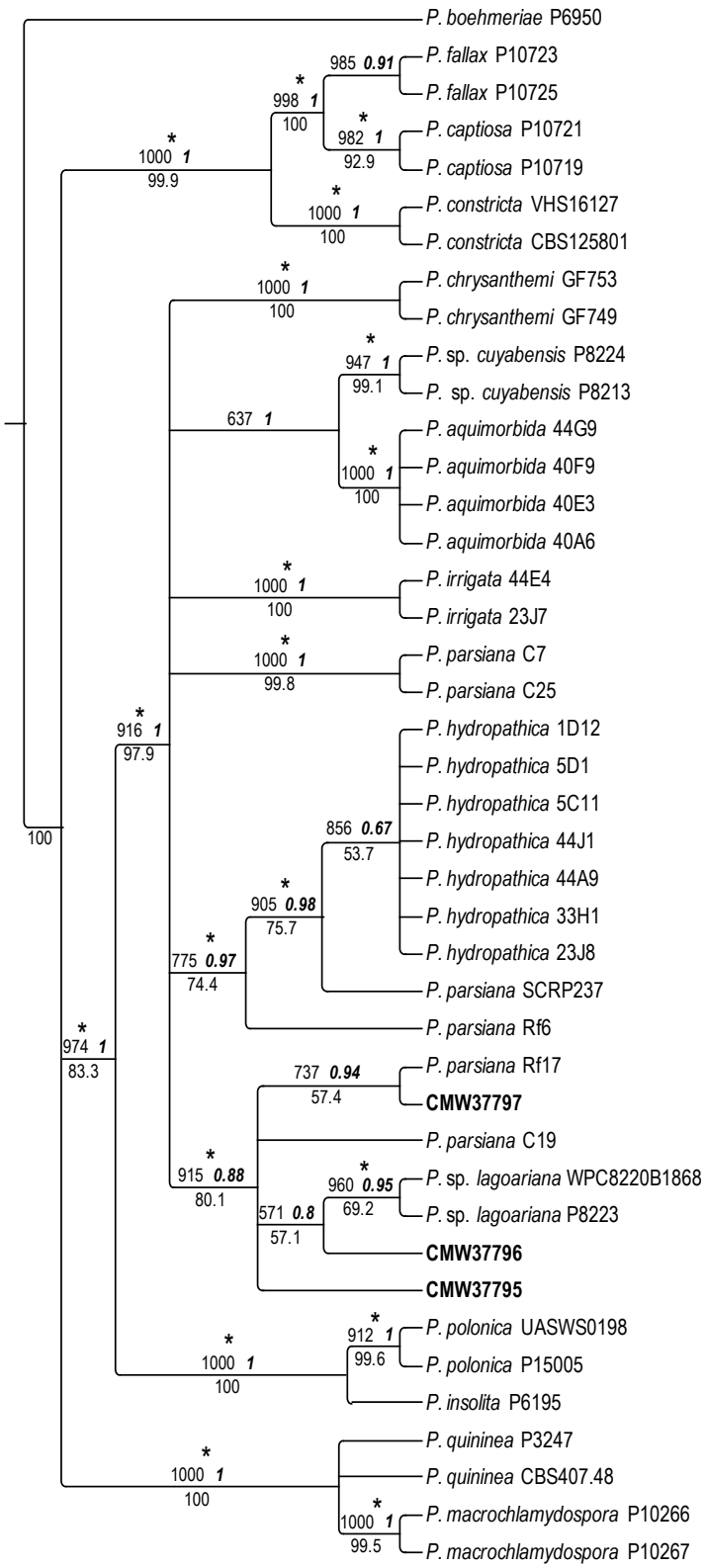

Figure 2.4 Cladogram derived from maximum likelihood analyses of the ITS region of various clade 9 *Phytophthora* spp. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from Bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

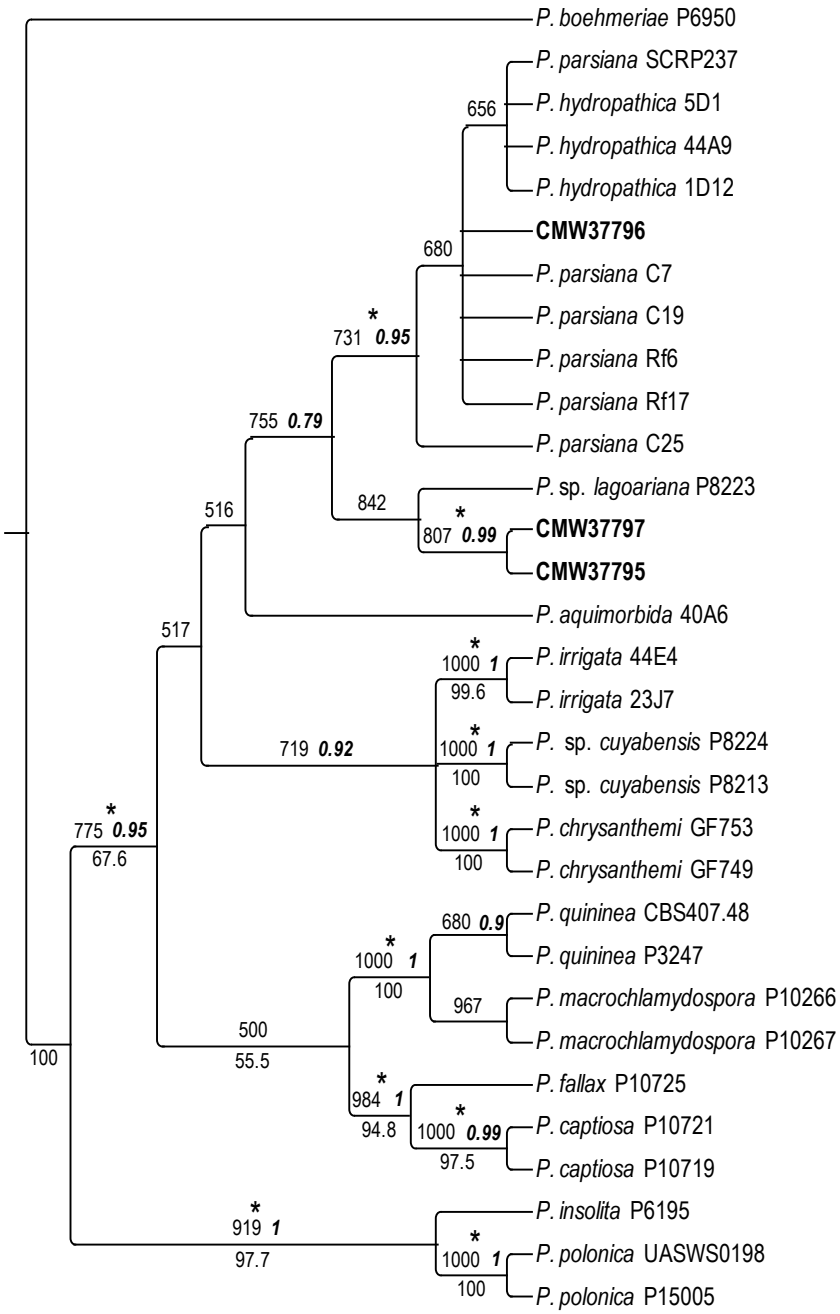

Figure 2.5 Cladogram derived from maximum likelihood analyses of the β-*tub* region of various clade 9 *Phytophthora* spp. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from Bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

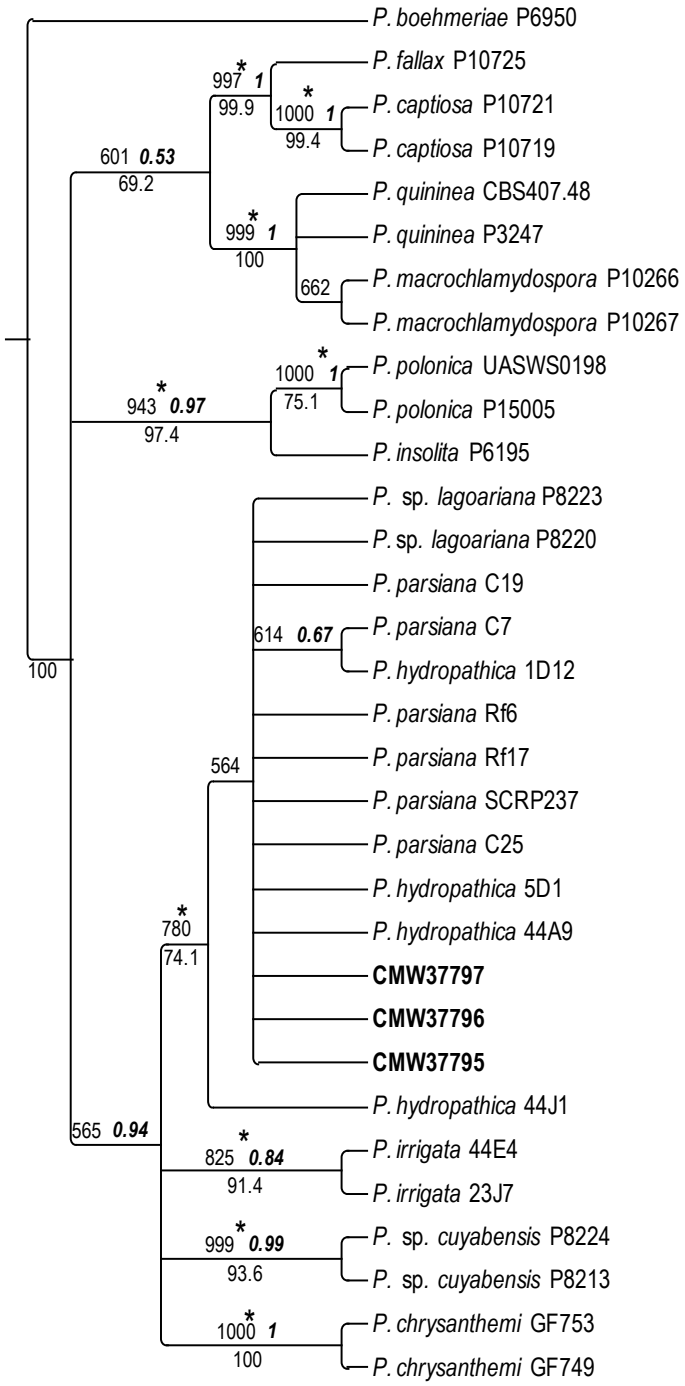

Figure 2.6 Cladogram derived from maximum likelihood analyses of the *EF*-1α region of various clade 9 *Phytophthora* spp. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from Bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

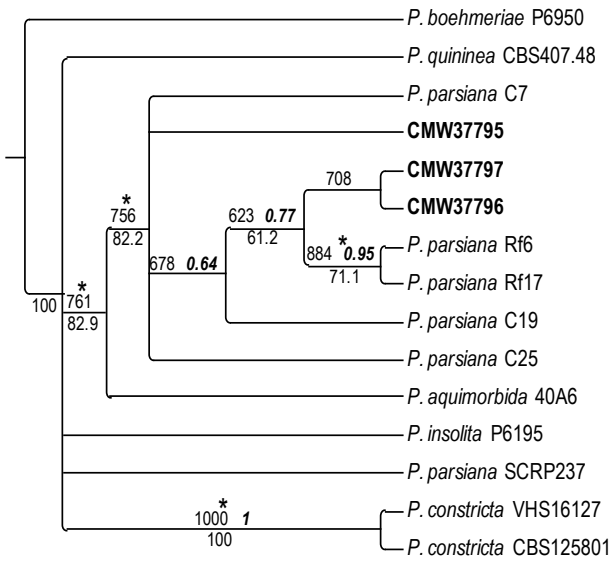

Figure 2.7 Cladogram derived from maximum likelihood analyses of the *cox*I region of various clade 9 *Phytophthora* spp. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from Bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

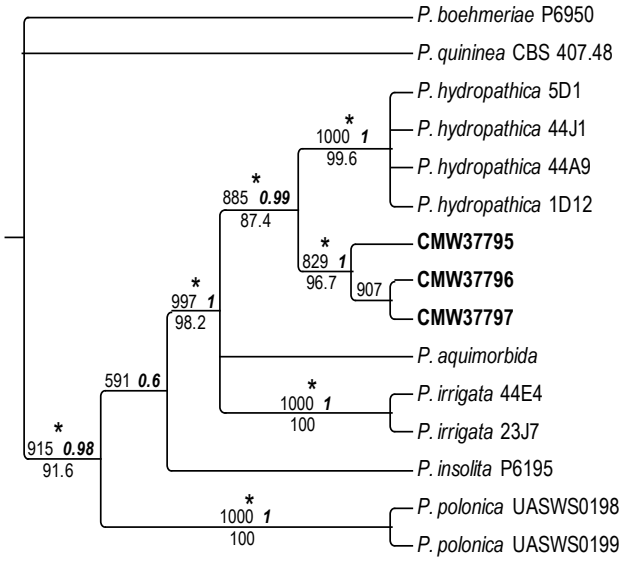

Figure 2.8 Cladogram derived from maximum likelihood analyses of the *nadh*1 region of various clade 9 *Phytophthora* spp. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from Bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

Table 2.4 Comparison of variable sites within ITS region of *Phytophthora* spp. with respect to unknown isolates and two least phylogenetically distant reference isolates.

Isolates	Variable sites (bp position in alignment)														
	53	92	99	128	135	152	415	418	420	421	623	624	650	675	678
P. parsiana Rf17	T	C	A	C	T	C	G	G	G	A	T	-	G	T	C
P. sp. *lagoariana* WPC8220	C	A	G	T	A	T	C	-	A	T	-	A	A	C	T
FI 36	C	A	G	T	A	T	-	G	G	A	-	A	G	C	T
CMW37796	C	A	G	T	A	T	-	G	A	A	-	A	A	C	T
FI 45	C	A	G	T	A	T	-	G	A	A	-	A	G	C	T
FI 44	C	A	G	T	A	T	-	G	C	A	-	A	G	C	T
FI 46	C	A	G	T	A	T	-	G	A	A	-	A	G	C	C
FI 30	T	A	G	T	A	T	-	G	A	A	-	A	G	C	C
FI 18	C	A	G	T	A	T	-	G	A	T	-	A	A	T	C
CMW37795	C	A	G	T	A	T	-	G	A	A	-	A	G	T	C
FI 26	C	A	G	T	A	T	-	T	C	T	-	T	G	T	C
FI 13	C	C	G	T	T	T	-	T	C	T	-	T	G	G	C
CMW37797	T	C	G	T	T	T	-	G	A	A	-	T	G	G	C
FI 6	C	C	G	T	G	T	-	G	A	A	-	A	A	T	C
FI 48	C	A	G	T	G	T	-	G	A	T	-	T	A	C	T
FI 5	C	C	G	T	T	T	-	G	T	T	T	G	T	C	G
FI 17	C	C	G	T	A	T	-	T	G	A	T	-	G	C	C

2.4.4 Pathogenicity trials

2.4.4.1 Confirmation of *Phytophthora* isolates' pathogenicity

Since CMW37795, CMW37796 and CMW37797 were found in association with dead and dying *Eucalyptus* roots, these isolates were evaluated with regards to their pathogenicity on *E. nitens*, which is susceptible to PRR in the field. The pathogenicity trial was repeated independantly in order to validate results. Stem inoculations with these isolates at 25°C resulted in browning and contained lesions around the inoculation sites (Figure 2.9 C-E). Lesions were visibly larger than the mock-inoculated negative controls, but smaller than those on the plants inoculated with the *P. cinnamomi* isolate (Figure 2.9 A-B). No tree mortality or symptoms other than the stem lesions were observed when inoculated at 25°C.

The ITS sequence of re-isolates from lesion margins for CMW26130, CMW37796 and CMW37797-inoculated plants verified that they were identical to that of the original isolate. ITS sequences for CMW37795 re-isolates from both of the replicated trials repeatedly yielded very mixed signal peaks upon sequencing. Culture morphology of these isolates are the same as the original inoculum.

Lesion length data was not normally distributed according to the Shapiro-Wilk normality test. The null hypothesis that lesions for control and inonculated plants are the same, was rejected

since the p-value for the Mann-Whitney test statistic was less than 0.05. Therefore, the *P. cinnamomi* isolate, CMW37795, and CMW37797 caused significantly larger lesions than those of the control, but this was not the case for CMW37796 (Figure 2.10). Results from the replicate trials were similar, with the exception that the lesions of those in the second trial (Figure 2.10 B) were approximately 15 mm longer than those in the other trial. Seasonal variation is a likely cause of this, since the first trial was performed during early winter, whereas the second was conducted in summer, which is more conducive for pathogenicity.

2.4.4.2 Effect of temperature and host on virulence

Both *P. hydropathica* and *P. parsiana* are high-temperature *Phytophthora* spp., with optimal temperatures of 30°C (Hong *et al.*, 2010; Mostowfizadeh-Ghalamfarsa *et al.*, 2008). Since the isolates group closely with these species, they are likely to have a high optimal temperature, especially considering that they were isolated from Tzaneen, where the average summer temperatures range between 28-30°C. The performance of CMW37796 (the least virulent isolate) and CMW37797 (the most virulent isolate) was evaluated at 30°C, in order to mimic the natural conditions under which the isolates were found more accurately. CMW37795 was not included in the trial due to limited plant availability. *E. grandis* is normally fairly resistant to PRR, and usually only succumbs to the disease when subjected to stress. The host of origin for the isolates in question had a probable *E. grandis* background so an *E. grandis* clone at our disposal was used to evaluate whether these *Phytophthora* isolates are virulent on *E. grandis*. Therefore the virulence of CMW37796 and CMW37797 and CMW26310 (*P. cinnamomi*) at 25°C and 30°C on both *E. nitens* and *E. grandis* was compared.

Figure 2.11 depicts the symptoms associated with the inoculations on *E. nitens* at 30°C. Most of the controls displayed the normal control phenotype (Figure 2.11 A), with only slight browning around the wounded area of the stem. However, there were purple lesions similar to those caused by *Phytophthora* distributed along the length of the stem and branches of two mock-inoculated control plants (Figure 2.11 B). Re-isolations verified that there was no *Phytophthora* present in these lesions, so it may be an abiotic response to the high temperature the plants were subjected to. These sporadic purple lesions were present on a few of the inoculated plants in the trial as well.

Interestingly, the CMW37796- and CMW37797-inoculated plants displayed symptoms not observed at 25°C, or on the mock-inoculated control and *P. cinnamomi*-inoculated plants at 30°C. These symptoms included the leaves dying from the bottom of the plant, proximal to the site of inoculation (Figure 2.11 D), purple-brown irregularly shaped lesions on leaves, particularly close to the petiole, and death of parts of the leaves (Figure 2.11). Moreover, there

was occasional mortality of entire trees (Figure 2.11). These symptoms were not observed on the *E. grandis* trees at 30°C. CMW37796 and CMW37797 are virulent on *E. nitens* to some degree, but only at a raised temperature which is optimal to the pathogen and is unnaturally high for the host.

Figure 2.9 *E. nitens* stems 6 wpi at the region surrounding the site of inoculation with (A) sterile agar plugs (negative control) (B) CMW26310 (*P. cinnamomi,* positive control) (C) CMW37795 (D) CMW37796 and (E) CMW37797 colonized mycelial plugs.

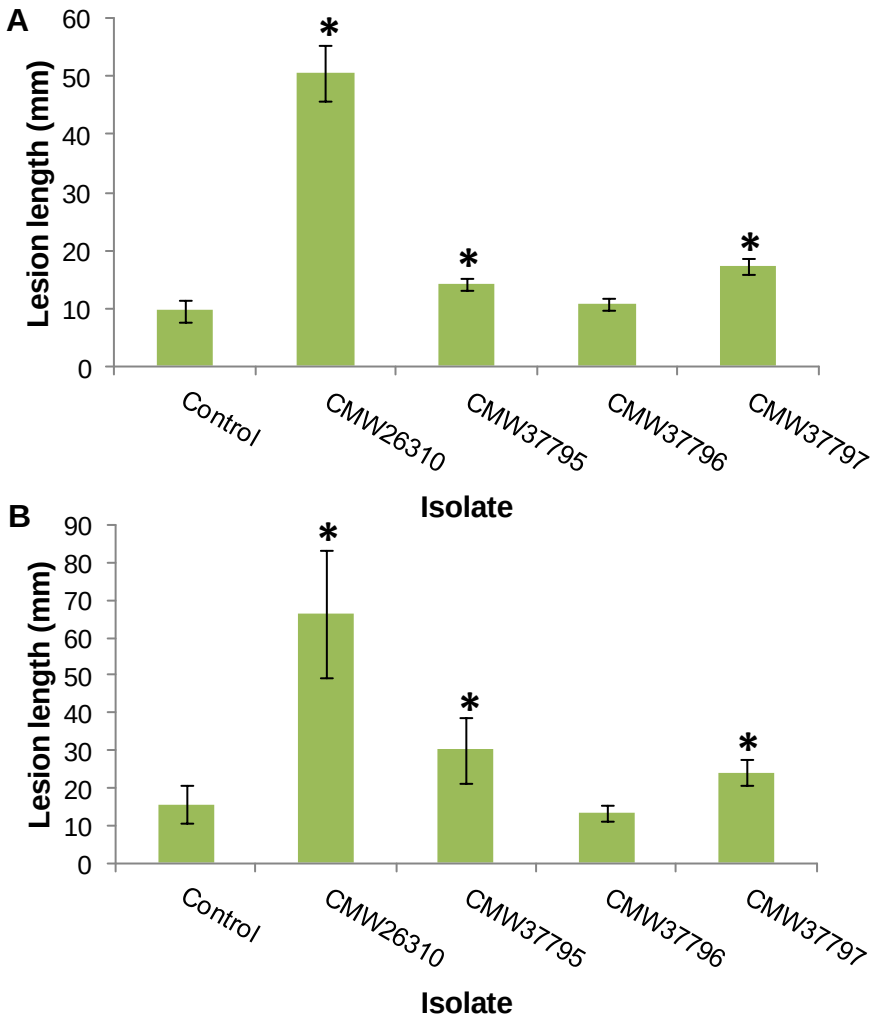

Figure 2.10 Lesion lengths recorded on *E. nitens* stems 6 wpi with various *Phytophthora* isolates (CMW26310 is a *P. cinnamomi* isolate). Two independent replicates of the trial are depicted in (A) (n=10) and (B) (n=13). Bar length is the mean lesion length of all individuals, with error bars calculated using standard error. Significantly larger lesions as compared to the control ($p<0.05$) using the Mann-Whitney test is indicated with *.

Figure 2.11 Symptoms on *E. nitens* plants inoculated at 30°C with various *Phytophthora* isolates. (A) Mock inoculated control with no symptoms (B) Mock inoculated control plant with purple lesions on the stem (C) *P. cinnamomi* inoculated positive control with a lesion around the site of inoculation and no other observable symptoms on the leaves (D - G) CMW37796 inoculated plants showing (D) leaves dying (E) brown spots on the leaves (F) parts of leaves dying (G) mortality of an entire tree (H-J) CMW37797-inoculated plants showing (H) extensive partial leaf mortality (I) brown spots on leaves (J) a dead tree. Black arrows indicate areas with specific symptoms, such as brown spots on leaves or purple lesions on stems.

In order to compare species and temperature effect of isolate virulence, the Mann-Whitney test was employed to compare lesion lengths with mock-inoculated control lesions (Figure 2.12). Two-way ANOVA analyses could not be performed due to the non-parametric nature of the data. *P. cinnamomi* and CMW37797 displayed significantly larger lesions (at a confidence level of 95%) than those of their respective controls in both hosts and at both temperatures. An expected observed trend is the higher average lesion length in susceptible *E. nitens* as opposed to tolerant *E. grandis* at the same temperature (Linde *et al.*, 1994b). At 30°C on *E. nitens*, CMW37796 caused very large lesions, indicating that the combination of temperature and host could be responsible for increased virulence of this isolate.

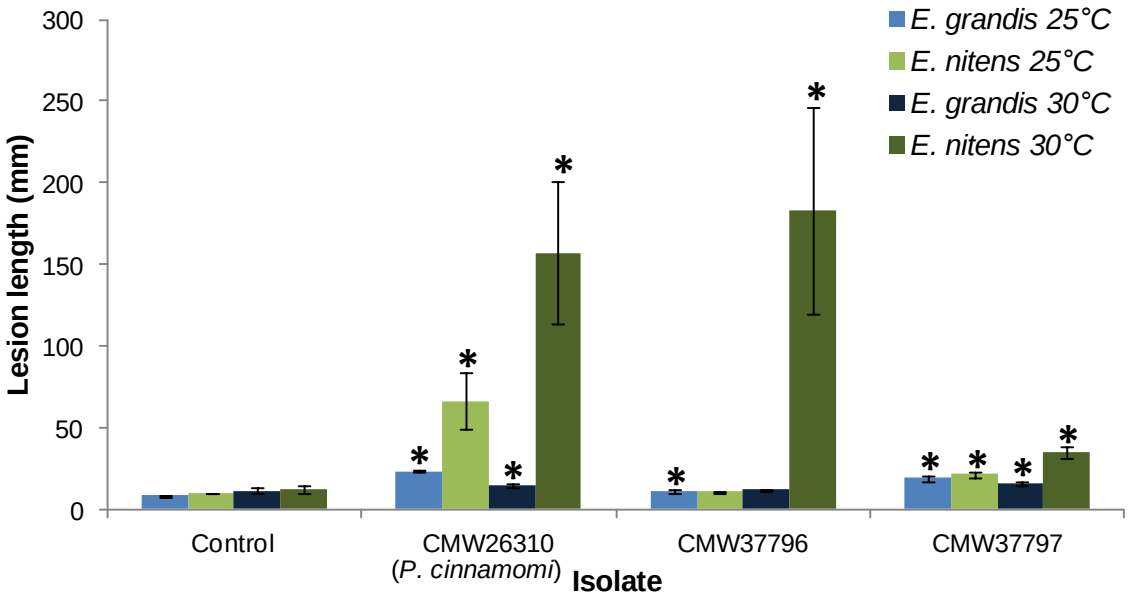

Figure 2.12 Lesion lengths recorded on *E. nitens* and *E. grandis* stems 6 wpi with various *Phytophthora* isolates at 25°C and 30°C. Bar length is the mean lesion length of all individuals, with error bars calculated using standard error. Significantly larger lesions as compared to that treatment's control ($p<0.05$) using the Mann-Whitney test is indicated with *.

The ITS sequence of re-isolates from inoculated individuals representing different treatment groups verified that the original isolates were the causal agents of the lesions. Even though the *P. cinnamomi* isolate has been stored for a long time, it resulted in the most consistent infection, with higher average lesion lengths than those of the isolates obtained from the field at 25°C, and lesions comparable to CMW37796 at 30°C. Therefore it was decided to continue with a pathosystem between *E. nitens* and *P. cinnamomi* for further host profiling experiments.

2.4.5 Confirmation of *P. cinnamomi* hyphal penetration

The use of a *P. cinnamomi-E. nitens* stem inoculation pathosystem requires confirmation of hyphal contact with host tissue at certain time-points. Additionally, the time-frame of disease progression needs to be determined in order to choose a valuable time-point for harvesting host tissue for gene expression profiling. There is clear progression of disease symptoms in stem tissue harvested at 24 hpi, 48 hpi, 96 hpi and 1 wpi (Figure 2.13). At 24 hpi (Figure 2.13 A) there is very slight browning visible in the wounded part of the inoculated stem as opposed to the control. By 48 hpi (Figure 2.13 B), the browning has intensified and spread beyond the immediate inoculation site. This is fairly rapid, considering that the mycelia have grown from the agar plug onto the host tissue and at least 1-2 mm beyond the inoculation site within this time. By 96 hpi (Figure 2.13 C) the inoculated stem is blackened by the necrosis, and the lesion is still extending. The 1 wpi (Figure 2.13 D) inoculated stem depicts advanced necrosis, with some white visible on the stem surface which may be hyphae, or some secondary infection.

Confocal microscopy of 48 hpi tissue (Figure 2.14) indicates that there is extensive colonization of host tissue distal from the inoculation site by *P. cinnamomi* at this time-point. This has important implications for sampling of host tissue, since this provides assurance that there is contact between the pathogen and host by this time, so any time-point subsequent to this would provide some degree of responses by the host.

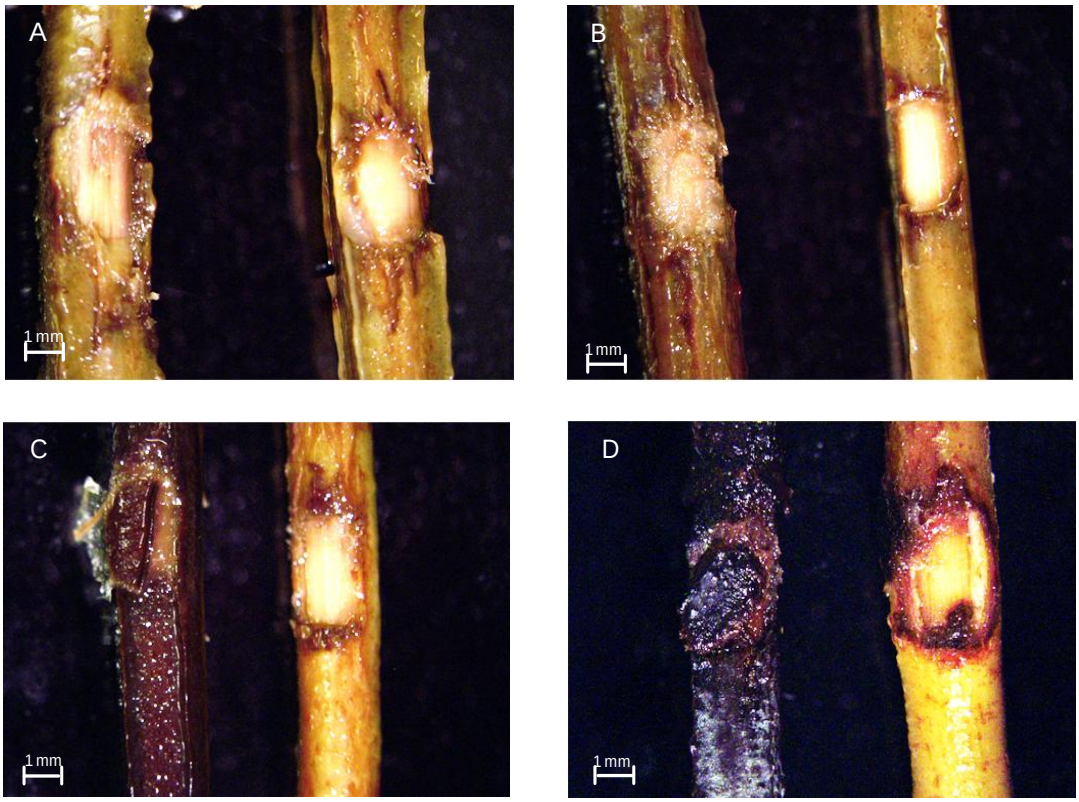

Figure 2.13 Stereo microscope photographs of *E. nitens* stems (A) 24 hpi (B) 48 hpi (C) 96 hpi and (D) 1 wpi with *P. cinnamomi* (stem on the left of each picture) or mock-inoculated (stem on the right). The progression of infection by the pathogen is evident from the increase in stem necrosis over the time-course.

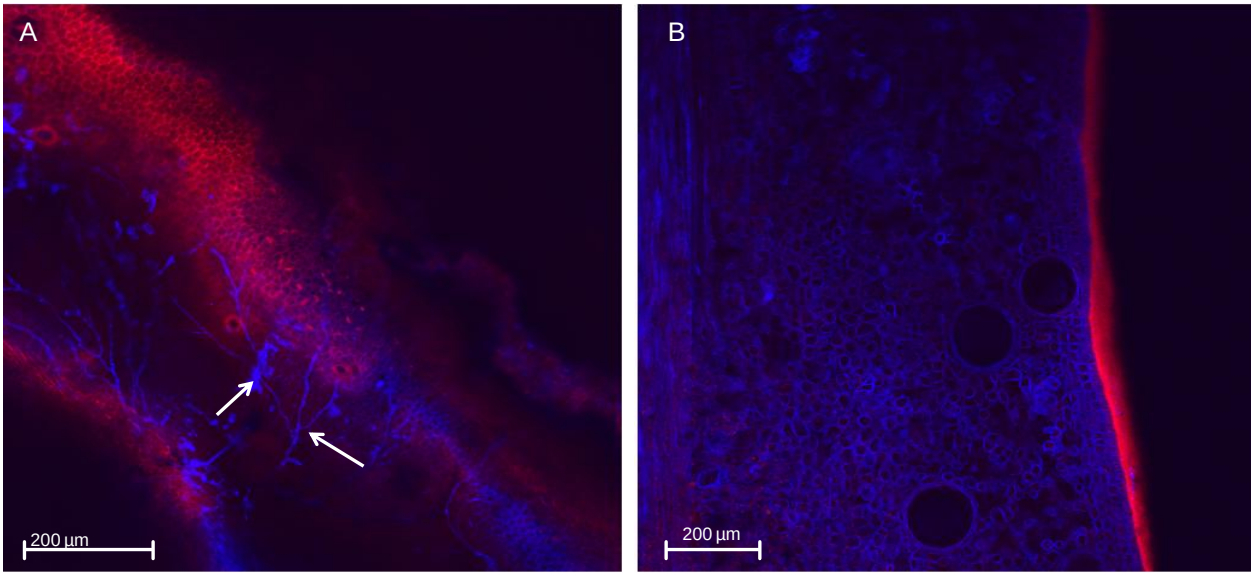

Figure 2.14 Longitudinal sections of *E. nitens* stem tissue (A) with white arrows indicating the presence of hyphae (blue thread-like structures) 48 hpi with *P. cinnamomi* (B) and the absence of hyphae in the mock-inoculated control.

2.5 Discussion

2.5.1 Identity of *Phytophthora* isolates

The establishment of a pathosystem requires a virulent pathogen and a susceptible host. The search for a virulent *Phytophthora* isolate with which to challenge *Eucalyptus* yielded fifteen *Phytophthora* isolates. These were found associated with dead and dying *Eucalyptus* trees in Tzaneen, a warm subtropical region in South Africa. Phylogenetic analysis of the ITS region places the isolates in clade 9, in close relation to *P.* sp. *lagoariana* and *P. parsiana* isolates Rf17 and C19 (Figure 2.3). FI5 and FI17 are probably distinct from the other isolates and CMW37797 belongs to a distinct clade with *P. parsiana* Rf17 (Figure 2.4). It is unlikely that they are *P.* sp. *lagoariana*, since the isolates belonging to this species group in a separate, well-supported clade.

Analysis of the β-*tub* region supported this distinction from *P.* sp. *lagoariana*, since CMW37797 and CMW37795 form a neighbouring, but once again, distinct clade. CMW37796 belongs to a clade containing all the *P. hydropathica* and *P. parsiana* isolates, but there was no resolution in this clade that could provide an idea of which species it is. The *EF*-1α region provided even less resolution, since all three of the above isolates clustered with *P. hydropathica, P. parsiana* and *P.* sp. *lagoariana*, so it is not useful to distinguish which species the isolates belong to. The *cox*I region indicated that CMW37796, CMW37797 and CMW37795 are associated with various *P. parsiana* isolates. The *nadh*1 region provided some clarity with regards to the relation of the isolates to *P. hydropathica,* since the *P. hydropathica* and unknown isolates group into two distinct and well-supported clades, suggesting that they are not the same species. *P. hydropathica* and *P. parsiana* cannot be compared in relation to the isolates based on mitochondrial gene regions since sequence data for the same region for both species is not available.

Many *Phytophthora* spp. belonging to clade 9 have been described in the last 15 years (Kroon *et al.*, 2012), and it could even be divided into various sub-clades in future (T. Burgess, personal communication). Species identification in the clade is difficult at this time though, since many species remain undescribed. It is likely that CMW37795, CMW37796 and CMW37797 represent three different species, especially considering that there are noticeable differences between them in culture morphology and virulence on *Eucalyptus*. Phylogenetic analysis indicates that they are probably not *P. hydropathica* or *P.* sp. *lagoariana*, but they may belong to a currently unknown or undescribed species. It is most likely that the isolates under consideration are part of the *P. parsiana* complex. The *P. parsiana* isolates described in the original paper by Mostowfizadeh-Ghalamfarsa *et al.* (2008) are best described as a species complex (Hong *et al.*, 2010; Kroon *et al.*, 2012), since some of the isolates are actually very

different from the type strain. The isolates from the current study group away from the type strain C25, but do show close relation to some of the other isolates belonging to the species complex (Rf17 and C19). There are three sub-clades visible in the original *P. parsiana* species description (Hong *et al.*, 2010; Mostowfizadeh-Ghalamfarsa *et al.*, 2008), and these same groups are also easily observable and very distinct in the ITS trees of this study, which lends further support to the *P. parsiana* complex hypothesis.

Since there is very large between-isolate variation in this study, the possibility exists that none of the isolates are a single species, but that they are hybrids. Hybrid formation is not uncommon to *Phytophthora* spp., and has been noted to a large extent in some of the clade 6 *Phytophthora* spp. (Burgess *et al.*, 2012; Burgess *et al.*, 2010; Kroon *et al.*, 2012). The possibility of the isolates being part of a hybrid swarm was investigated through analysis of ITS variability between isolates. Based on the results in this study, the isolates are not part of a hybrid swarm, unless the actual parental species have not been sequenced and described yet. If the two supposed parents (the phylogenetically closest, *P. parsiana* Rf17 and *P.* sp. *lagoariana*) were the parents, there would have been a clear inheritance pattern of parts of the ITS region from one parent or the other. There were some challenges with sequencing the ITS region directly from PCR product, since mixed signal is produced and sequencing of multiple clones is required in order to capture any allelic variance and to obtain a complete picture of the variability within the region, which was beyond the scope of the present study. Therefore, the possibility of these isolates belonging to a hybrid swarm cannot be ruled out completely.

2.5.2 Pathogenicity of *Phytophthora* isolates on *Eucalyptus*

CMW37795, CMW37796 and CMW37797 were found in association with the roots of dying *Eucalyptus* trees. The ability of these isolates to cause infection on *Eucalyptus* was confirmed by inoculating *E. nitens*, which is a host of *Phytophthora* in South Africa at 25°C. Lesions on CMW37796-inoculated stems did not vary significantly from the necrosis around the wound site of the controls, although it was successfully re-isolated from lesion margins in both replicates of the trial. Lesions caused by CMW37795 and CMW37797 were significantly larger than those of mock-inoculated control trees, but lesions caused by *P. cinnamomi* exceeded those of the unknown isolates by an average of several centimeters. Therefore *P. cinnamomi* remains the most aggressive *Phytophthora* spp. on *Eucalyptus* in South Africa to date (Linde *et al.*, 1994b; Maseko *et al.*, 2007). The variation in virulence between these isolates supports the possibility that they do not all belong to the same species. The slightly lower average lesion lengths recorded in the first trial (Figure 2.10 A) as opposed to the repeated trial (Figure 2.10 B) could be attributed to seasonal effects, since the first trial was performed in winter, and *Phytophthora* is able to infect better during summer (Maseko, 2010; Tippett *et al.*, 1983).

The ability of the *Phytophthora* isolates to cause disease on *E. nitens* through stem inoculations is not surprising, given the severity of the inoculation procedure, which can result in severe symptoms (Hüberli *et al.*, 2002). The use of stem inoculations makes it difficult to infer that the isolates are necessarily virulent to *Eucalyptus* in the field and are necessarily the causal agent of the disease. However, there is value in stem inoculations to prove a degree of virulence. Maseko *et al*. (2007) used this method to prove the virulence of two newly described *Phytophthora* spp. on *Eucalyptus*. In the current study, *E. grandis* remained highly tolerant to *Phytophthora* (Figure 2.12) so there are successful resistance mechanisms that remain in place in tolerant/resistant hosts despite the severity of the inoculation technique. A previous study by Hajebrahimi and Banihashemi (2011) tested the virulence of three *P. parsiana* isolates (one of which is C19, which is closely related to the isolates of this study) on *E. globulus* stems and found the interaction to be incompatible. The outcome is different from the current study, most likely since *E. globulus* is not known to be very susceptible to *Phytophthora*. It must also be kept in mind that the isolates investigated in this study could be an entirely different species from *P. parsiana* and would therefore also have a different host range. Lesion development by CMW37795 and CMW37797 is an indication that they have potential to cause disease, given favourable conditions and a susceptible host in the field.

A raised temperature (30°C) is the probable optimum for these isolates, since they are closely related to the high temperature species, *P. parsiana* and *P. hydropathica*. Furthermore, this temperature is similar to summer temperatures in the Tzaneen area where they were obtained. Inoculations with CMW37796 and CMW37797 at 30°C resulted in symptoms on the leaves of *E. nitens* trees that were similar to those observed on the dying trees the isolates were obtained from originally. These symptoms included brown spots on leaves, partial death of leaves, and mortality of some individuals. The same symptoms were not present in *P. cinnamomi*-inoculated plants to a noteworthy degree, and were completely absent in the control group. Raised temperature resulted in increased lesion lengths on *E. nitens,* but not a large difference in virulence on *E. grandis*, the tolerant host.

The increased virulence of CMW37796 and CMW37797 at 30°C is expected, since it is their probable optimum, and 30°C is at the far upper end of *P. cinnamomi*'s optimal temperature spectrum, which accounts for its increased virulence (Zentmyer *et al.*, 1976). Additionally, resistance can break down at higher temperatures, making the *Eucalyptus* more susceptible to *Phytopthora* (Hüberli *et al.*, 2002). It has been noted that increased temperatures associated with climate change may increase the impact of certain *Phytophthora* spp. on hosts (Brasier and Scott, 1994; Hardy, 2007). Since a higher temperature increased virulence of

Phytophthora isolates on *Eucalyptus* in this study, it is speculated that they could pose a threat to *Eucalyptus* plantations and natural vegetation in warm regions such as Tzaneen and possibly cooler regions, as climate change continues. Fortunately the eucalypts planted in warmer regions, such as *E. grandis* clones, are generally not prone to PRR and therefore will probably only be affected in very small, localized areas, such as waterlogged soil with young trees. To the best of our knowledge, this is the first report of any clade 9 *Phytophthora* spp. in South Africa causing disease on *Eucalyptus*.

Though the studies are not directly comparable, lesions in these trials (in the range of 5-7 cm) are slightly smaller compared to those from other South African studies using *P. cinnamomi*. Lesion averages of approximately 13 cm were observed on field-inoculated *E. grandis* and *E. dunnii* (Linde *et al.*, 1994b; Maseko *et al.*, 2007), and 10-30 cm in *E. marginata* (Tippett *et al.*, 1985). The smaller lesions caused by the unknown isolates could be simply due to the fact that they are not extremely virulent on *Eucalyptus*, and the lower virulence of the *P. cinnamomi* isolate could be attributed to the age of the culture used. Furthermore, it needs to be kept in mind that *E. nitens* is not considered to be very susceptible to PRR, since it does not belong to the *Monocalyptus* subgenus (Brown, 1977). As explained earlier, this is one of the most susceptible *Eucalyptus* spp. available for the study of PRR in South Africa, and susceptibility is most likely due to conditions in the field, which might not be adequately replicated under greenhouse conditions. *E. nitens* was proven far more susceptible than *E. grandis* in this study, so despite the age of the *P. cinnamomi* culture, this species was chosen as the most suitable inoculum due to its high virulence and numerous past scientific studies (Erwin and Ribeiro, 1996).

2.5.3 Establishment of a pathosystem for transcriptome profiling of host responses

The stem inoculation method used during the course of this study has proven highly repeatable and consistent. It provides numerous benefits with regards to sampling RNA for host responses. A large number of trees can be inoculated in a short space of time so that a time-course can be accurately conducted, the amount of inoculum can be controlled, the area which is sampled from each individual can be standardized, and the material is soil-free, making RNA extraction easier. RNA extraction from roots is difficult in some species due to the presence of high amounts of polyphenols and polysaccharides (Loomis, 1974; Salzman *et al.*, 1999) such as in *Camellia sinensis* and *Vitis* spp. (Gambino *et al.*, 2008; Muoki *et al.*, 2012). In *Eucalyptus* spp. root RNA extractions have proven problematic and the protocol has not been optimized in our laboratory, therefore stem material is a better option for profiling at this stage.

A suitable time-point to profile host responses was chosen based on several factors within this pathosystem. The chosen inoculum, mycelia, is probably slower in establishing disease than zoospores (Cahill *et al.*, 1989). It needs to be considered that different parts of the stem are in different stages of the infection, depending on distance from the site of inoculation. As infection progresses, tissue at the lesion margin should be initiating some of the early responses to infection. Tissue closer to the inoculation site will be further along the infection cycle and will display more advanced stages of defence responses. It has been proven that there is a definite difference in defence responses, depending on whether the area is close or distal to the inoculation site, and these responses are still occurring several days after inoculation (Deflorio *et al.*, 2011; Karlsson *et al.*, 2007).

Tissue sampled at 24 hpi would not provide sufficient contact of the host and pathogen to warrant profiling of responses at this time, since responses in the small infected area would be overshadowed by responses in the uninfected material, which is in the majority. Both the 48 hpi and 96 hpi time-points (or anything close to these) could be valuable for profiling host responses. Sampling at 48 hpi should provide information about a large number of early responses, but the majority of tissue will be healthy (especially since lesions usually only develop on the inoculated side of the stem). Healthy tissue will only display responses due to signals from neighbouring cells that have come into contact with the pathogen. The 96 hpi time-point ensures that most of the material sampled (approximately 50%) is displaying symptoms of contact with the pathogen, but there is still a fair amount of healthy tissue at the back of the stem (non-inoculated side) and above and below the lesion margins. Therefore material harvested at this time-point provides a range of responses, from early to late infection. Tissue sampled at one wpi would provide enough infected material, but the larger part of that is dead material where most of the defence responses have already played out. Lesion extension slows down somewhat after one wpi, but at this stage infection is still active and the host is still susceptible and unsuccessful in combating the pathogen. Time-points before one wpi provide an opportunity to see the attempted defence by the host and may even provide some insight into some targets that the pathogen is manipulating. Tree pathosystems often involve sampling points several days after inoculation, such as in *Pinus sylvestris* infected with *Heterobasidion annosum* and *Fagus sylvatica* and *P. citricola* (Adomas et al., 2007; Fleischmann et al., 2005). Thus, it is predicted that a later time-point around 96 hpi would be suitable for further investigation with RNA-seq.

2.6 Conclusion

A clade 9 *Phytophthora* species has been found to cause disease on stressed *Eucalyptus* seedlings in the Tzaneen region. The isolates cannot be positively identified, and further description of new clade 9 species, along with possible re-classification of some of the *P. parsiana* isolates, would enable more accurate molecular identification. The isolates are able to cause disease on *E. nitens* at 25°C, and disease symptoms are aggravated by raising the temperature to 30°C, which is the temperature in the pathogen's natural habitat. *P. cinnamomi* proved to be most virulent on *E. nitens*, so this host-pathogen combination has been chosen to provide stem tissue that can be profiled in order to elucidate host responses using RNA-seq during a late stage of infection.

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Chapter 3

Whole transcriptome analysis of *Eucalyptus nitens* host responses upon challenge with *Phytophthora cinnamomi*

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This chapter has been written in the format of a BMC Genomics article and the final manuscript is in prepreparation for submission. I performed all the analysis associated with the RNA-seq data and wrote the manuscript. Sitha Ramsuchit performed the inoculations, sampling and RNA extractions and purification. Noëlani van den Berg provided guidance and insight during decision making for the duration of the project. Alexander A. Myburg provided practical insights and expertise with regards to RNA-seq analysis. Sanushka Naidoo was the main supervisor of the project. Noëlani van den Berg, Sanushka Naidoo and Alexander A. Myburg critically reviewed the chapter.

3.1 Abstract

Phytophthora spp. are responsible for root and collar rot, stem cankers, and dieback of various economically important *Eucalyptus* spp. in South Africa. The molecular basis for resistance and susceptibility in *Eucalyptus* spp. upon challenge with *Phytophthora* spp. is still poorly understood and the aim of this study was to determine the transcriptional profile of a compatible host, *E. nitens*, challenged with *P. cinnamomi*. Eighteen *E. nitens* plants were challenged with the pathogen and at five days post inoculation, at which point the pathogen was confirmed to be in contact with the host tissue microscopically, total mRNA was isolated from stem tissue. RNA-sequencing was performed and approximately 78% of the reads mapped successfully to the *E. grandis* genome, whereafter fragments per kilobase of transcript per million fragments (FPKM) values were calculated for annotated gene models. There were 1475 significantly differentially expressed genes in inoculated tissue compared to mock-inoculated tissue, and those with associated putative TAIR10 orthologs were submitted for gene ontology (GO) overrepresentation analysis. GO terms of genes up-regulated upon inoculation were related to defence hormone pathways such as jasmonic acid, ethylene, salicylic acid, and flavonoid biosynthesis and water stress. Gene ontologies related to cell wall synthesis, growth hormones, lignin biosynthesis and photosynthesis were overrepresented in the dataset of genes down-regulated upon inoculation. Generally, *E. nitens* seems to be up-regulating defence responses, whereas the down-regulated genes associated with growth indicate that resources are shifted from growth to defence. Numerous putative defence genes and pathogenesis related (*PR*) genes were found in the up-regulated dataset, including the *PR-1*, *PR-3, PR-4* and *PR-5* putative orthologs which are all strongly up-regulated. A putative ortholog of a specific peroxidase gene, previously identified as a potential cross-species effector target in different *Phytophthora* pathosystems, was down-regulated in *E. nitens*. This may be one of the important factors influencing susceptibility, together with the way hormone regulation takes place, and possibly the late timing of correct defence responses. Comparison with a resistant host will indicate more clearly which factors underpin compatibility and resistance. Knowledge of these mechanisms may provide gene targets for genetic modification that will enhance tolerance to *Phytophthora* in eucalypts.

3.2 Introduction

Over 18 million hectares of *Eucalyptus* spp. are cultivated world-wide (FAO, 2006) and eucalypts contribute significantly to South Africa's economy as well (Godsmark, 2009). These trees are grown to provide pulpwood for paper, chemical cellulose, and essential oils (Hillis and Brown, 1983). Numerous pests and pathogens threaten the productivity of eucalypt plantations around the world (FAO, 2007), including the oomycete genus, *Phytophthora* (Wingfield *et al.*, 2011). In South Africa, susceptible cold-tolerant *Eucalyptus* plantations have been affected by various *Phytophthora* spp. to such an extent that some valuable species such as *E. fastigata* and *E. fraxinoides* are not cultivated anymore (Linde *et al.*, 1994a; Wingfield and Kemp, 1994).

To date, *Phytophthora cinnamomi* is one of the most virulent *Phytophthora* spp. causing root rot of eucalypts in South African plantations (Linde *et al.*, 1994b; Maseko *et al.*, 2007). *Eucalyptus nitens* belongs to the subgenus *Symphyomyrtus*, which is generally considered resistant to *P. cinnamomi* (Brown, 1977). However, some eucalypts that are resistant to *P. cinnamomi* in their native environment still succumb to the pathogen in South Africa (Cahill *et al.*, 2008), and *E. nitens* is one of these. *P. cinnamomi* has been isolated from diseased roots of this species (Maseko, 2010), and certain *E. nitens* stands are severely affected by *Phytophthora* root rot (J. Roux, personal communication).

P. cinnamomi is a devastating pathogen with a very broad host range (Shearer *et al.*, 2004). *Phytophthora* spp. include hemi-biotrophic pathogens (Hein *et al.*, 2009). *P. cinnamomi* has been called a necrotroph, but it may act as a hemi-biotroph under some conditions (Cahill *et al.*, 2008; O'Gara *et al.*, 1997). The pathogen releases zoospores from the roots of infected plants. Once a zoospore has recognized a host, attached and germinated, hyphae colonize the host (Hardham, 2005). *P. cinnamomi* infects best under flooding, or high water potential conditions (Tippett *et al.*, 1987). Hyphae primarily invade the phloem of eucalypts, and the xylem to a lesser extent (Davison *et al.*, 1994; Hardham, 2005). Infection with *P. cinnamomi* commonly causes die-back, root-rot and in some cases, collar-rot, most likely because it interferes with the plant's transpiration (Hardham, 2005).

The recent expansion in genomics resources for *Eucalyptus* could be key to successfully combating pathogens such as *Phytophthora*. The *E. grandis* v1.1 genome has been sequenced with eight times coverage and 36376 gene models have been identified (publicly available at www.phytozome.net/eucalyptus.php). *Eucalyptus* genetics have been explored with regards to gene expression and quantitative trait loci (QTL) of wood properties and some biotic and abiotic stresses (Grattapaglia and Kirst, 2008; Neale and Kremer, 2011). Recently

some full transcriptome sequencing studies have been performed, with the first NGS RNA-sequencing (RNA-seq) and whole-transcriptome assembly performed on an *E. grandis* x *E. urophylla* hybrid (Mizrachi *et al.*, 2010; Villar *et al.*, 2011). A study investigating the host responses of *E. grandis* upon challenge with the gall wasp, *Leptocybe invasa*, has been performed (C. Oates, personal communication) and serves as the first RNA-seq study of biotic stresses in *Eucalyptus* spp.

RNA-seq has contributed knowledge toward several host responses during pathogen challenge. This includes the responses of *Arabidopsis thaliana*, soybean, wheat, cotton, citrus and cucumber to their respective pathogens (Dowen *et al.*, 2012; Martinelli *et al.*, 2012; Savory *et al.*, 2012; Tremblay *et al.*, 2012; Xu *et al.*, 2012; Xu *et al.*, 2011). Two host response studies to *Phytophthora* spp. have been undertaken to date. The host responses of raspberry to *P. rubi*, and those of potato tubers to *P. infestans* have been successfully profiled using RNA-seq (Gao *et al.*, 2013; Ward and Weber, 2012).

RNA-seq provides an overview of the entire interaction transcriptome, which makes it possible to identify major trends and to compare the regulation of different components of defence within a single experiment. This type of analysis provides a detailed picture of a host plant's responses to a pathogen, which tells us what artillery the plant has at its disposal, and what its preferred defence mechanisms under particular conditions are. Host defence responses occur at different levels and there are numerous different mechanisms involved (Agrios, 2005). The timing and degree to which these responses are activated could determine the outcome of the interaction between a plant and pathogen (Tao *et al.*, 2003). An advantage of RNA-seq data over microarrays is that it provides information about the transcripts differentially expressed by the pathogen. Differentially expressed genes produced by the pathogen during a successful infection could reveal a lot about virulence factors, and it is a valuable tool to elucidate effectors and information concerning other *Phytophthora* life-style mechanisms such as the switch from biotrophy to necrotrophy (Pais *et al.*, 2013).

Hormone signaling is a key part of defence, and the role of hormones such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) has been studied in detail. These hormones control defence networks, including the expression of pathogenesis-related (*PR*) genes (Bari and Jones, 2009; Pieterse *et al.*, 2009; Sudisha *et al.*, 2012). In a simplified model, JA and SA form the backbone of defence signaling, and they are modulated by ET, auxin and abscisic acid, which are SA-antagonists, as well as cytokinins and gibberellic acid (GA) which negatively affect the JA pathway (Pieterse *et al.*, 2012). Though these SA and JA modulators are responsible for growth and metabolic processes as well, their role in defence has come to the

forefront in recent years (Robert-Seilaniantz *et al.*, 2011). The exact hormones involved in resistance to *Phytophthora* spp. has not been elucidated, since hormone regulation varies between the different pathosystems (Attard *et al.*, 2010; Halim *et al.*, 2009; Kawamura *et al.*, 2009; Moy *et al.*, 2004).

PR gene expression is closely linked with hormone signaling. In *A. thaliana*, *PR-1*, *PR-2* and *PR-5* are considered to be markers of the SA pathway, while *PR-3*, *PR-4* and *PDF1.2* are considered JA markers (Thatcher *et al.*, 2005; Thomma *et al.*, 1998). However, this is not always the case, since Ferreira (2007) also mentions that each class has an acidic and basic subclass, and that SA could regulate the acidic PR proteins, while JA regulates the basic ones. *PR* genes are usually induced upon contact with a pathogen and have varying mechanisms of action, including direct antimicrobial activity (Ferreira *et al.*, 2007). Multiple *PR* gene classes are differentially regulated against *Phytophthora* and are thought to be important for successful defence (Attard *et al.*, 2010; Moy *et al.*, 2004; Schlink, 2009) and the overexpression of specific *PR* genes have conferred tolerance against various *Phytophthora* spp. (Acharya *et al.*, 2013; Alexander *et al.*, 1993; Fagoaga *et al.*, 2001; He *et al.*, 2013; Pushin *et al.*, 2010; Sarowar *et al.*, 2009).

The phenylpropanoid pathway is responsible for synthesis of metabolic and defence compounds. Its role in defence includes involvement in SA biosynthesis, production of phenolic compounds such as lignin, which strengthen cell walls against pathogen invasion, and flavonoid production, which includes the production of flavonoid phytoalexins (Dixon, 2001; Hématy *et al.*, 2009; Vogt, 2010). Phytoalexins may play an important role in *Phytophthora*-host interactions, as seen in the need for camalexin-mediated resistance in *A. thaliana* against *P. brassicae* and *P. capsici* (Schlaeppi *et al.*, 2010; Wang *et al.*, 2013). The phenylpropanoid pathway is also involved in cell wall fortification by lignin and protein components that are oxidatively polymerized by peroxidases, making them stronger and less susceptible to hyphal penetration (Brisson *et al.*, 1994; Naoumkina *et al.*, 2010).

The host responses elicited by *P. cinnamomi* have been studied in various species. This includes *Eucalyptus* spp. (Cahill *et al.*, 2008), maize (Allardyce *et al.*, 2013), avocado (Mahomed and Van den Berg, 2011), *A. thaliana* (Rookes *et al.*, 2008) and chestnut (Dinis *et al.*, 2011). Some of the major findings in these studies involve the importance of correctly regulated hypersensitive response and reactive oxygen species and synthesis of phenylpropanoid pathway-related substances such as flavonoids and lignin. *PR*-1 and *PR*-5 feature in several interactions as possible resistance factors. Eucalypts specifically, display early water-stress during infection and this is linked to lowered cytokinin levels and possible

disturbance of ABA levels (Cahill *et al.*, 1986).

Successful defence by a host plant often depends on the timing of defence responses, as well as the degree to which the responses are activated. Compatible and incompatible interactions often reveal that similar defence components are regulated upon pathogen challenge, but the timing of compatible interactions is often delayed and the magnitude of responses is smaller than the incompatible interaction (Desender *et al.*, 2007; Gao *et al.*, 2013; Huibers *et al.*, 2009; Tao *et al.*, 2003). This failed attempt at defence provides an opportunity to observe the defence artillery activated by the host, which could be very similar to a resistant interaction.

Despite the similarities, certain components of a compatible interaction may differ quite notably from the resistant interaction. Pathogens are able to manipulate their hosts by means of effectors, and thus target certain host defence components (Cheng *et al.*, 2012; Jones and Dangl, 2006; Schlink, 2010). The difference between host down-regulation and manipulation by the pathogen cannot be clearly determined in just a susceptible interaction, but often, manipulation is observed as down-regulation of certain defence responses that are expected to be up-regulated. For example, *P. infestans* manipulates its host to suit its life-style by suppressing the hypersensitive response (HR) in potato during its biotrophic phase, and then manipulating the induction of HR during the necrotrophic phase (Bos *et al.*, 2010; Gilroy *et al.*, 2011).

This study aimed to profile the transcriptional responses activated by *E. nitens* upon challenge with *P. cinnamomi*. The use of RNA-seq provided the first glimpse of the whole transcriptome response of the compatible *E. nitens-P. cinnamomi* interaction. Previous work (see chapter 2) has confirmed the establishment of *P. cinnamomi* in *E. nitens* stem tissue by a late time-point (four days post-inoculation). It is hypothesized that the stem tissue sampled at a late time-point will contain a variety of differentially regulated genes related to a late attempted defence response. Particularly, regulation of *PR* genes, hormone signaling and the phenylpropanoid pathway is expected, along with some host target manipulation by the pathogen.

3.3 Materials and Methods

3.3.1 Inoculated plant material

Eucalyptus nitens cuttings obtained from Sappi Forests Research, Shaw Research Centre, KwaZulu-Natal, were maintained and stem inoculated with *P. cinnamomi* CMW26310 as in section 2.3.1.2 and 2.3.8.1 respectively. Stems were inoculated twice, 10 cm apart. For both the mock-inoculated control and inoculated trees, stem tissue was harvested from 18 trees (which consisted of three pooled biological replicates of six trees each) at 5 days post inoculation (dpi). Sampling was performed in a balanced block design. Three centimetres of stem tissue was harvested per inoculation site, with 1.5 cm of stem tissue below and above the centre of the site. Harvested material was immediately frozen in liquid nitrogen and stored at -80°C.

Since sampling was destructive, nine extra trees were used to observe symptom development for six weeks following inoculation. Beneath-bark lesions were measured in other trials to statistically validate the effectivity of inoculation. For these lesions, a Shapiro-Wilk test was performed in GraphPad Prism 6 (Motulsky, 1999) and the non-parametric Mann-Whitney test was used to assess whether inoculated plants had significantly larger lesions than mock-inoculated plants at a 95% confidence level.

3.3.2 RNA extraction and quality analysis

A modified cetyltrimethyl ammonium bromide (CTAB) RNA extraction protocol was performed on 3 g tissue per sample, with the chloroform:isoamyl alcohol extraction step repeated three times (Chang *et al.*, 1993). On-column DNAse treatment with 10 units DNaseI (Fermentas, Ontario, Canada) and RNA purification was performed with the RNeasy® Mini kit (Qiagen, Valencia, California). The purified RNA was eluted with 60 µl RNase free water. One µg total RNA (based on Nanodrop concentration values) was resolved on a 2% (w/v) agarose gel to confirm the presence of intact rRNA subunits and further quality analysis was done on a 2100 Bioanalyzer (Agilent, Santa Clara, California).

First strand cDNA synthesis was performed on 2 µg RNA, using the ImProm-II™ Reverse Transcriptase kit (Promega, Madison, Wisconsin, USA) with a modified protocol and 1 µg random hexamer primer (Invitrogen Life Technologies, Ontario, Canada). The 14 µl template-primer mixture was incubated at 70°C for 5 min, and 4°C for 5 min. Reverse transcription was performed by mixing 1X reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTP's, 1 U/µl RiboLock™ (Fermentas) and 2 µl reverse transcriptase to a final volume of 26 µl. After adding the template-primer mixture, incubation at 25°C for five min, 42°C for 1 hr, and 70°C for 15 min completed the protocol.

A check for gDNA contamination was performed using an intron-spanning primer pair that targeted the EgrADP ribosylation factor gene. Two µl cDNA template each for control (C1, C2, C3) and inoculated (I1, I2, I3) biological replicates was added to a 23 µl PCR reaction mix containing 5 µl 5X buffer (Bioline, London, UK), 0.5 U MyTaq™ (Bioline) and 10 mM each of forward (5'-TTCTGGTGCCATGCTGAGAA-3') and reverse (5'-GATGCTGTGTTGCTCGTCTT-3') primers. A no-template control and positive *E. nitens* gDNA control was included. PCR cycling conditions were as follows: denaturation for 2 min at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension of 7 min at 72°C. The PCR products were resolved on a 1% (w/v) agarose gel alongside a 100 bp O'GeneRuler (Fermentas) and visualized under UV light.

3.3.3 RNA sequencing

Approximately 20 µg total RNA for each sample was submitted for sequencing at Beijing Genomics Institute (BGI, Beijing, China). mRNA sequencing was performed with random fragmentation of the mRNA and adapter ligation. Fifty bp reads were obtained using an Illumina HiSeq 2000 (Illumina, San Diego, CA).

3.3.4 Bioinformatic analysis

3.3.4.1 Quality analysis and filtering

Adaptors, low quality reads, and reads with more than 10% unknown nucleotides were removed from the dataset by BGI. The Galaxy platform (Blankenberg *et al.*, 2010b; Giardine *et al.*, 2005; Goecks *et al.*, 2010) was used to analyze and process the RNA-seq reads. FASTQ Groomer (Blankenberg *et al.*, 2010a) and FASTQC (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) were used to format reads into the required format and to assess read quality.

3.3.4.2 Mapping and transcript expression analysis

Mapping to the *E. grandis* v1.1 genome was done with Bowtie (Langmead *et al.*, 2009) and Tophat v1.3.1 (Trapnell *et al.*, 2009), allowing for 2 bp mismatches per 50 bp read and a maximum intron length of 10000 bp. Mapping statistics were verified using SAMtools flagstat (Li *et al.*, 2009). Assembly of mapped reads and calculation of expression values of predicted *E. grandis* transcripts as fragments per kilobase of transcript per million fragments (FPKM) was performed by Cufflinks (Trapnell *et al.*, 2010). Significant differential expression analysis was done with Cuffdiff (Trapnell *et al.*, 2010), where parameters settings were changed to allow for a minimum alignment count of 1000, a false discovery rate of 0.05, as well as quartile normalization and bias correction. Additional parameter settings were set to an average fragment length of 49 bp and a standard deviation of 10 bp for the fragment lengths.

3.3.4.3 Gene ontology overrepresentation analysis

Microsoft Excel 2007 (Microsoft, Redmond, WA) was used to match significantly differentially expressed *E. grandis* v1.1 gene and transcript models to TAIR10 and TAIR9 identifiers based on a previous reciprocal BLAST analysis. The significantly differentially expressed genes (and transcripts for which the representative gene model was not differentially expressed) with TAIR10 putative orthologs were divided into up- and down-regulated datasets. These were submitted for gene ontology (GO) overrepresentation analysis in BiNGO v2.44 (Maere *et al.*, 2005) using the Cytoscape v2.8.3a platform (Shannon *et al.*, 2003). Overrepresentation was evaluated against the *A. thaliana* genome in the categories for "biological processes," "molecular function," and "cellular component." A hypergeometric test with a Benjamini & Hochberg FDR correction of 0.05 was used. Output for the GO biological processes category was submitted to REViGO (Supek *et al.*, 2011) for a simplified visual representation. Understanding of biological pathways was aided by MapMan v3.5.1R2 (Thimm *et al.*, 2004) which placed the differentially expressed TAIR10 ID's in context of various metabolic pathways such as biotic stress and cell functions. MapMan was used to map TAIR10 ID's to the secondary metabolism pathway and phenylpropanoid pathway involved in lignin synthesis. These figures were used to derive a table and detailed diagram of the lignin biosynthesis pathway overlaid with the putative orthologs from *E. nitens* that were differentially expressed.

3.3.4.4 *PR* gene comparative literature search

Putative differentially expressed *Eucalyptus PR* gene orthologs were selected from the differentially up- and down-regulated datasets based on the descriptions of the matching TAIR annotations. The putative *PR* genes from this study were compared to other studies investigating host responses to *P. cinnamomi* where expression of the same TAIR ID (based on reciprocal BLAST analysis) was noted. Additionally, a putative cross-species effector peroxidase target, was identified in three previous *Phytophthora*-host interactions (Porter *et al.*, 2009). The ortholog of *Carica papaya* (EL784270), was submitted for BLASTn analysis using default parameters on Phytozome v9.1 (Goodstein *et al.*, 2012) to determine whether any of the *E. grandis* genes are potential orthologs.

3.4 Results

3.4.1 Inoculation with *Phytophthora cinnamomi*

Phytophthora cinnamomi-infected tissue for RNA-seq profiling was obtained by inoculating the stems of *E. nitens* seedlings in two places with agar plugs. At 5 days post inoculation (dpi), slight oxidation was visible around inoculation sites of control plants, whereas lesions of inoculated plants extended to the boundaries of the three centimeter sampled area (Figure 3.1 A). Lesions developed on one side of the stem only, so by harvesting the whole section of stem a range of responses was captured – from the five day-colonized necrotic tissue in the centre, to the lesion margin where the *P. cinnamomi* was still actively extending the lesion, as well as healthy tissue on the uninfected side of the stem.

Since destructive sampling was used, expected symptom development after six weeks was confirmed on some un-sampled trees. The method of inoculation was well established prior to the current trial. The efficacy of inoculation was verified by the presence of hyphae in stem tissue at 48 hpi and lesion development over a four day time-course (refer to Figures 2.13 and 2.14). Additionally, clear lesions were visible at an advanced time-point (Figure 3.1 B).

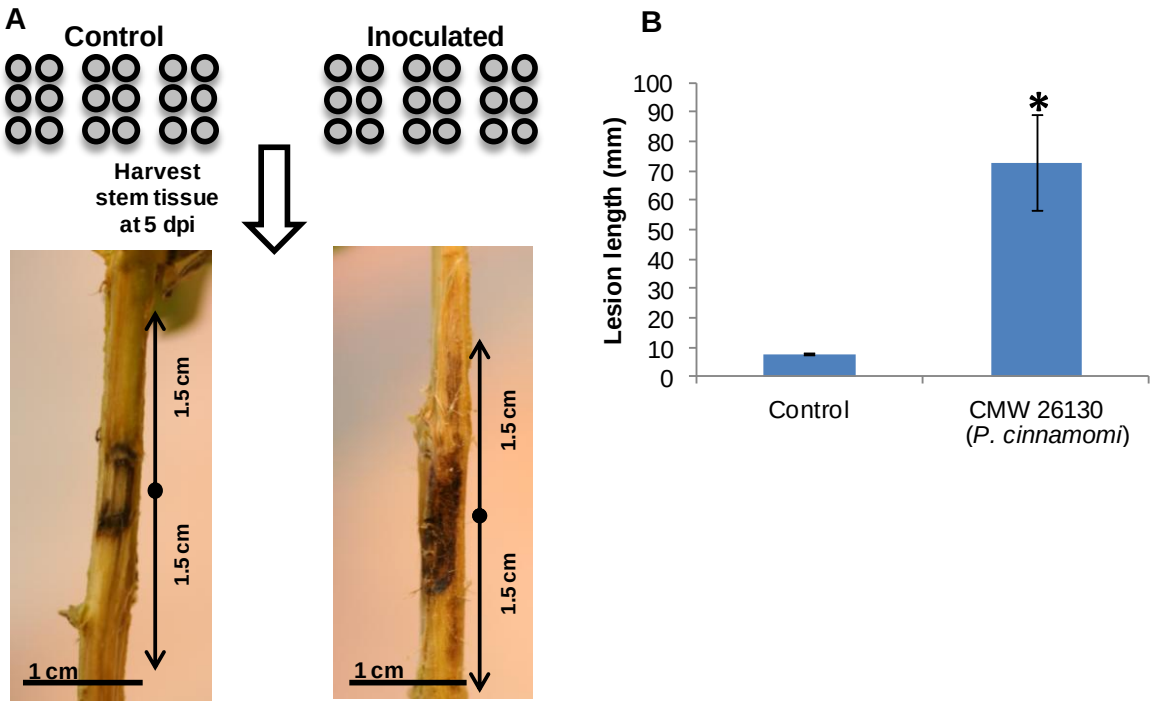

Figure 3.1 (A) Schematic overview of the sampling strategy. Six *E. nitens* individuals (represented by circles) were pooled per biological replicate, with three biological replicates each for inoculated and control. A section of 1.5 cm stem tissue was harvested below and above the site of inoculation (two inoculation sites per tree) at 5 dpi. **(B)** Lesions on *E. nitens* seedlings 6 wpi with CMW26310 (*P. cinnamomi*). The * indicates that lesions on inoculated seedlings were significantly larger than the mock-inoculated negative control at $p<0.05$ using the Mann-Whitney test for non-parametric data. Error bars show standard error based on n=12 replicates.

3.4.2 RNA quality validation

Total RNA was successfully isolated from the three biological replicates of control and inoculated stem tissue. RNA Integrity Number (RIN) values for RNA samples were in the acceptable range for RNA-seq (Table 3.1). Gel electrophoresis revealed intact 28S and 18S ribosomal subunits, which served as further confirmation that the RNA was undegraded (Figure 3.2 A). The intron-spanning primer pair of *EgrADP* amplified a ~250 bp fragment in cDNA samples, whereas the ~1.5 kb intron-containing fragment was amplified only in the genomic DNA positive control (Figure 3.2 B). This indicated that there was no residual genomic DNA in the cDNA, or the RNA that the cDNA was made from.

Table 3.1 Agilent Bioanalyzer quality control values for total RNA samples of *E. nitens*

Sample name	Total RNA concentration (ng/µl)	RIN	28S:18S	OD 260/280	OD 260/230
Control 1	2022	9.1	1.2	2.2	2.42
Control 2	682	8.3	1.1	2.09	1.85
Control 3	2132	9.1	1.6	2.15	2.18
Inoculated 1	1340	9.7	1.6	1.98	1.93
Inoculated 2	1226	9.5	1.5	2.16	2.06
Inoculated 3	1758	9.7	1.6	2.15	2.22

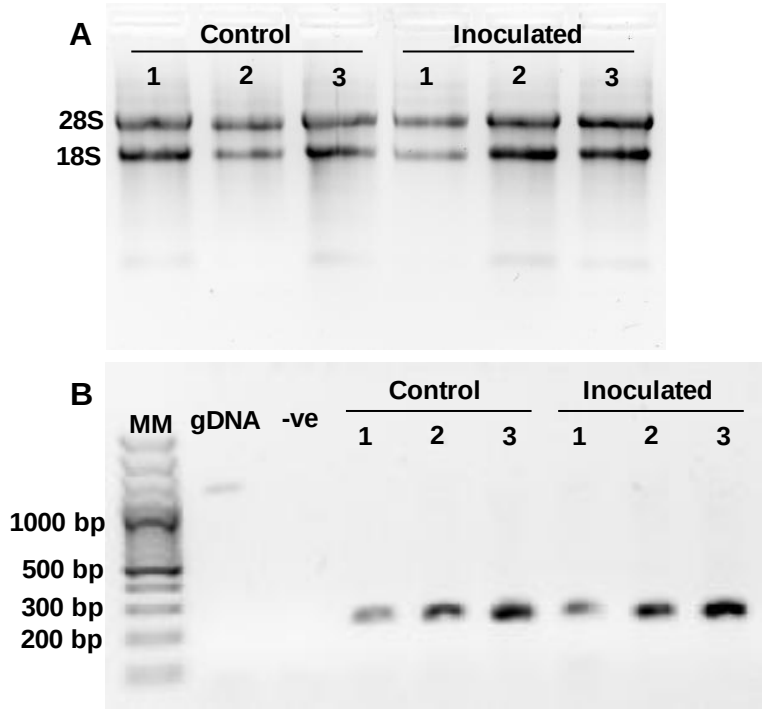

Figure 3.2 Quality control agarose gel images of (A) total RNA for the six samples analyzed with RNA-seq (B) *EgrADP* PCR amplicons to check for residual genomic DNA within cDNA samples. MM – Fermentas O'GeneRuler 100 bp ladder; gDNA – genomic DNA; 1, 2, 3 represents the different biological replicates for the control and inoculated samples; -ve indicates the no-template control.

3.4.3 RNA-sequencing and mapping

After the RNA was sequenced, quality analysis was performed on the reads. A quality phred score was assigned to each nucleotide, and these were summarized in boxplots, where a boxplot was constructed for each base-pair position of all the reads in a sample. Figure 3.3 shows the quality of the forward and reverse reads of control 1 in a boxplot distribution. Other samples show similar distributions (Appendix E). The reads did not require trimming, since the median values at each nucleotide position were far above the quality score threshold of 20.

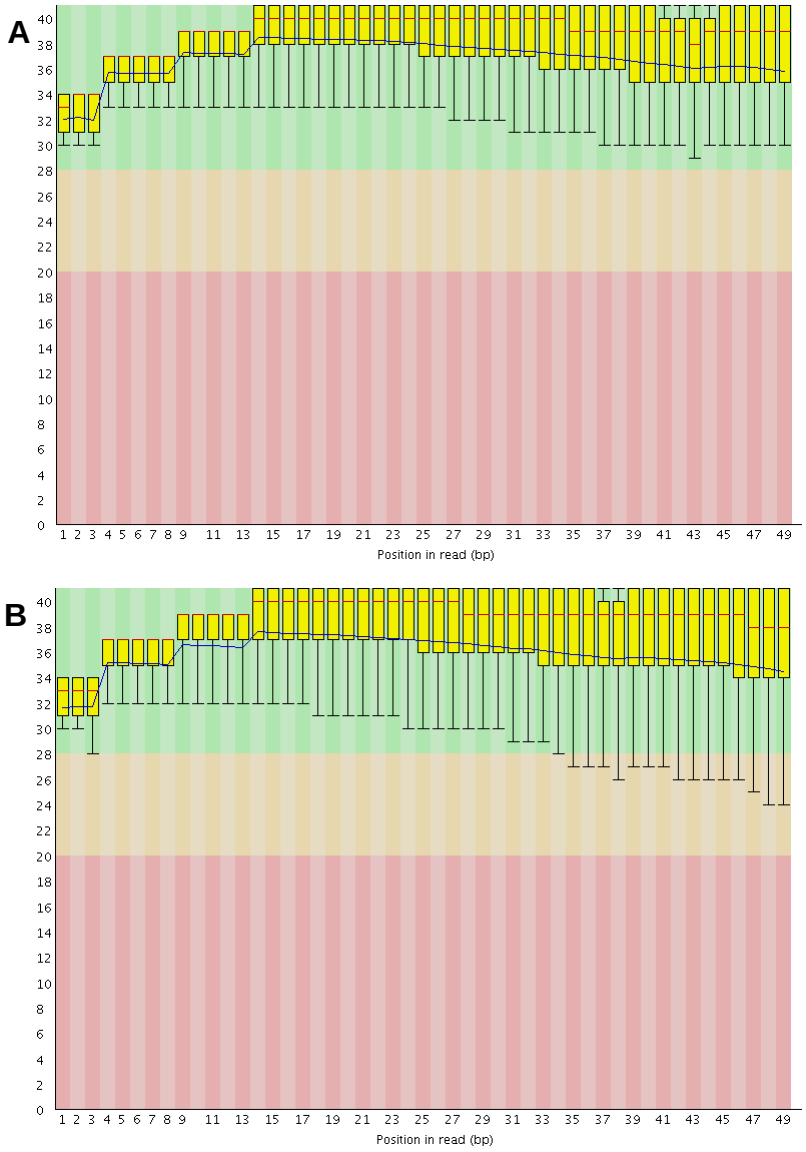

Figure 3.3 FastQC boxplots depicting the quality of RNA-seq read data for control biological replicate 1 (A) forward and (B) reverse reads. The y-axis represents quality scores whereas base pair position is plotted on the x-axis.

Approximately 36 million forward/reverse reads were obtained per sample, and Tophat was used to map the reads to the *E. grandis* genome. About 67% of the reads mapped as proper pairs, and another ~11% mapped as singletons (Table 3.2). Therefore, approximately 78% of the *E. nitens* transcripts mapped to the *E. grandis* genome, which is within the expected range. The number of expressed genes and the average FPKM values were similar across samples, which provided confidence that the samples were treated in a consistent manner and the results are comparable across data sets.

Table 3.2 Flagstat and FastQC RNA-seq statistics after mapping the *E. nitens* reads to the v1.1 *E. grandis* genome.

Sample name	Total reads	Properly paired (%)[1]	Singletons mapped (%)[2]	GC content (%)	Expressed genes	Average FPKM
Control 1	37444809	66.44	10.24	50	29024	493598
Control 2	36111678	68.02	10.16	50	29250	492972
Control 3	37060251	68.76	8.9	49	29135	467006
Inoculated 1	37234371	66.13	11.65	49	29429	471923
Inoculated 2	36622434	67.30	12.48	49	29407	497171
Inoculated 3	36022978	68.19	10.18	49	29576	473466

[1] Number of proper pairs in proportion to the total reads mapped
[2] Number reads where one from a pair in proportion to the total mapped

3.4.4 Differentially expressed genes

After mapping to the genome, Cuffdiff was used to compare pathogen-inoculated samples with the mock-inoculated controls. More gene and transcript models were significantly differentially up-regulated than down-regulated at a false discovery rate (FDR) of 0.05 (hereafter use of the terms “up-regulated” and “down-regulated” implies significant differential expression). Annotated Cuffdiff and Cufflinks files, as well as annotated lists of all the significantly differentially expressed genes are available in Appendix F and G respectively. A histogram depicting the frequency of log2 fold change categories can be found in Appendix H.

Of the 890 up-regulated and 585 down-regulated gene models (859 and 557 transcript respectively), only ~25% did not have an assigned TAIR10 ID, which meant those could not be used in BiNGO and MapMan (Figure 3.4). There are multiple transcripts matching certain gene models due to alternative splicing. Differential expression of a particular gene model is taken into account using expression data from all transcripts for that gene. Separate differential expression of transcripts was also taken into account, and the trends were very similar to that of the gene models.

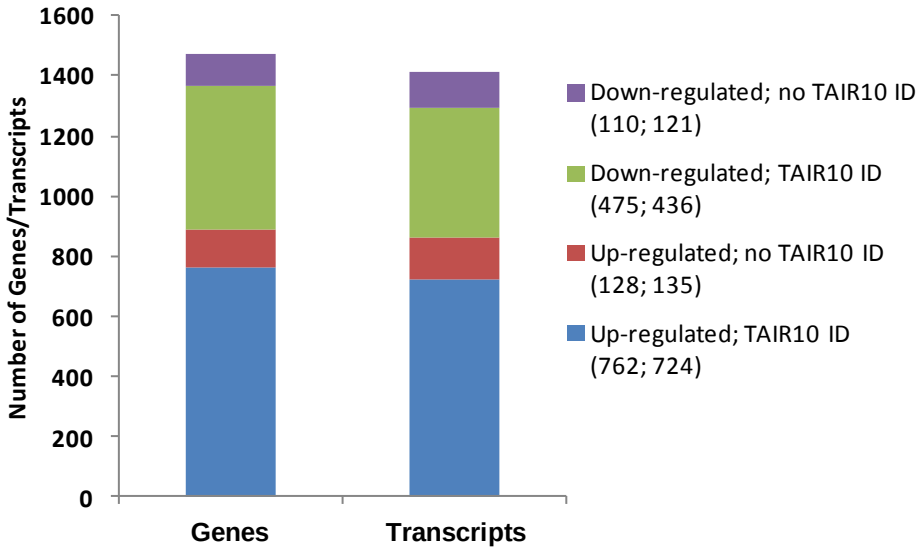

Figure 3.4 Number of gene models and transcripts that are significantly up- or down-regulated in inoculated versus control *E. nitens* stems. Some *E. grandis* gene models do not have an associated TAIR10 ID and the number without IDs in this data subset is indicated. Exact numbers are indicated in brackets of the figure legend with the number of genes first; transcripts second.

Among the top up-regulated genes (Table 3.3) were putative orthologs of five different thaumatin (AT1G20030) and two osmotin (AT4G11650) *E. grandis* genes, which are members of the *PR-5* family. Though there are different *E. grandis* gene models for these, they were annotated to the same TAIR accessions. The presence of seven *PR-5* genes within the top up-regulated genes indicates that regulation of this defence class may be of importance in this interaction. There were also five RmlC-like cupin superfamily genes (AT3G05950 and AT5G39160; which bind manganese, and contain a germin domain), as well as three Kunitz family trypsin and protease inhibitor proteins (AT1G17860), which are implicated in defence.

Some defence-associated putative orthologs were found in the down-regulated top table as well (Table 3.4). These include a major facilitator involved in response to JA (AT2G26690), a UDP-Glycosyltransferase (AT1G22400) involved in HR, SA and JA response, a metallothionein (AT3G09390), an integrase-type DNA-binding protein (AT1G19210) which is responsive to chitin, a phloem protein (AT4G19840) involved in flavonoid synthesis and the HR, an MLP-like protein (AT1G24020) involved in defence, and lastly, a disease resistance-responsive protein (AT4G23690) involved in defence, sterol and lignan production. There were also putative orthologs related to secondary cell wall, growth and xylan synthesis. The full table of differentially up- and down-regulated genes can be found in Appendix G.

Table 3.3 Top-table of 30 up-regulated gene models in *E. nitens* challenged with *P. cinnamomi*.

Gene	log2 (fold change)	p-value	q-value	TAIR ID	TAIR description
Eucgr.H00312	7.38	1.61E-13	3.31E-12	None	None
Eucgr.I01194	7.34	0	0	AT3G19000.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Eucgr.H04195	6.46	0	0	AT3G05950.1	RmlC-like cupins superfamily protein
Eucgr.B03654	5.90	0	0	AT2G44480.1	Beta glucosidase 17
Eucgr.E01382	5.42	0	0	AT1G20030.2	Pathogenesis-related thaumatin superfamily protein
Eucgr.L02860	5.32	0	0	AT5G12020.1	17.6 kDa class II heat shock protein
Eucgr.E01384	5.31	0	0	AT1G20030.2	Pathogenesis-related thaumatin superfamily protein
Eucgr.E01463	5.28	0	0	AT2G45220.1	Plant invertase/pectin methylesterase inhibitor superfamily
Eucgr.H00189	5.23	0	0	AT4G24000.1	Cellulose synthase like G2
Eucgr.L00636	5.23	0	0	AT4G15550.1	Indole-3-acetate beta-D-glucosyltransferase
Eucgr.E01389	5.23	0	0	AT1G20030.2	Pathogenesis-related thaumatin superfamily protein
Eucgr.A01726	5.20	0	0	AT3G22060.1	Receptor-like protein kinase-related family protein
Eucgr.C03071	5.19	0	0	AT5G12020.1	17.6 kDa class II heat shock protein
Eucgr.E01385	5.13	0	0	AT1G20030.2	Pathogenesis-related thaumatin superfamily protein
Eucgr.G03072	5.10	0	0	AT2G40180.1	Phosphatase 2C5
Eucgr.H03863	5.09	0	0	AT4G11650.1	Osmotin 34
Eucgr.J01959	5.03	0	0	AT5G59720.1	Heat shock protein 18.2
Eucgr.G02100	5.00	0	0	AT1G14870.1	PLANT CADMIUM RESISTANCE 2
Eucgr.K02326	4.97	0	0	AT1G17860.1	Kunitz family trypsin and protease inhibitor protein
Eucgr.L03158	4.97	0	0	AT1G17860.1	Kunitz family trypsin and protease inhibitor protein
Eucgr.E01381	4.96	0	0	AT1G20030.2	Pathogenesis-related thaumatin superfamily protein
Eucgr.H03865	4.93	0	0	AT4G11650.1	Osmotin 34
Eucgr.K02323	4.89	0	0	AT1G17860.1	Kunitz family trypsin and protease inhibitor protein
Eucgr.H04192	4.88	0	0	AT3G05950.1	RmlC-like cupins superfamily protein
Eucgr.H04176	4.87	0	0	AT5G39160.1	RmlC-like cupins superfamily protein
Eucgr.H04193	4.86	0	0	AT5G39160.1	RmlC-like cupins superfamily protein
Eucgr.F00032	4.82	0	0	AT2G15780.1	Cupredoxin superfamily protein
Eucgr.H03864	4.77	0	0	AT4G11650.1	Osmotin 34
Eucgr.H04194	4.74	0	0	AT5G39160.1	RmlC-like cupins superfamily protein
Eucgr.C03541	4.70	0	0	AT2G06050.3	Oxophytodienoate-reductase 3

Table 3.4 Top-table of 25 down-regulated gene models in *E. nitens* challenged with *P. cinnamomi*.

Gene	log2 (fold change)	p-value	q-value	TAIR ID	TAIR description
Eucgr.K00177	-3.62	5.03E-13	9.57E-12	AT1G65680.1	Expansin B2
Eucgr.J00937	-2.70	0	0	AT1G15480.1	Tetratricopeptide repeat (TPR)-like superfamily protein
Eucgr.C04214	-2.67	1.45E-13	3.00E-12	AT2G26690.1	Major facilitator superfamily protein
Eucgr.F02269	-2.41	3.34E-08	3.53E-07	AT5G59845.1	Gibberellin-regulated family protein
Eucgr.J01276	-2.40	4.67E-12	8.00E-11	AT1G22400.1	UDP-Glycosyltransferase superfamily protein
Eucgr.L00264	-2.40	2.44E-10	3.32E-09	AT1G09240.1	Nicotianamine synthase 3
Eucgr.H04069	-2.35	4.51E-09	5.29E-08	AT1G47960.1	Cell wall / vacuolar inhibitor of fructosidase 1
Eucgr.H04336	-2.28	5.99E-09	6.95E-08	AT5G43700.1	AUX/IAA transcriptional regulator family protein
Eucgr.J02054	-2.27	1.44E-14	3.34E-13	AT3G09390.1	Metallothionein 2A
Eucgr.K02050[1]	-2.20	3.19E-08	3.62E-07	AT3G10080.1[2]	RmlC-like cupins superfamily protein
Eucgr.G00485	-2.20	4.81E-11	7.15E-10	AT2G23690.1	Unknown protein; Involved in: N-terminal protein myristoylation
Eucgr.G00483	-2.19	2.40E-10	3.28E-09	AT2G23690.1	Unknown protein; Involved in: N-terminal protein myristoylation
Eucgr.L02839	-2.17	1.23E-08	1.38E-07	AT1G47960.1	Cell wall / vacuolar inhibitor of fructosidase 1
Eucgr.C00780	-2.16	3.91E-08	4.07E-07	AT1G19210.1	Integrase-type DNA-binding superfamily protein
Eucgr.G01889	-2.15	9.95E-11	1.43E-09	AT2G23690.1	Unknown protein; Involved in: N-terminal protein myristoylation
Eucgr.E01070	-2.14	1.20E-11	1.95E-10		
Eucgr.H02288	-2.12	3.14E-10	4.19E-09	AT4G19840.1	Phloem protein 2-A1
Eucgr.J00938	-2.12	3.49E-13	6.91E-12	AT5G60490.1	FASCICLIN-like arabinogalactan-protein 12
Eucgr.B02546	-2.09	6.68E-11	9.83E-10	AT1G24020.2	MLP-like protein 423
Eucgr.K02050	-2.09	0	0	AT3G10080.1[2]	RmlC-like cupins superfamily protein
Eucgr.F01203	-2.09	0	0	AT3G15050.1[2]	IQ-domain 10
Eucgr.G00484	-2.04	4.02E-07	3.48E-06	AT2G23690.1	Unknown protein; Involved in: N-terminal protein myristoylation
Eucgr.E00809	-2.00	3.24E-10	4.30E-09	AT4G23690.1	Disease resistance-responsive (dirigent-like protein) family protein
Eucgr.B03333	-1.98	1.21E-05	7.98E-05	AT1G23760.1	BURP domain-containing protein
Eucgr.G01683	-1.96	4.59E-10	6.01E-09	AT4G34530.1	Cryptochrome-interacting basic-helix-loop-helix 1

[1]Indicates a transcript, for which the gene model is not down-regulated, only this single splice-variant.
[2]TAIR9 ID, since no matching TAIR10 ID is annotated

3.4.5 Overrepresented Gene Ontologies

Differentially expressed genes with matching TAIR10 ID's were used in BiNGO to test for overrepresentation against the *A. thaliana* genome as background and in MapMan to infer which *A.thaliana* annotated metabolic pathways are active in schematic diagrams (Appendix L). Overrepresented GO terms related to the plasma membrane, cell wall and vacuole were common to both the up- and down-regulated datasets (Figure 3.5). Photosystem I and tubulin complex terms were overrepresented as part of the down-regulated terms. Overrepresented molecular function GO terms for the up-regulated dataset included defence-related terms such as oxidoreductase activity and NAD(P)H oxidase, as well as transcription-related terms such as promoter binding and transcription activator activity (Figure 3.6 A). In the down-regulated dataset, structural and cell wall chemistry-related terms such as structural constituent of cytoskeleton, xyloglucan:xyloglucosyl transferase and laccases were present. Terms related to water channel activity were apparent in the down-regulated gene set (Figure 3.6 B).

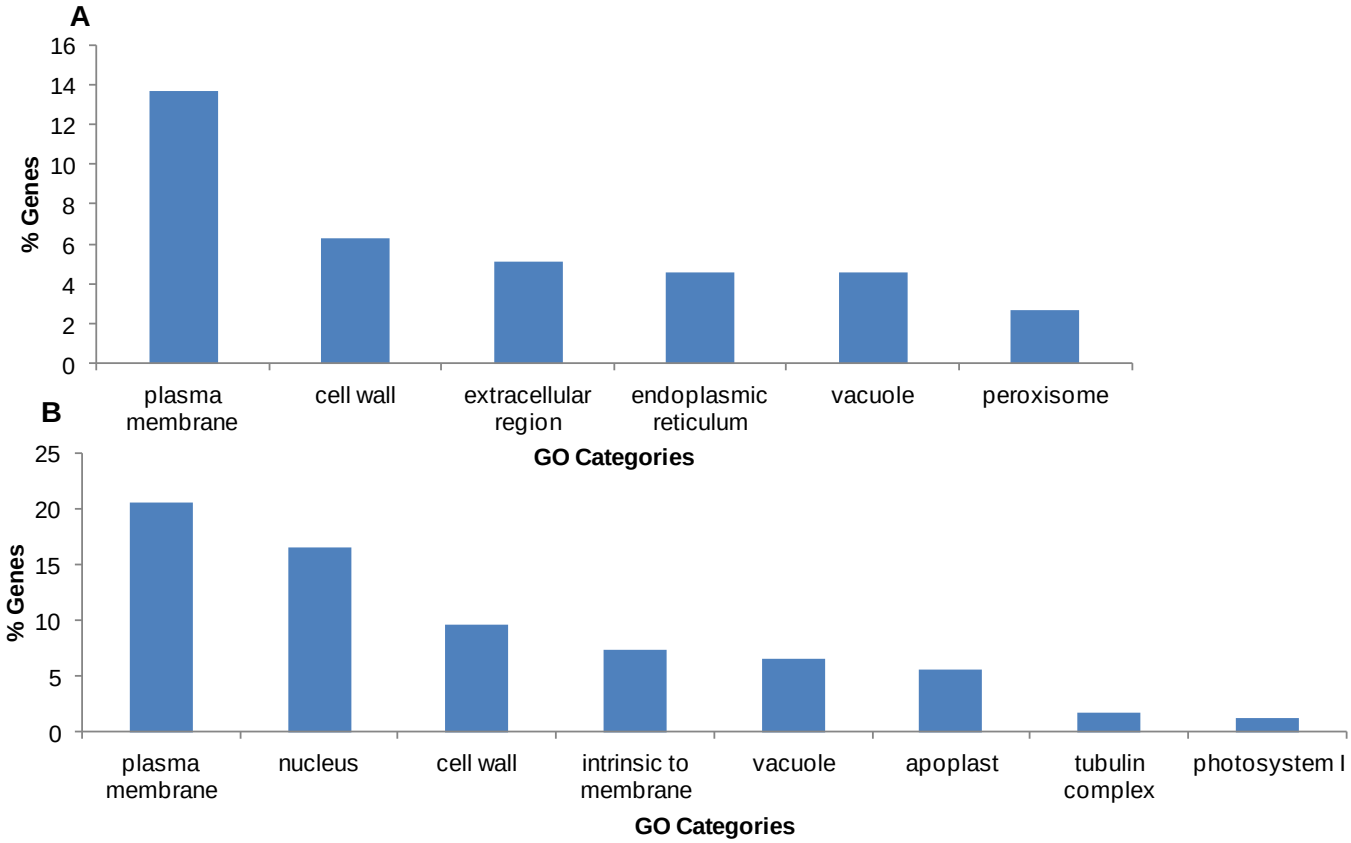

Figure 3.5 GO terms related to cellular components obtained from over-representation analysis in BiNGO for (A) up-regulated and (B) down-regulated TAIR10 ID's from the differentially regulated genes. "% Genes" indicates the number of query genes mapped to a gene set in relation to the total number of genes from the query list mapped to the background list (up-regulated 411, down-regulated 301). Therefore the y-axis indicates the percentage of differentially expressed genes in the query list that are annotated to each significantly enriched GO category.

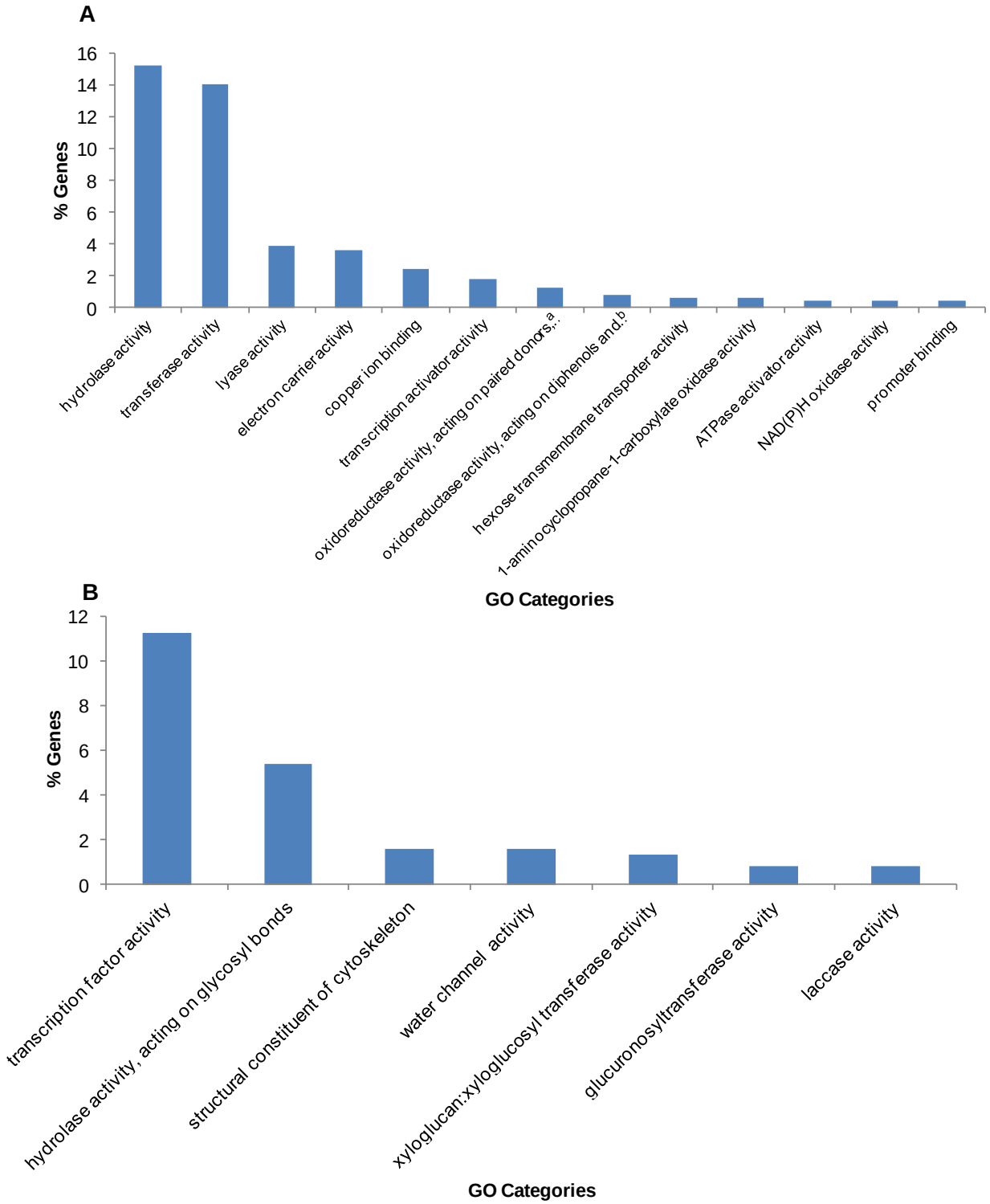

Figure 3.6 GO terms related to molecular function obtained from over-representation analysis in BiNGO for (A) up-regulated and (B) down-regulated TAIR10 ID's from the differentially regulated genes. "% Genes" indicates the number of query genes mapped to a gene set in relation to the total number of genes from the query list mapped to the background list (up-regulated 494, down-regulated 373). Therefore the y-axis indicates the percentage of differentially expressed genes in the query list that are annotated to each significantly enriched GO category.

[a]oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors
[b]oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor

The BiNGO output for biological processes was submitted to REViGO for simplification and visual representation of GO categories. In the up-regulated dataset for biological processes, the majority of overrepresented GO terms were related to defence (Figure 3.7 and Appendix I). Several of the categories involved JA and ET signalling, and there were a few terms related to SA. There were also responses to chitin and reactive oxygen species, as well as callose deposition. Phenylpropanoid pathway terms and aromatic compound synthesis related to flavonoid biosynthesis were also found in this dataset (Table 3.5 and Figure 3.9). Another prominent up-regulated response is water deprivation/water stress terms, and some physiological terms such as syncytium formation, cell wall loosening and organ senescence were noted.

Overrepresented GO terms in the down-regulated dataset for biological processes (Figure 3.8 and Appendix I) were predominantly related to growth, cell wall modifications, and cell wall chemistry. Syncytium formation, response to chitin and the phenylpropanoid pathway featured again. The phenylpropanoid pathway terms were related to lignin biosynthesis, as opposed to flavonoid synthesis in the up-regulated dataset (see Table 3.5 and Figure 3.9). There were minimal biotic stress-related terms, and hormone-related terms in this dataset such as auxin and gibberellin could be present as part of the growth responses. Photosynthesis-related terms were also overrepresented.

The stem discolouration on mock-inoculated controls, could be attributed to the wounding method used during inoculation, or there may be a response to the agar plug used. There weren't a large number of wounding responses in the up-regulated or down-regulated dataset, indicating that the inoculation method did not affect the genes responding differentially.

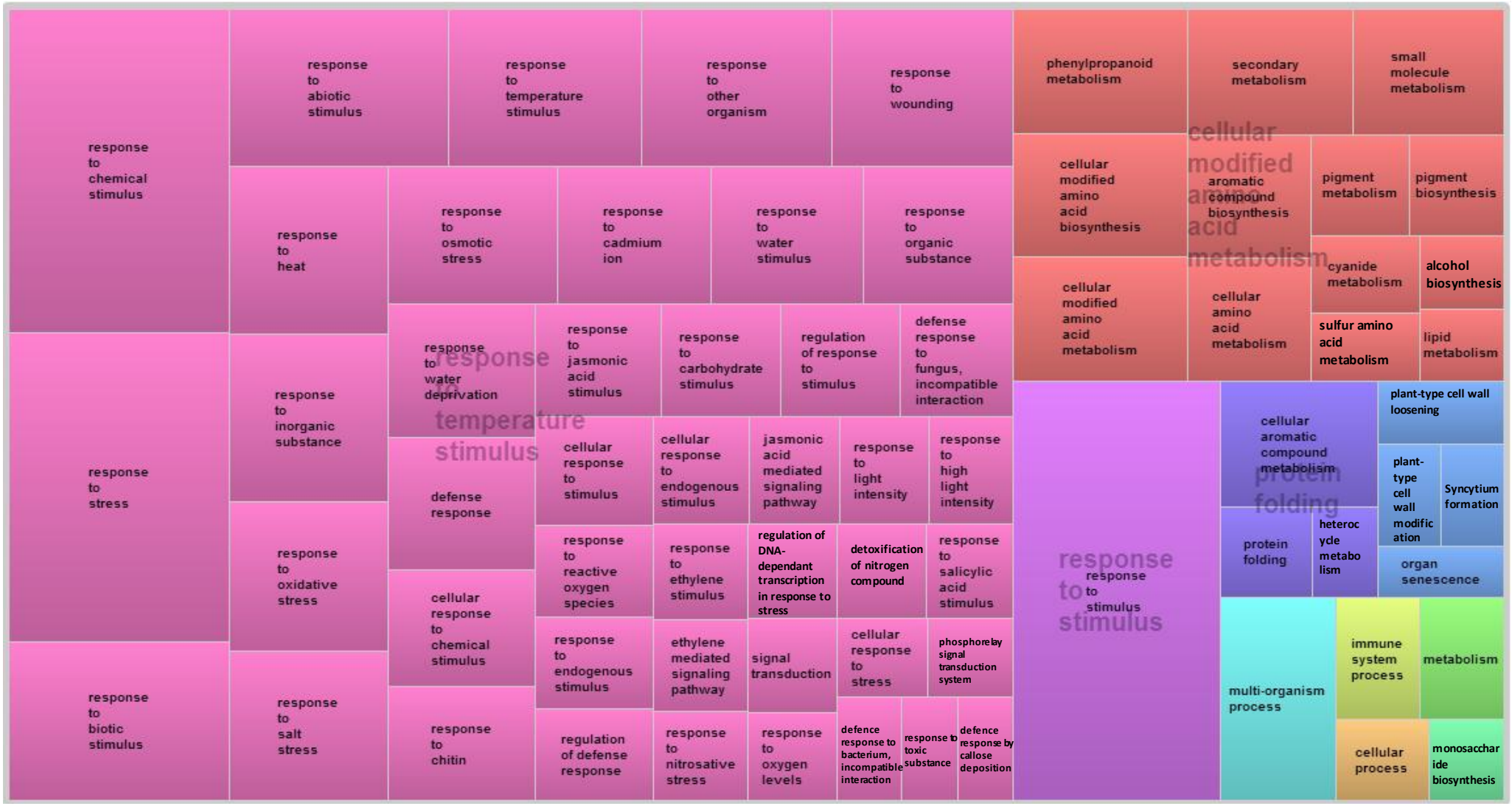

Figure 3.7 Over-represented GO terms obtained for up-regulated biological processes from BiNGO. The figure was produced using REViGO and the size of each block is inverse to the p-value associated with the term.

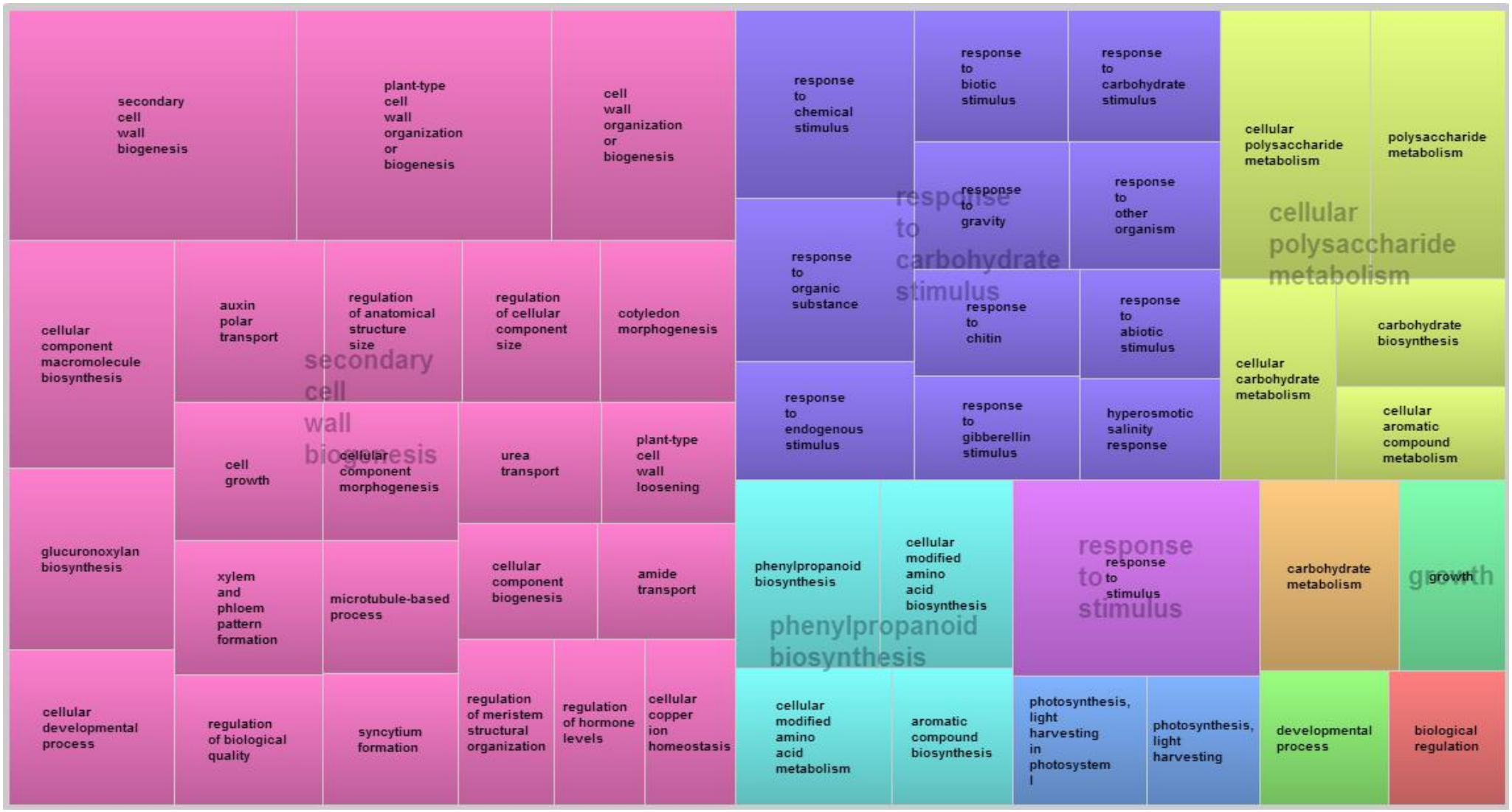

Figure 3.8 Over-represented GO terms obtained for down-regulated biological processes from BiNGO. The figure was produced using REViGO and the size of each block is inverse to the p-value associated with the term.

3.4.6 Regulation of *E. nitens* putative orthologs involved in secondary metabolite biosynthesis

Secondary metabolites include terpenoids and phenylpropanoid pathway products such as flavonoids, lignin, and phenols. Putative gene orthologs encoding the enzymes that catalyze some of the reactions in the secondary metabolism pathways were identified with the use of MapMan. Many of the TAIR10 orthologs represented in these pathways were associated with multiple putative orthologs in *E. nitens*. These multiple orthologs were often not consistently regulated, since some were up-regulated and others were down-regulated.

Several genes involved in terpenoid metabolism were identified as differentially expressed in *E. nitens*, with the majority found in the up-regulated dataset (Table 3.5). Putative gene orthologs encoding enzymes involved in flavonoid biosynthesis were all up-regulated, except for *chalcone and stilbene synthase family protein* (AT5G13930.1) which had five associated putative *E. nitens* orthologs, all of which were strongly down-regulated. Genes encoding enzymes with similar annotated functions generally had similar expression trends (Table 3.5). Gene orthologs involved in phenol metabolism (all encoding laccases) were down-regulated, with the exception of laccase 14, which was strongly up-regulated (Table 3.5).

General phenylpropanoid biosynthesis (including enzymes involved in lignin biosynthesis – see Figure 3.9) was both up- and down-regulated. There were three putative orthologs of *O-methyltransferase 1* (AT5G54160.1), with one up-regulated and the other two down-regulated along with the down-regulation of an *O-methyltransferase family protein* (AT4G35160.1).The putative orthologs encoding *phenylalanine ammonium lyase* are both up-regulated, which could indicate an increase in flux for the entire phenylpropanoid pathway (Table 3.5). Lignin biosynthesis could be down-regulated in *E. nitens* since most genes encoding enzymes involved in biosynthesis of the coniferyl alcohol and sinapyl alchol subunits are down-regulated. Some of the TAIR10 accessions with multiple putative orthologs have mixed expression profiles, so it is hard to tell whether the pathway is up- or down-regulated. Table 3.5 is included in Appendix J along with the average FPKM values of mock-inoculated controls and inoculated tissue to clarify expression levels of the various putative orthologs.

Table 3.5 Expression of *E. nitens* putative orthologs in the secondary metabolism MapMan pathway.

Eucalyptus ID	log2 (fold change)	TAIR ID	TAIR description
		Terpenoids	
Eucgr.H00849	0.69	AT5G48230.2	Acetoacetyl-CoA thiolase 2
Eucgr.D01931	1.11	AT4G11820.2	Hydroxymethylglutaryl-CoA synthase / HMG-CoA synthase
Eucgr.H05007	2.22	AT1G76490.1	Hydroxy methylglutaryl CoA reductase 1
Eucgr.I01544	2.14		
Eucgr.F03606	-0.75		
Eucgr.J00991	0.90	AT3G54250.1	GHMP kinase family protein
Eucgr.A01783	1.03	AT3G02780.1	Isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase 2
Eucgr.E03836	1.12	AT5G47770.1	Farnesyl diphosphate synthase 1
Eucgr.A02022	1.49	AT1G06570.1	Phytoene desaturation 1
Eucgr.K00294	-0.96	AT4G25700.1	Beta-hydroxylase 1
		Flavonoids	
Eucgr.G03066	2.95	AT5G05600.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Eucgr.A00518	1.72		
Eucgr.J01008	2.00	AT5G65550.1	UDP-Glycosyltransferase superfamily protein
Eucgr.H00584	3.50	AT1G59960.1	NAD(P)-linked oxidoreductase superfamily protein
Eucgr.B02637	2.77		
Eucgr.D01635	-1.30	AT5G13930.1	Chalcone and stilbene synthase family protein
Eucgr.D01632	-1.32		
Eucgr.L02670	-1.33		
Eucgr.K01978	-1.37		
Eucgr.D01640	-1.51		
Eucgr.K00040	1.94	AT5G24530.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Eucgr.K00447	1.87		
Eucgr.J02808	1.61	AT5G07990.1	Cytochrome P450 superfamily protein
Eucgr.C01023	3.28	AT4G25310.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Eucgr.E02899	2.57	AT1G17020.1	Senescence-related gene 1
Eucgr.C01029	3.33	AT4G25300.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Eucgr.C01285	3.14		
		Phenylpropanoids	
Eucgr.K00951	-1.10	AT4G35160.1	O-methyltransferase family protein
Eucgr.B02801	1.25	AT5G01210.1	HXXXD-type acyl-transferase family protein
Eucgr.G02850	2.24	AT3G53260.1	Phenylalanine ammonia-lyase 2
Eucgr.G02852	0.75		
Eucgr.I01134	-0.70	AT4G34050.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Eucgr.C00924	2.45	AT4G26220.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Eucgr.J02393	-1.18	AT4G36220.1	Ferulic acid 5-hydroxylase 1
Eucgr.F02624	2.06	AT5G54160.1	O-methyltransferase 1
Eucgr.E03874	-1.65		
Eucgr.E03875	-1.78		
Eucgr.G01350	0.97	AT3G19450.1	GroES-like zinc-binding alcohol dehydrogenase family protein
		Phenols	
Eucgr.G03098	-0.93	AT2G40370.1	Laccase 5
Eucgr.F02650	4.04	AT5G09360.1	Laccase 14
Eucgr.L01345	3.55		
Eucgr.F02652	1.73		
Eucgr.A01282	-1.00	AT5G60020.1	Laccase 17
Eucgr.B02316	-1.14		
Eucgr.K03111	-1.63		
Eucgr.K02996	-0.86	AT2G38080.1	Laccase/Diphenol oxidase family protein

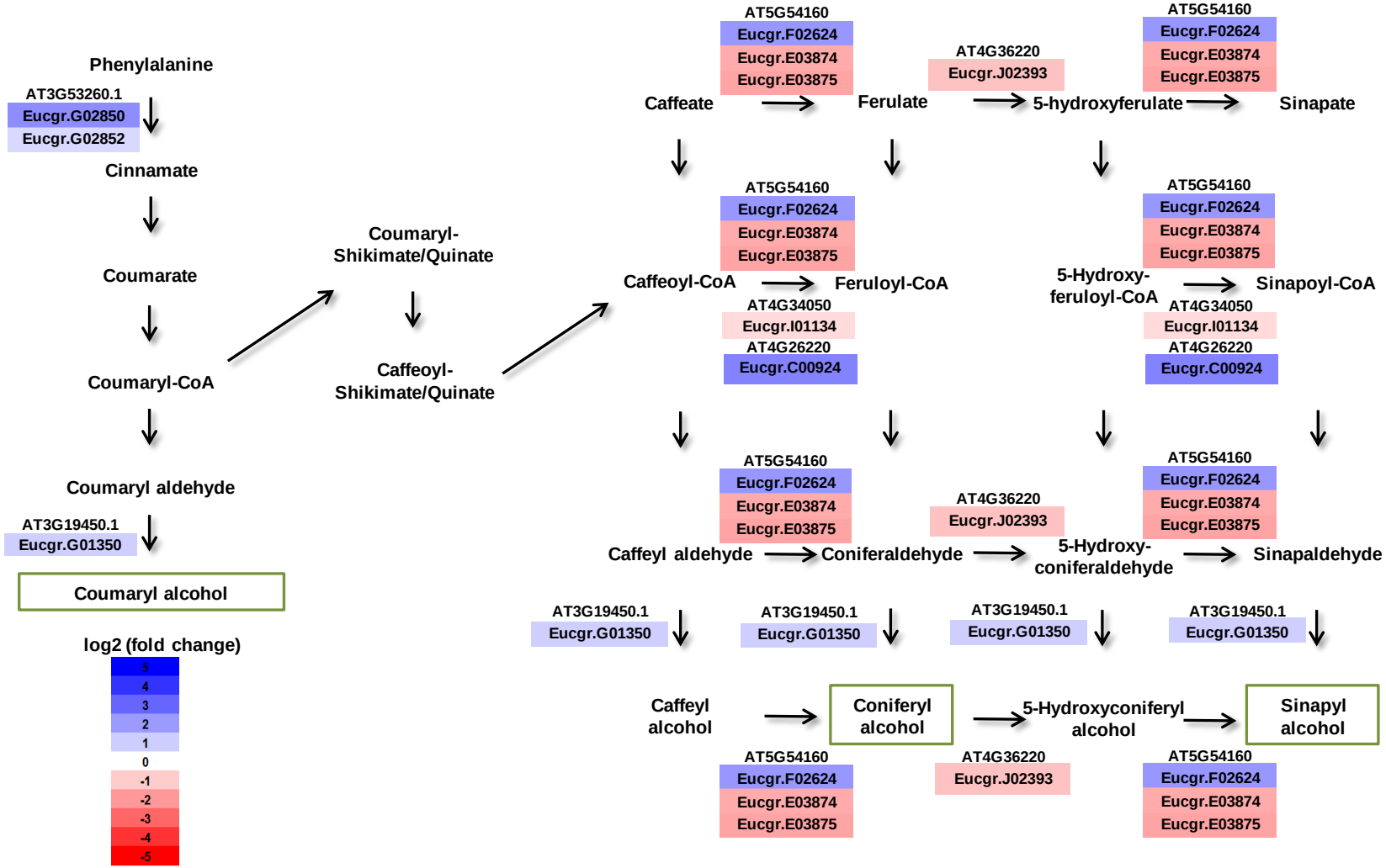

Figure 3.9 Summary of *E. nitens* gene expression of putative orthologs involved in the lignin biosynthetic pathway (figure derived from phenylpropanoid pathway in MapMan). The subunits that lignin is composed of are marked with a green border. The *A. thaliana* gene encoding various enzymes in the pathway is depicted next to the step that it's product catalyzes. The putative ortholog/s in *E. nitens* are shown underneath their *A. thaliana* counterparts. *E. grandis* ID's are colour coded to indicate the fold change on the log2 scale that was recorded after challenge with *P. cinnamomi*.

3.4.7 Comparison of *PR* gene expression across host-*P. cinnamomi* and other *Phytophthora* spp. interactions

One of the noteworthy aspects of the RNA-seq data obtained was that several putative *PR* genes displayed particularly high fold-change values (mostly in the up-regulated dataset). Therefore a summary table was constructed to list all the possible *PR* genes in this *E. nitens* dataset, and comparisons with other *P. cinnamomi*-host interactions were made based on TAIR ID's that matched (Table 3.6) Appendix K has the average FPKM values added to Table 3.6 to clarify specific expression values. The lack of matching TAIR ID's across studies is not necessarily due to different interactions – but rather due to a lack of large-scale data. The avocado transcriptome (a tolerant interaction analysed using 454 sequencing at various timepoints after inoculation - genes indicated in table were present in tissue, but weren't necessarily differentially expressed) (Reeksting, 2013) showed many similarities with the current *E. nitens* study. The incompatible maize interaction (profiled using microarrays of 6 hpi and 24 hpi tissue) (Allardyce *et al.*, 2013) was difficult to compare, since there weren't appropriate *A. thaliana* orthologs for many of the *PR* genes in that dataset.

Within the *E. nitens-P. cinnamomi* interaction, the most prominent putative *PR* genes are *PR-1*, *PR-3* (chitinase), and *PR-5* (thaumatin-like and osmotin). These are not only consistently highly up-regulated in inoculated tissue, but there are multiple *E. nitens* putative orthologs per *A. thaliana* gene that are all regulated at similar fold-change levels. Putative *E. nitens* orthologs of *PR-4* (chitin-binding), *PR-8* (chitinase class III), and *PR-12* (defensins) were up-regulated. The *PR-9* (peroxidase) and *PR-10* (ribonuclease-like) classes contained a mix of up- and down-regulated putative orthologs. A potential peroxidase target for effectors from *Carica papaya* (EL784270) was submitted for BLAST analysis on Phytozome to determine whether it matched one of the *E. grandis* putative orthologs. The putative *PR-9* ortholog Eucgr.E04056 was the best match for the papaya peroxidase, with an E-value of $3.1e^{-104}$ and a positive similarity of 80.4%. This peroxidase was noted to be down-regulated in several other *Phytophthora*-host interactions, and the *E. nitens* putative ortholog was downregulated by a 1.85 fold-change on a log2 scale in the *P. cinnamomi*-infected tissue. *PR-14* (lipid transfer proteins) and *PR-15* (oxalate oxidase/germin) putative orthologs were all down-regulated.

Phytophthora cinnamomi-inoculated avocado tissue displayed the presence of similar *PR* gene classes compared with *E. nitens*, except *PR*-1. In maize there were two of the same *PR-3* and *PR-5* putative orthologs, and the same *PR-1* was detected in two *A. thaliana* studies [the study by Rookes *et. al* (2008) involved a non-host interaction with *A. thaliana* (Col-O) where a northern blot was done for 12, 26, 48 and 72 hpi tissue and the interaction by Eshragi *et. al* (2011) with phosphite-induced resistance profiled *A. thaliana* (Ler) using RT-qPCR at 7 days post treatment] (Table 3.6).

Table 3.6 Possible *PR* genes found to be differentially regulated in *E. nitens* challenged with *P. cinnamomi*. TAIR ID's corresponding to the *E. grandis* putative *PR* genes were matched to genes from other studies involving *P. cinnamomi*.

Eucalyptus ID	log2 (fold change)	TAIR ID	TAIR description	*Persea americana*[a]	*Zea mays*[b]	*A. thaliana* (Col-0)[c]	*A. thaliana* (Ler)[d]
General							
Eucgr.F02733	3.19	AT1G78780.2	Pathogenesis-related family protein	+			
PR-1							
Eucgr.D01552	4.56	AT2G14580.1	Basic pathogenesis-related protein 1				
Eucgr.D01560	4.36						
Eucgr.G01134	3.34						
Eucgr.G01140	4.40						
Eucgr.G01148	4.37						
Eucgr.G01137	3.24	AT2G14610.1	Pathogenesis-related gene 1			+	+
Eucgr.G01171	4.31						
Eucgr.L01707	3.13						
Eucgr.L02505	3.14						
PR-3 **- Chitinase class I, II, IV, VI, VII**							
Eucgr.L00941	4.02	AT3G12500.1	Basic chitinase	+	+		
Eucgr.J02519	3.94						
Eucgr.L00938	3.93						
Eucgr.L00939	3.73						
Eucgr.L00937	3.47						
Eucgr.I01495	3.11						
Eucgr.H04034	-1.33	AT3G16920.1	Chitinase-like protein 2	+			
Eucgr.H00455	-0.75	AT1G05850.1	Chitinase family protein (TAIR 9)				
Eucgr.K02166	2.53	AT3G54420.1	Homolog of carrot EP3-3 chitinase	+	+		
Eucgr.K02166	2.21						
Eucgr.H00326	3.75						
Eucgr.H00321	3.75						
Eucgr.H00328	3.57						
Eucgr.A00020	1.43						
PR-4 **- Chitin-binding**							
Eucgr.B02124	3.42	AT3G04720.1	Pathogenesis-related 4	+			
Eucgr.L03258	3.32						
Eucgr.B02122	3.06						
PR-5 **- Thaumatin-like and osmotin**							
Eucgr.H03863	5.09	AT4G11650.1	Osmotin 34	+			
Eucgr.H03865	4.93						
Eucgr.H03864	4.78						
Eucgr.L01962	4.69						
Eucgr.E00557	4.43						
Eucgr.D01888	3.57						
Eucgr.D01892	3.34						
Eucgr.D01887	3.31						
Eucgr.E00560	2.97						

Table 3.6 (continued)

Eucalyptus ID	log2 (fold change)	TAIR ID	TAIR description	*Persea americana*[a]	*Zea mays*[b]	*A. thaliana* (Col-0)[c]	*A. thaliana* (Ler)[d]
***PR-5* - Thaumatin-like and osmotin (continued)**							
Eucgr.A01474	1.03	AT5G38280.1	PR5-like receptor kinase				
Eucgr.A01470	0.96						
Eucgr.A01478	0.78						
Eucgr.E01382	5.42	AT1G20030.2	Pathogenesis-related thaumatin super-family protein	+			
Eucgr.E01384	5.31						
Eucgr.E01389	5.23						
Eucgr.E01385	5.13						
Eucgr.E01381	4.96						
Eucgr.B00944	-1.36	AT1G73620.1	Pathogenesis-related thaumatin super-family protein	+	+		
Eucgr.J02061	-1.18	AT2G28790.1					
Eucgr.G01772	-1.33	AT4G38660.1					
***PR-8* - Chitinase class III**							
Eucgr.E00091	2.40	AT5G24090.1	Chitinase A	+			
Eucgr.L03478	1.52						
***PR-9* - Peroxidase**							
Eucgr.D01857	3.08	AT4G11600.1	Glutathione peroxidase 6 (TAIR 9)	+			
Eucgr.F04198	2.34	AT1G71695.1	Peroxidase superfamily protein	+			
Eucgr.F04195	1.46						
Eucgr.L02740	1.39						
Eucgr.J02352	3.65	AT4G37530.1	Peroxidase superfamily protein	+			
Eucgr.A01385	1.83	AT1G05260.1		+			
Eucgr.J02173	-1.23	AT5G40150.1					
Eucgr.F03724	-1.76	AT5G42180.1					
Eucgr.E04056	-1.85	AT4G21960.1		+			
***PR-10* - Ribonuclease-like**							
Eucgr.F03953	0.83	AT1G80780.3	Polynucleotidyl transferase, ribonuclease H-like superfamily protein	+			
Eucgr.J00535	-1.46	AT5G22250.1					
***PR-12* - Defensins**							
Eucgr.K03440	2.72	AT4G11393.1	Defensin-like (DEFL) family protein				
***PR-14* - Lipid transfer proteins**							
Eucgr.B00824	-1.00	AT3G18280.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	+			
Eucgr.K03041	-1.66	AT5G05960.1					
Eucgr.H00727	-0.85	AT5G48485.1					
Eucgr.F03514	-1.31	AT5G55460.1					
Eucgr.I02679	-0.95	AT5G64080.1					
Eucgr.K01283	-1.21	AT5G59320.1	Lipid transfer protein 3				
Eucgr.A00746	-1.60						
Eucgr.K01282	-1.76						
***PR-15* - Oxalate oxidases (Germin)**							
Eucgr.A00990	-1.18	AT3G62020.1	Germin-like protein 10	+			

"+" signs indicate where the same TAIR ID ortholog was found to be expressed in that study.

[a] Reeksting *et al.,* 2013 and Bianca Reeksting, personal communication

[b] Allardyce *et al.,* 2013

[c] Rookes *et al*., 2008

[d] Eshragi *et al.,* 2011

3.5 Discussion

3.5.1 Responses in *E. nitens* tissue after inoculation with *P. cinnamomi*

E. nitens plantations are susceptible to root rot caused by *Phytophthora*. In order to gain a better understanding of a compatible pathosystem between *Eucalyptus* spp. and *Phytophthora spp*., an RNA-seq study was undertaken to profile gene expression in *E. nitens* stem tissue five days after inoculation with *P. cinnamomi* hyphae.

According to the optimal defence hypothesis the type and amount of defence within plants is orchestrated according to the value of an organ. Due to its critical role in plant physiology, the stem is defined as a high-value organ, where especially structural defences may be important (Meldau *et al.*, 2012; Zangerl and Bazzaz, 1992). Stems do not produce precisely the same defence responses as roots (Okubara and Paulitz, 2005), but they provide clean, plentiful amounts of tissue for RNA isolation. Profiling stem tissue provides a way to investigate responses ranging from necrotic tissue that has been in contact with the pathogen for 5 days (near the inoculation site), up to tissue at the extending lesion margin where the pathogen is still actively invading, as well as distal non-infected tissue. This range provides a global view of host transcription during the infection process from which the interaction can be studied further, since the timing of this particular pathosystem is not well established.

Since this is a compatible pathosystem, the defence responses observed are likely to be highly similar to a resistant interaction, but with delays in initiation of these responses. Additionally, manipulation of the host by the pathogen can be observed, making a compatible interaction ideal for a broad, preliminary study of a pathosystem. A late (5 dpi) time-point was chosen specifically so that we could be sure the mycelia had made contact with host tissue and we were capturing a range of responses in the stem, but also so that the delayed responses inherent to a compatible interaction could be captured.

The outcomes of this RNA-seq study provided some valuable insights into *Eucalyptus* defence, as well as specific host defence mechanisms utilized against *P. cinnamomi*. After mapping the *E. nitens* transcript reads to the *E. grandis* genome, trends within the transcriptome were analysed by using putative *A. thaliana* orthologs. Since the majority of differentially expressed genes had matching TAIR10 putative orthologs, it was possible to assign tentative identities and test for overrepresentation of GO categories for a large portion of the dataset. This analysis could not be done for the *Eucalyptus* genome, since there were not sufficiently annotated files available at the time of analysis. Overrepresentation of GO terms provides a way of seeing the likely responses of this non-model plant on a global level, though the overrepresented terms are not necessarily biologically significant and many significant genes

are not reflected as overrepresented terms.

More genes were significantly up-regulated than down-regulated. Most of the up-regulated genes belong to GO terms related to a biotic stress defence response in the form of *PR* genes, hormone signaling and flavonoid synthesis via the phenylpropanoid pathway. In contrast, the largest component of the differentially down-regulated dataset consists of terms associated with growth, as well as cell wall biosynthesis and photosynthesis. Taken together, these responses are indicative of a host that is actively attempting to combat infection, despite it being at a late stage of a compatible interaction. Obviously, any activated defence mechanisms are not sufficient to confer resistance, and there may be some critical responses missing. We observed differential expression of various defence responses, and in each case we provide possible reasons for the susceptible outcome of the interaction.

3.5.2 Defence hormone signaling

JA-, SA- and ET-related GO terms are upregulated in this interaction, suggesting that they could play a role in defence signalling for this interaction. SA-regulated genes such as putative orthologs of *PR-1* and *PR-5* are up-regulated, but so are JA-responsive orthologs belonging to *PR-3*, *PR-4* and *PR-12*. There are numerous JA-related GO terms in this interaction, meaning that JA may have a prominent role, and this is supported by the up-regulation of thaumatin-like putative orthologs, and the suppression of putative lipid-transfer proteins and germin-like proteins, which was also noted in a microarray study involving JA treatment on *A. thaliana* (Schenk *et al.*, 2000). In their dataset, SA and JA did not seem to act antagonistically, so this could indicate a form of co-ordinated defence (Schenk *et al.*, 2000; Van Wees *et al.*, 2000). Other studies involving hosts inoculated with *Phytophthora* spp. have also shown mixed hormone responses (Attard *et al.*, 2010; Shibata *et al.*, 2010). JA may be needed for successful defence against *P. cinnamomi*, in maize, a resistant monocot (Allardyce *et al.*, 2013). Other studies indirectly support this view by hypothesizing that suppression of JA and activation of SA may be a compatibility factor (Moy *et al.*, 2004; Restrepo *et al.*, 2005). Therefore, the preference for JA signalling-associated genes in the current study could be part of a successful defence mechanism against *P. cinnamomi*, since it is primarily a necrotrophic pathogen (Glazebrook, 2005; Hardham, 2005). However, since the interaction is compatible, this is only attempted defence that may be happening too late, at too low levels.

3.5.3 Regulation of putative *PR* genes

A comparison of putative *PR* gene orthologs from *E. nitens* with other *P. cinnamomi*-inoculated hosts (Table 3.6) was performed, and for the purposes of discussion, a summary of literature with *Phytophthora*-host interactions involving *PR* genes is included to enable wider

comparison with host defence against other *Phytophthora* spp. (Table 3.7). Gene models identified as *PR* genes in this dataset are putative orthologs of *A. thaliana PR* genes. For many of these genes, there are multiple differentially expressed *E. grandis* gene models matching to one *A. thaliana* putative ortholog. Expression of these multiple *PR* gene transcripts in *E. nitens* could indicate that some of the orthologs have slightly different functions and are all used during a defence response. The multiple putative orthologs of *PR-1*, chitinase, chitin-binding protein, as well as thaumatin-like protein and osmotin, may indicate an important role for these genes in defence specifically against *P. cinnamomi* (Table 3.6). Since *PR* genes are markers of defence hormone signaling, the different putative *PR* genes expressed reflect the mix of JA and SA signalling noted in the overrepresentation analysis.

Transcription of *PR-1*, chitinase (*PR-3*), chitin-binding protein (*PR-4*) and thaumatin-like protein/osmotin (*PR-5*) putative orthologs is highly up-regulated in *E. nitens*. Interestingly, *PR-1*, *PR-3*, and *PR-5* are reported to respond to challenge with *Phytophthora* spp. most frequently in previous studies (Table 3.7). It has been hypothesized that *PR-1* may be important in defence against oomycetes, although it's mechanism of action has not been elucidated (Van Loon *et al.*, 2006). A *Phytophthora*-resistant potato expresses *PR-1* constitutively (Ali *et al.*, 2012) and constitutive expression of *PR-1* in transgenic tobacco confers resistance to *P. parasitica* (Alexander *et al*., 1993). Putative orthologs of thaumatin-like proteins and osmotin belonging to the *PR-5* class, are up-regulated at particularly high fold-change levels in this study. Transgenic plants overexpressing *PR-5* genes have been shown to increase resistance to *P. citrophthora* and *P. infestans* (Acharya *et al.*, 2013; Fagoaga *et al.*, 2001; Pushin *et al.*, 2010). PR-5 proteins are said to act synergistically with cell-wall degrading proteins, which could be a partial explanation of the presence of the high levels of putative chitinase orthologs in this interaction (Lorito *et al.*, 1996). The evidence from other *Phythophthora*-host interactions where *PR-1, PR-3*, *PR-4,* and *PR-5* enhanced resistance, motivates that the high transcript levels in the current interaction should also contribute to resistance. It is possible that these genes would be part of successful defence in a resistant *Eucalyptus* host, but that they are not expressed quickly enough after inoculation, which results in a compatible outcome. Additionally, there was no differential regulation of putative β-1,3-glucanases (*PR-2*) orthologs, which was expected, since oomycete cell walls are primarily composed of β-1,3-glucans, the targets of PR-2 proteins. This is another factor possibly influencing the successful advancement of *P. cinnamomi*.

The peroxidase (*PR-9*) and ribonuclease-like protein (*PR-10*) classes have putative orthologs, some of which are up-regulated, and some down-regulated. Both classes have roles in metabolism as well as defence (Almagro *et al.*, 2009; Van Loon *et al.*, 2006), so it is possible

that not all of the putative orthologs are truly defence-related. The down-regulated peroxidases are associated with categories in this dataset such as giberellin (GA) and secondary cell wall-associated terms, and the strongly up-regulated orthologs all have GO functions related to oxidative stress. Peroxidases are noted fairly often in other *Phytophthora*-host interactions as well (Table 3.7). Peroxidases are potential cross-species *Phytophthora* effector targets, since a certain *C. papaya* peroxidase and its putative orthologs have been suppressed in different hosts upon inoculation with *P. sojae, P. palmivora* and *P. infestans* (Moy *et al.*, 2004; Porter *et al.*, 2009; Restrepo *et al.*, 2005). An *E. grandis* gene, Eucgr.E04056, is highly similar to the *C. papaya* ortholog, and is also strongly suppressed in the current interaction. *P. cinnamomi* may very well also possess an effector that manipulates the peroxidase. The *A. thaliana* accession matching Eucgr.E04056, AT4G21960.1, is a peroxidase that responds to oxidative stress, so it could be required for cross-linking cell wall components and strengthening cell walls. Another putative *PR-9* ortholog with defence-associated GO terms, Eucgr.J00535, is also down-regulated. A comparison with a resistant *Eucalyptus* interaction would provide evidence whether down-regulation of these peroxidases could be a factor that contributes to compatibility.

Another down-regulated *PR* gene family that could contribute to susceptibility in this interaction, is lipid transfer proteins (*PR-14*). In a resistant interaction between *P. colocasiae* and taro, *PR-14* is up-regulated (Sharma *et al.*, 2009) and enhanced resistance to *P. nicotianae* was conferred by a pepper lipid transfer protein overexpressed in tobacco (Sarowar *et al.*, 2009). Lipid transfer proteins bind to the same receptors as elicitins from *Phytophthora* spp. (Buhot *et al.*, 2001), and may therefore play a role in the lipid-related interplay between oomycetes and plants (Blein *et al.*, 2002) so reduced *PR-14* expression could have a negative influence on defences against *Phytophthora*.

PR-15 has a proven role in resistance to *Phytophthora* spp., since taro (*Colocasia esculenta*) transformed with a wheat oxalate oxidase gene conferred increased resistance to *P. colocasiae* (He *et al.*, 2013). Germin-like oxalate oxidases produce hydrogen peroxide which is toxic to pathogens, enhances defences responses, and plays a role in cell-wall strengthening (Ferreira *et al.*, 2007; Van Loon *et al.*, 2006). In *E. nitens*, a germin-like protein associated with xylan synthesis was down-regulated, but since the TAIR annotation does not mention oxalate oxidase activity, it cannot be assumed that it is an actual *PR-15*. Germins belong to the cupin superfamily (Bernier and Berna, 2001), for which there are several putative orthologs amongst the most up-regulated transcripts.

Table 3.7 *PR* genes implicated in defence in various host-*Phytophthora* interactions

Publication	Host	*Phytophthora* spp.	*PR*-1	*PR*-3	*PR*-4	*PR*-5	*PR*-9	*PR*-10	*PR*-12	*PR*-14	*PR*-15	Experimental evidence
Acharya *et al.*, 2013	*Solanum tuberosum*	*P. infestans*				+						Overexpression of *Camellia sinensis* TLP provides resistance
Alexander *et al.*, 1993	*Nicotiana tabacum*	*P. nicotianae*	+									Constitutive overexpression of *PR*-1 provides tolerance
Ali *et al.*, 2012	*S. tuberosum*	*P. infestans*	+									Resistant genotype; SDS-PAGE
Attard *et al.*, 2010	*A. thaliana*	*P. parasitica*	+	+	+				+			RT-qPCR at 0, 2.5, 6, 10.5, 30 hpi
Bengtsson *et al.*, 2013	*S. tuberosum*	*P. infestans*	+									BABA induced tissue at 2 dpi
Beyer *et al.*, 2001	*S. tuberosum*	*P. infestans*		+			+					SSH and Northern analysis at 48 hpi.
Birch *et al.*, 1999	*S. tuberosum*	*P. infestans*	+	+								SSH and cDNA-AFLP and EST sequencing
Chen *et al.*, 2008	*Glycine max*	*P. sojae*			+			+				RT-PCR at 0, 1, 3, 6, 12, 24, 48 hpi
Chen and Halterman, 2011	*S. tuberosum*	*P. infestans*	+			+						RT-qPCR at 0, 48, 96 hpi
Dellagi *et al.*, 2000	*S. tuberosum*	*P. infestans*		+								Northern blot at 15, 48, 72 hpi
Fagoaga *et al.*, 2001	*Citrus sinensis*	*P. citrophthora*				+						Constitutive expression of tomato *PR*-5 provides resistance
Gao *et al.*, 2013	*S. tuberosum*	*P. infestans*		+		+						RNA-seq at 0, 24, 48 hpi
García-Pineda *et al.*, 2010	*Persea americana*	*P. cinnamomi*					+					Enzyme assay at 0, 48, 96 hpi
Gyetvai *et al*., 2012	*S. tuberosum*	*P. infestans*	+	+		+						DeepSAGE 0, 1, 3 dpi
He *et al.*, 2013	*Colocasia esculenta*	*P. colocasiae*									+	Expression of wheat oxalate oxidase transgenic improves resistance
Korgan *et al.*, 2011	*S. tarijense*	*P. infestans*					+					Enzyme assay at 0, 24, 48, 72 hpi
Lim *et al*., 2013	*S. tuberosum*	*P. infestans*	+	+	+	+	+					iTRAQ proteomics; phosphite and *P. cinnamomi* treated 0, 4 dpi
Meins and Ahl, 1989	*N. tabacum*	*P. nicotianae*		+								Immunoassay and Northern blots at 0, 2, 4, 7 dpi
Moreno-Chacón *et al.*, 2013	*Elaeis guineensis*	*P. palmivora*					+					Enzyme assay at various stages of infection
Moushib *et al.*, 2013	*S. tuberosum*	*P. infestans*	+									SDS-PAGE 1 day after sugar beet extract treatment
Moy *et al.*, 2004	*G. max*	*P. sojae*	+	+								Microarray at 3, 6, 12, 24, 48 hpi

Table 3.7 (continued)

Publication	Host	*Phytophthora* spp.	*PR*-1	*PR*-3	*PR*-4	*PR*-5	*PR*-9	*PR*-10	*PR*-12	*PR*-14	*PR*-15	Experimental evidence
Nakazawa-Ueji *et al.*, 2010	*Capsicum chinense*	*P. capsici*						+				Dot blot at 48 hpi
Núñez-Pastrana *et al.*, 2011	*Capsicum chinense*	*P. capsici*	+					+				RT-qPCR at 0, 4, 8, 12, 24, 48, 72 hpi
Orłowska *et al*., 2012	*S. tuberosum*	*P. infestans*		+		+	+					RT-qPCR at 1, 4, 17, 24, 30, 41, 65 hpi
Porter *et al.*, 2009	*Carica papaya*	*P. palmivora*					+					Northern blot at 1, 12, 24, 48 hpi
Pushin *et al.,* 2010	*S. lycopersicum*	*P. infestans*				+						Expression of strawberry and kiwi PR-5 improves resistance
Restrepo *et al.*, 2005	*S. tuberosum*	*P. infestans*		+		+						Microarray at 6, 12, 24, 48, 72 hpi
Ronning *et al.*, 2003	*S. tuberosum*	*P. infestans*		+								EST sequencing of various tissues and timepoints
Ros *et al.*, 2004	*S. tuberosum*	*P. infestans*	+	+		+	+					SSH and cDNA array hybridization at 16 and 72 hpi
Ros *et al.*, 2005	*S. tuberosum*	*P. infestans*	+			+	+					cDNA array hybridization at 72 hpi
Sarowar *et al.*, 2009	*Nicotiana tabacum*	*P. nicotianae*								+		Constitutive expression of pepper LTP improves resistance
Schlink, 2009	*Fagus sylvatica*	*P. citricola*	+	+		+		+				SSH and EST sequencing at 6 dpi
Sharma *et al.*, 2009	*Colocasia esculenta*	*P. colocasiae*		+			+			+		SSH 8, 12, 24, 36, 48 hrs after symptom
Silvar *et al.*, 2008	*Capsicum anuum*	*P. capsici*	+				+					RT-qPCR at 8 and 24 hpi
Sugano *et al.*, 2012	*G. max*	*P. sojae*	+		+				+			BTH and ACC treated - RT-qPCR at 0, 4, 6, 8 hpi
Tian *et al.*, 2006	*S. tuberosum*	*P. infestans*	+	+		+						Microarray at 24, 48, 72 hpi
Trognitz *et al.*, 2002	*S. phureja x tuberosum*	*P. infestans*				+	+	+				QTL's associated with resistance
Wang *et al.*, 2005	*S. tuberosum*	*P. infestans*	+		+	+						Microarray at 2, 4, 6, 8, 12, 24, 36, 48, 72 hpi
		Total studies:	**18**	**16**	**5**	**15**	**12**	**5**	**2**	**2**	**1**	

"+" signs indicate that the relevant *PR* gene was either responding to pathogen challenge or shown to enhance resistance

ACC: 1-aminocyclopropane-1-carboxylic acid; BABA: DL-β-aminobutyric acid; BTH: benzothiadiazole; cDNA-AFLP: cDNA-amplified fragment length polymorphism; EST: expressed sequence tag; iTRAQ: isobaric tag for relative and absolute quantification QTL: quantitative trait loci; RT-PCR: Reverse transcription- polymerase chain reaction; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; SAGE: serial analysis of gene expression SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSH: subtractive hybridization; TLP: thaumatin-like protein

3.5.4 Regulation of the phenylpropanoid pathway

The phenylpropanoid pathway is another critical component of defence, represented in this study by multiple overrepresented GO terms in both the up-regulated and down-regulated datasets. Two putative orthologs of the entry point enzyme (Blount *et al.*, 2000) for the pathway, phenylalanine ammonium lyase, were up-regulated, so flux into the entire pathway could be increased upon inoculation with *P. cinnamomi*. However, distinct components of this pathway are present in both the up- and down-regulated datasets.

There are several up-regulated genes with GO annotations associated with flavonoid biosynthesis. Flavonoids can be anti-microbial (Dixon, 2001). For example, soybean flavonoids genistein, quercitin and naringenin inhibit *P. sojae* growth (Rivera-Vargas *et al.*, 1993) and certain *Citrus* flavonoids have an antimicrobial action against *P. citrophthora* (Del Río *et al.*, 2004). Susceptible *Lupinus angustifolius* up-regulated genistein in response to *P. cinnamomi* (Gunning *et al.*, 2013). Phenolics are important in responses to *P. cinnamomi*, and key phenylpropanoid enzymes are involved in resistance mechanisms (Coelho *et al.*, 2011; Jackson *et al.*, 2000). Therefore the up-regulation of flavonoid biosynthesis-related terms in *E. nitens* responding to *P. cinnamomi* may very well be a form of attempted defence. However, there are five putative *E. nitens* orthologs of chalcone synthase (AT5G13930.1), which are strongly down-regulated. Chalcone synthase catalyzes the first committed step of the flavonoid biosynthesis pathway where p-coumaroyl-CoA and malonyl-CoA molecules are joined to form chalcones (Naoumkina *et al.*, 2010; Taiz and Zeiger, 2002). Since five different orthologs are all down-regulated it is possible that *P. cinnamomi* is manipulating an upstream regulator in order to suppress the flux into the flavonoid biosynthetic pathway.

Several laccases were down-regulated upon inoculation with *P. cinnamomi* in *E. nitens*. The role of most laccases has not been clearly elucidated yet, but the available data implicates their involvement in cross-linking of lignin subunits (Gavnholt and Larsen, 2002; Turlapati *et al.*, 2011). Three putative *E. nitens* orthologs of laccase 14 (AT5G09360.1), which is annotated as being involved in lignin catabolic function, were up-regulated.

In the down-regulated dataset, GO terms associated with lignin synthesis via the phenylpropanoid pathway are overrepresented. This can also be observed in the number of down-regulated orthologs represented in the metabolic pathway leading to lignin production. There were three putative orthologs of *O-methyltransferase 1* (AT5G54160.1), with one up-regulated and the other two down-regulated along with the down-regulation of an *O-methyltransferase family protein* (AT4G35160.1).

For lignin synthesis, most genes encoding enzymes involved in biosynthesis of the coniferyl alcohol (G subunit) and sinapyl alcohol (S subunit) are down-regulated, although genes encoding enzymes catalyzing the synthesis of coumaryl alcohol (H subunit) are up-regulated. In angiosperms, the S and G are most prevalent, and this has been confirmed in *Eucalyptus* spp. (Gavnholt and Larsen, 2002; Nunes *et al.*, 2010). Since synthesis of these subunits is possibly suppressed, lignin biosynthesis could be down-regulated in *E. nitens*. Further studies are required to establish whether all of the putative orthologs for each enzyme are functional, and whether the regulation of this pathway on transcriptional level is influencing the actual lignin levels in the plant.

Lignin is associated with strengthening of cell-walls and helps prevent penetration by a pathogen (Bechinger *et al.*, 1999), and down-regulation of monolignols can compromise host resistance (Naoumkina *et al.*, 2010). Lignin synthesis plays a role in raspberry responses to challenge with *P. rubi* (Ward and Weber, 2012) and transgenic potato plants with limited phenylpropanoid substrates had increased susceptibility to *P. infestans* (Yao *et al.*, 1995). Along with the down-regulation of some peroxidase putative orthologs, decreased lignin synthesis in this study could implicate a strategy by *P. cinnamomi* to ensure that cell walls are not strengthened. This could ensure easier access to plant cells during necrosis. However, this could also form part of the general down-regulation of growth and cell-wall synthesis related terms within this dataset.

3.5.5 Regulation of growth hormones

The growth hormones, GA and auxin, were both down-regulated in this dataset. Recently, a mechanism has been elucidated whereby JA-mediated defence pathways are prioritized over GA-mediated growth (Hou *et al.*, 2013; Yang *et al.*, 2012). Treatment of soybean with GA increased susceptibility to *P. sojae* (Sugano *et al.*, 2013) and it has been proposed that GA influences defence against necrotrophs by repressing resistance (Mengiste, 2012). Therefore, the observed down-regulation of GA-related terms and simultaneous positive regulation of JA signalling by *E. nitens* may be a correct defence mechanism specific against the necrotrophic nature of *P. cinnamomi,* although the extent and timing or nature of the defence mechanism is not enough to stop the pathogen in this interaction.

Auxin is one of the main plant growth hormones, responsible for growth and cell differentiation (Balmer and Mauch-Mani, 2012), but it is also involved in defence and modulates defence hormones (Bari and Jones, 2009; Pieterse *et al.*, 2012). Its importance in defence is emphasized by pathogens that produce it, or manipulate its levels in the host (Robert-Seilaniantz *et al.*, 2011). For example, auxin is potentially manipulated by an effector of *P.*

parasitica which suppresses cell-death resulting from elicitin recognition (Evangelisti *et al.*, 2013). *P. cinnamomi* is primarily a necrotroph, so it is unlikely to attempt to suppress cell-death. Defence has a high metabolic cost, so resources may be shunted away from growth toward defence while a plant is under threat from a pathogen (Herms and Mattson, 1992; Walters and Heil, 2007). This is a likely explanation for why terms related to growth hormones auxin and GA were found in the down-regulated dataset and those related to defence hormones JA, SA and ET were up-regulated.

3.5.6 Down-regulation of secondary cell-wall formation

This priority given to defence at the cost of growth is reflected in a large number of down-regulated terms related to growth, secondary cell-wall biosynthesis and cell-wall sugar metabolism. Some of the terms are indicative of cell-wall loosening and re-organization of the cell walls, such as the terms related to syncytium formation. Syncytia (multinucleate cells which form nursing cells that feed the nematodes) are formed by cyst nematodes (Jones and Northcote, 1972). Syncytium formation is controlled by certain host proteins, such as expansin, which is involved in cell-wall loosening and growth (Taiz and Zeiger, 2002; Wieczorek and Grundler, 2006). It is unlikely that syncytia are actually formed, since it has never been noted in microscopic studies of host responses to *P. cinnamomi*, but several putative orthologs of expansin were found in the down-regulated datasets. The down-regulation of expansin transcription could be linked to the observed suppression of auxin signaling. *Xanthomonas oryzae pv. oryzae* induces auxin expression in rice, which is linked to increased production of expansins which are responsible for cell wall loosening. When auxin signaling was suppressed in rice (conferring resistance), expansin expression was also suppressed (Ding *et al.*, 2008). Decreased cell wall loosening could be part of a mechanism used by *E. nitens* to attempt defence against *P. cinnamomi*, since it is a necrotroph and intact plant cell walls will delay pathogen progress.

3.5.7 Regulation of water-stress and photosynthesis-related terms

Some physiological responses to *P. cinnamomi* include the down-regulation of photosynthesis-related terms and up-regulation of water stress terms. This is expected in an interaction with *Phytophthora*, where necrosis and invasion of the phloem and xylem affects water metabolism directly by interfering with water transport. Phloem blockage can also cause product-inhibited photosynthesis, which results in stomatal closure, which affects water metabolism indirectly (Koehl *et al.*, 2007). Various tree species inoculated with *P. cinnamomi* show declines in stomatal conductance and photosynthesis. In *Eucalyptus sieberi*, this decline is associated with susceptibility, since these factors decreased less severely in resistant *Eucalyptus sideroxylon* (Dempsey *et al.*, 2012). In *Quercus suber*, photosynthesis and stomatal

conductance also decreased after inoculation (Medeira *et al.*, 2012), and dehydrins are responsive to *Phytophthora* spp. and water stress (Schlink, 2010; Turco *et al.*, 2004). A putative dehydrin, Eucgr.I02395, is up-regulated in this study as well.

Photosynthesis is not isolated from defence mechanisms, so a reduction in this important process may have direct implications for successful defence. Manter *et al.* (2007) noted the prevalence of photosynthetic and stomatal conductance decrease in *Phytophthora*-host interactions. They showed that photosynthetic decline could be caused by elicitins in the absence of water stress. Maintaining adequate photosynthetic levels may assist tolerance or resistance, which is a possible explanation of why lower photosynthetic rates and stomatal conductance is associated with an increase in pathogen colonization (Portz *et al.*, 2007) and lower tolerance to *Phytophthora* spp. (Farrow *et al.*, 2011). The noted down-regulation of genes related to photosynthesis in *E. nitens* could indicate that photosynthesis is being suppressed in some way. This down-regulation could be either a secondary physiological response as part of the plant's down-regulation of growth and up-regulation of defence, or it could be a target of manipulation by the pathogen. There is a chloroplast gene, resistance to *Phytophthora* 1 (*RPH1*), which is a possible effector target to *Phytophthora* (Belhaj et al., 2009), but the *E. nitens* putative ortholog, Eucgr.K03409, was not significantly down-regulated. Since stem tissue was profiled, regulation of photosynthesis related transcripts probably does not provide an entirely accurate reflection of what is happening to photosynthesis in the leaves.

3.6 Conclusion

Stem tissue of *E. nitens* displays an active attempt to combat the pathogen at five days after inoculation with *P. cinnamomi*. This is evident in the up-regulation of putative orthologs involved in flavonoid synthesis, JA, ET and SA hormone signaling and key *PR* genes such and *PR-1*, *PR-3* and *PR-5*. There is a strong prioritization of defence over growth, since terms related to growth hormones GA and auxin, as well as putative orthologs associated with cell wall biosynthesis and re-organization are down-regulated. There are several factors that could play a role in conferring susceptibility. Importantly, a putative ortholog of a cross-species *Phytophthora* effector target was down-regulated, indicating that *P. cinnamomi* effectors may be manipulating it in this interaction. Down-regulated lipid transfer protein orthologs, a decrease in photosynthesis-related terms, as well as decreased lignin biosynthesis terms may also play a role in susceptibility. Up-regulated terms related to defence hormone signaling, *PR* gene regulation and flavonoid biosynthesis probably represent genes that could be effective in combating *P. cinnamomi* when timing and intensity of regulation is correct. Figure 3.10 summarizes the data from *E.nitens* after inoculation with *P. cinnamomi*. Comparison with a resistant interaction is required to provide an indication of host targets manipulated by *P.*

cinnamomi and to enhance understanding of the defence pathways required for resistance. Further studies are also needed to confirm that regulation of key defence factors on the transcriptional level is present on biochemical and protein level as well. Once the pathogen's virulence and *E. nitens*' susceptibility and resistance factors are identified, possible targets for resistance engineering and breeding will emerge.

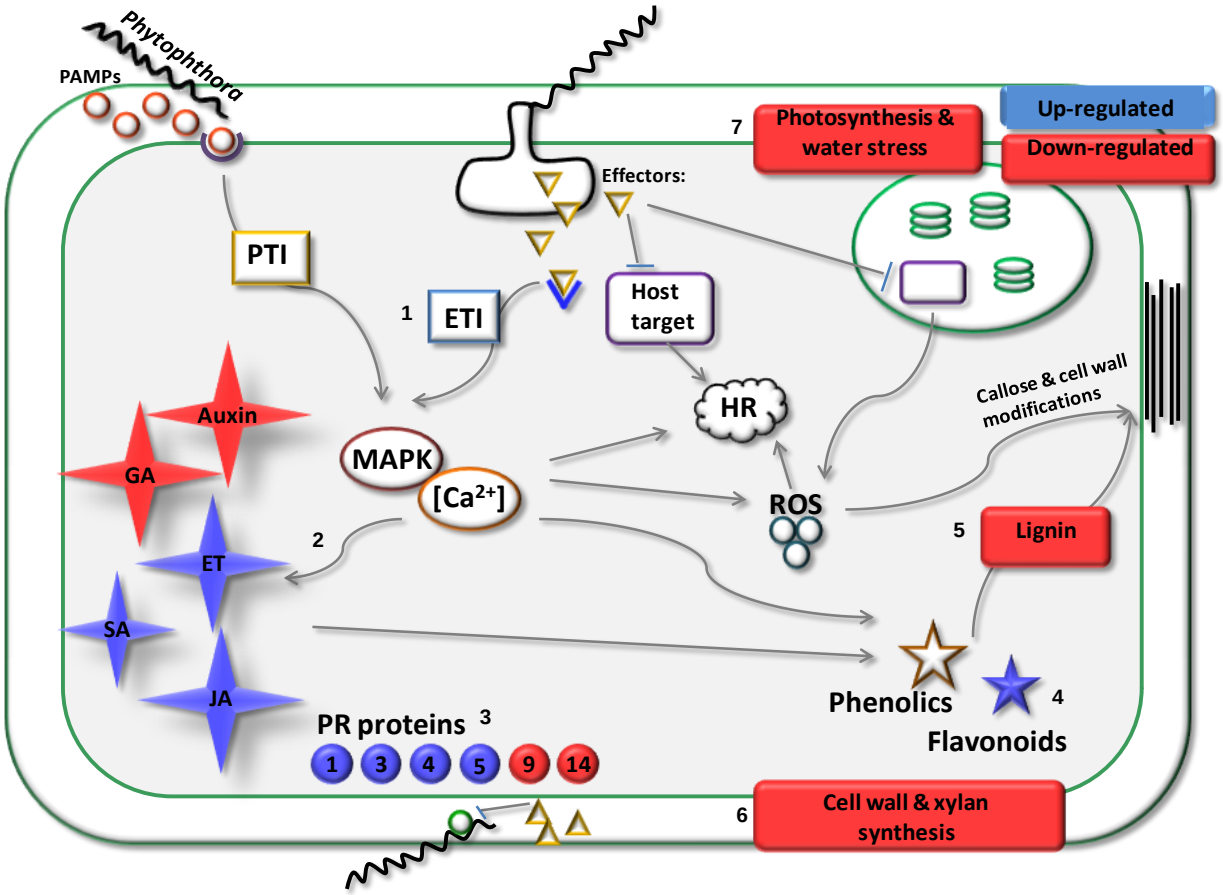

Figure 3.10 Model of defence responses involved in various *Phytophthora*-plant interactions from Chapter 1, with the main findings from the *E. nitens-P. cinnamomi* interaction superimposed. Differentially down-regulated defence categories are marked as red, while up-regulated is marked in blue. [1]When effectors are detected by host resistance (*R*) genes, effector triggered immunity (ETI) results. Several of the down-regulated defence categories may be manipulated by effectors, most notably, a putative *PR-9* ortholog. [2]Hormone signalling was regulated by up-regulation of SA, ET, and JA-related terms and down-regulation of growth-related hormones [3]*PR* gene classes *PR-1*, *PR-3*, *PR-4* and *PR-5* were up-regulated and certain putative orthologs of *PR-9* as well as *PR-14* orthologs were down-regulated. [4]Flavonoid biosynthesis is mainly up-regulated, though one putative gene encoding a key enzyme is down-regulated. [5]Numerous lignin biosynthesis-associated terms are down-regulated, which could result in weaker cell walls [6]Cell wall biosynthesis and growth-related terms are down-regulated, which could imply that defence processes are favoured above growth, despite the fact that it is a compatible interaction. [7]Photosynthesis-and water stress related terms are down-regulated, which could be an indirect physiological response to infection, or may favour the presence of the pathogen in the host.

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Chapter 4

Concluding Remarks

Cold-tolerant eucalypts, specifically *Eucalyptus nitens* plantations in South Africa, are subject to die-back and root rot caused by *Phytophthora* spp. More research on the molecular basis of responses to *Phytophthora* spp. in *Eucalyptus* is required in order to elucidate resistance mechanisms for selection of more tolerant trees. *P. cinnamomi* is one of the most virulent *Phytophthora* spp. on *Eucalyptus* at the present time, and though host responses to this species have been investigated in *E. marginata* (Cahill *et al.*, 2008), this species is not routinely cultivated in South Africa.

During the search for a suitable *Phytophthora* spp. to inoculate *E. nitens*, isolates belonging to a clade 9 *Phytophthora* spp. was found associated with dead and dying *Eucalyptus* seedlings in the Tzaneen area. Though the regions encoding the internal transcribed spacer (ITS), β-tubulin (β-*tub*), translation elongation factor 1 alpha (*EF*-1α), NADH dehydrogenase subunit 1 (*nadh*1) and cytochrome c oxidase subunit 1 (*cox*I) were sequenced for three of the isolates, and maximum likelihood, parsimony and Bayesian inference methods were used to construct phylogenetic trees, the species identity of the isolates remained unclear. This is partially due to a lack of sequence information for all the above mentioned gene regions within clade 9, but it is also due the presence of a so-called *P. parsiana* complex, which may contain multiple species and therefore confounds the phylogeny of the clade. Since the phylogenetic analysis was performed, a new species, *P. macilentosa*, has been added to the clade (Yang *et al.*, 2013), and some undescribed/unidentified clade 9 species have been found Australian and South African waterways (Hüberli *et al.*, 2013; Oh *et al.*, 2013). A study should be dedicated to resolve the phylogenetic relationship of species within clade 9, since identification of new isolates is difficult and confusing at the moment.

The unknown *Phytophthora* spp. was inoculated on *E. nitens* stems at a standard temperature, and a raised temperature due to the hot region where it was isolated and the high temperature tolerance of other species in clade 9. Though it was virulent on *E. nitens* at a higher temperature, *P. cinnamomi* remained the most virulent and an older isolate of this species was used to conduct further studies.

Through the use of confocal microscopy, *P. cinnamomi* hyphae were confirmed to be present in *E. nitens* stem tissue by 48 hpi. A late time-point of five days post-inoculation was chosen to profile the host responses to *P. cinnamomi,* since the timing of the system is not well-known and it was essential that most of the tissue sampled was in contact with pathogen hyphae. The use of 3 cm stem samples around the inoculation site capture a range of infection stages from the necrotrophic advanced infection in the centre, to the biotrophic lesion boundary. Future work could include a more targeted sample strategy to capture just cells on the extending lesion area, or those in advanced stages of necrosis. This

could be done through laser capture microdissection or cryo-sectioning of the desired regions, followed by RNA amplification. Profiling transcripts present in this specific tissue will enable more accurate analysis and ability to form hypotheses concerning host responses to the pathogen.

RNA-seq profiling revealed the up-regulation of defence-associated transcripts and simultaneous down-regulation of growth-associated ones at a late stage of infection. Protein and metabolite studies are required to confirm regulation of defence hormones, different *PR* genes and flavonoids in the interaction, as well as the down-regulation of proteins involved in lignin and cell wall biosynthesis. Many of the identified TAIR10 identifiers in this study correlated to multiple differentially regulated putative orthologs in *E. nitens* and protein level studies could help confirm whether all the transcripts are translated to be functional on protein level.

Since the differentially expressed transcripts from *E. nitens* were mapped to *E. grandis* and these were matched to putative orthologs in *A. thaliana,* functional studies of the *E. nitens* gene products are required before any firm conclusions can be drawn with regards to the role differentially regulated transcripts play in the interaction. One of the first candidates for such a functional study should be Eucgr.E04056, the down-regulated putative peroxidase ortholog with high sequence similarity to a papaya peroxidase and its putative orthologs which were down-regulated in papaya, potato and soybean in response to three different *Phytophthora* spp. (Moy *et al.*, 2004; Porter *et al.*, 2009; Restrepo *et al.*, 2005). The evidence from these pathosystems and the current *Eucalyptus* interaction strongly suggests that the peroxidase could be a cross-species host effector target for *Phytophthora*. Additionally, *PR-1, PR-3, PR-4,* and *PR-5* could be investigated by performing functional studies by transforming *A. thaliana PR* gene mutants with the putative orthologs from *E. nitens*.

MAPK signalling and Ca^{2+} signalling, as well the involvement of the oxidative burst and HR in defence against various *Phytophthora* spp. was noted in the literature review. These aspects of not been mined in the *E. nitens* interaction. Looking for specific genes related to these processes may uncover responses that have not been noticed yet. The dataset should also be mined for specific genes such as carbonic anhydrase (Birch *et al.*, 1999; Restrepo *et al.*, 2005) and berberine bridge enzyme (Moy *et al.*, 2004; Schlink, 2009) that have been observed in multiple studies and may play a role in defence. Furthermore, the RNA-seq data could be mapped to *P. cinnamomi* in order to elucidate genes that are involved in virulence, specifically those that could be acting as possible RXLR effectors.

Though the advanced stages of a compatible interaction with *P. cinnamomi* was profiled, it would seem that *E. nitens* is launching defence responses at the cost of growth. This reveals a possible resistance mechanism that is activated too late, and not strongly enough. Furthermore, the presence of certain down-regulated processes such as lignin biosynthesis and a putative peroxidase indicate possible manipulation by *P. cinnamomi* to establish infection and colonize the host. Comparative studies with a resistant *Eucalyptus* host will enable a hypothesis to be formed regarding the mechanisms specific to resistance and compatibility.

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Summary

Eucalyptus spp. are commercially important plantation trees, and in South Africa, *Phytophthora* spp. are problematic due to the die-back they cause on cold-tolerant species. Recently, next-generation sequencing technology such as RNA-seq has emerged as a useful tool to profile transcriptomes of non-model interactions, such as *Phytopthora*-infected *Eucalyptus*. *Phytophthora* species possess pathogen-associated molecular patterns that trigger basal defences, and effectors, which manipulate specific host targets responsible for defence. *Phytophthora* infection elicits various well-known defence responses, and the timing of defence activation seems to be critical in some cases due to *Phytophthora*'s hemi-biotrophic nature. In response to *P. cinnamomi* specifically, hosts often respond to infection through regulation of secondary metabolites and reactive oxygen species. The aim of this study was to set up a *Eucalyptus-Phytophthora* pathosystem and to determine the host responses to the pathogen at the molecular level.

The host *E. nitens* was selected to study the host-*Phytophthora* interaction due to its commercial importance in South Africa and the problems associated with *Phytophthora* infection in certain stands. The search for an appropriate *Phytophthora* spp. resulted in the discovery of a clade 9 *Phytophthora* spp. associated with the roots of dying eucalypts in the Tzaneen area. The identity of this isolate could not be confirmed due to a lack of sequence information for many gene regions of described species in clade 9. The isolate proved virulent on *E. nitens* at an elevated temperature, but *P. cinnamomi* remained more virulent in comparison. Microscopically and through re-isolation, *P. cinnamomi* was confirmed to be present in *E. nitens* stem tissue 48 hours post inoculation, indicative of successful infection.

To gain insight into attempted defence mechanisms and possible pathogen manipulation targets, RNA-sequencing of the compatible interaction between *E. nitens* and *P. cinnamomi* was performed by Beijing Genomics Institute. Stem tissue was sampled from mock-inoculated and inoculated trees at a late time point, 5 days post inoculation, and total RNA was extracted. RNA-seq reads were mapped to the *E. grandis* genome and 1475 differentially expressed genes were present in the inoculated samples compared to the

mock-inoculated controls. Overrepresented gene ontology (GO) terms in the differentially regulated dataset were used to analyse biologically significant pathways. RNA-sequencing revealed that *E. nitens* responds to inoculation with an overall shift from growth to defence related transcripts. GO terms related to defence mechanisms, such as jasmonic acid, ethylene and salicylic acid signalling, pathogenesis related (*PR*) genes, and flavonoid biosynthesis were found in the up-regulated dataset. In the down-regulated dataset, GO terms related to secondary cell wall and lignin biosynthesis, as well as giberellin and auxin signalling were found. A putative peroxidase, Eucgr.E04056 was down-regulated in in *E. nitens,* has previously been shown to be down-regulated in three different hosts challenged with different *Phytophthora* spp. This gene may represent a cross-species *Phytophthora* effector target.

Despite the late time after inoculation, *E. nitens* displays responses indicative of attempted defence. Comparison to a tolerant *Eucalyptus- P. cinnamomi* interaction will provide insight into which of these responses are important to mediate tolerance. This study has identified several potential host targets that may be under pathogen manipulation and further mining of the dataset for *P. cinnamomi* transcripts could identify expressed effectors during infection.

Appendices

Appendix A: Colony PCR confirmation of successful transformation

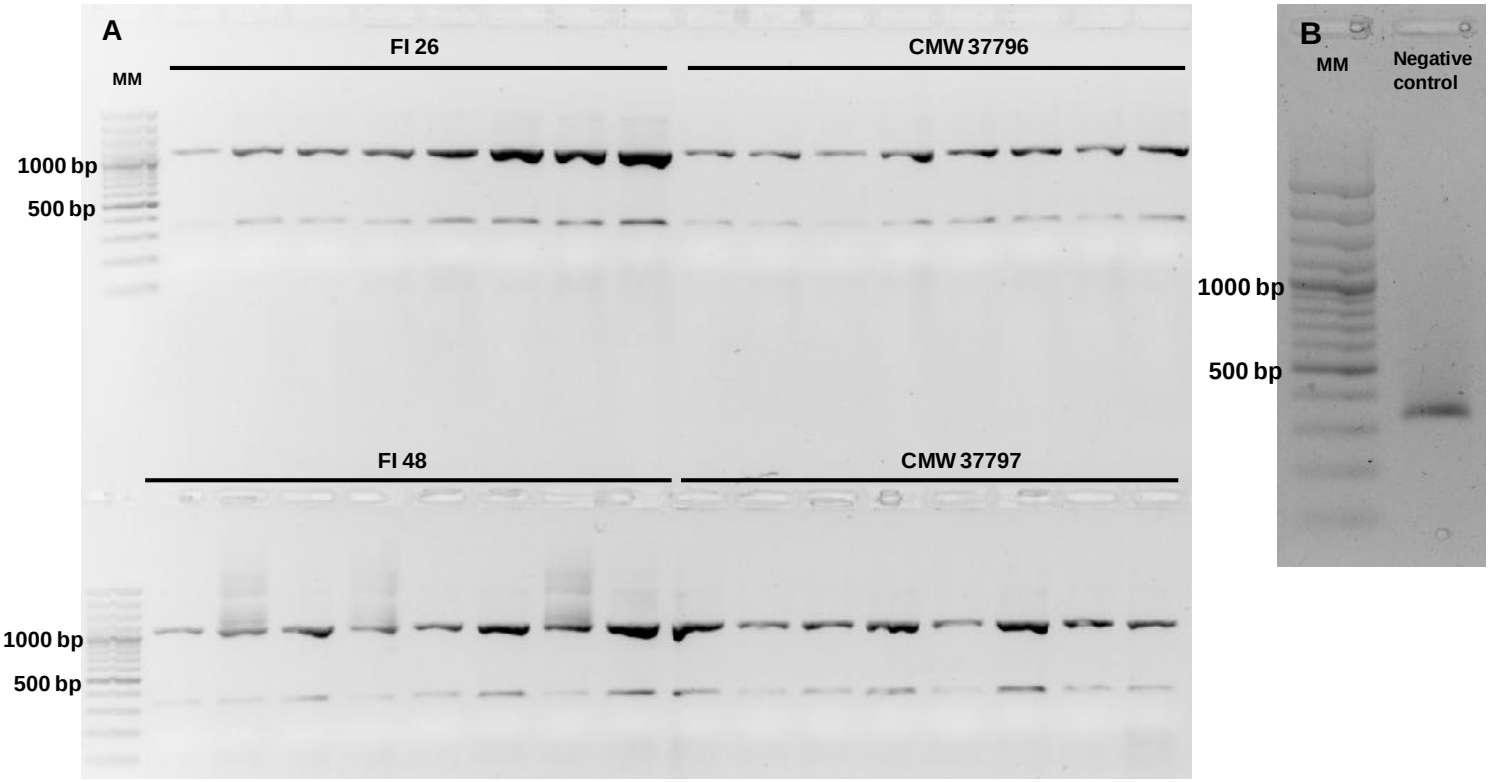

Figure A 2.1: M13 amplification of (A) recombinant colonies of clones containing the ITS region of FI 26, CMW 37796, FI 48 and CMW 37797. Eight colonies (each colony respresented by a lane) were screened per clone (B) a no-template negative control. Lanes marked MM contain the molecular marker - 100 bp Fermentas O'GeneRuler.

Appendix B: Sequence data of field isolates

Presented below is the sequence data obtained for field isolates during the course of the study. Data is represented in isolate order and FASTA format, with the gene region it pertains to displayed in the first line. This is also provided in electronic format on the attached CD (CD\File A1_Isolate sequences.docx)

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>CMW_37797 *cox*I
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>CMW37797 *nadh*1
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> FI 5 ITS

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>FI 13 ITS
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> FI 17 ITS
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>FI 26 ITS
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> FI 36 ITS
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> FI 44 ITS
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> FI 45 ITS
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> FI 46 ITS
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> FI 48 ITS
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Appendix C: Phylogenetic trees with *Phytophthora* spp. from all clades

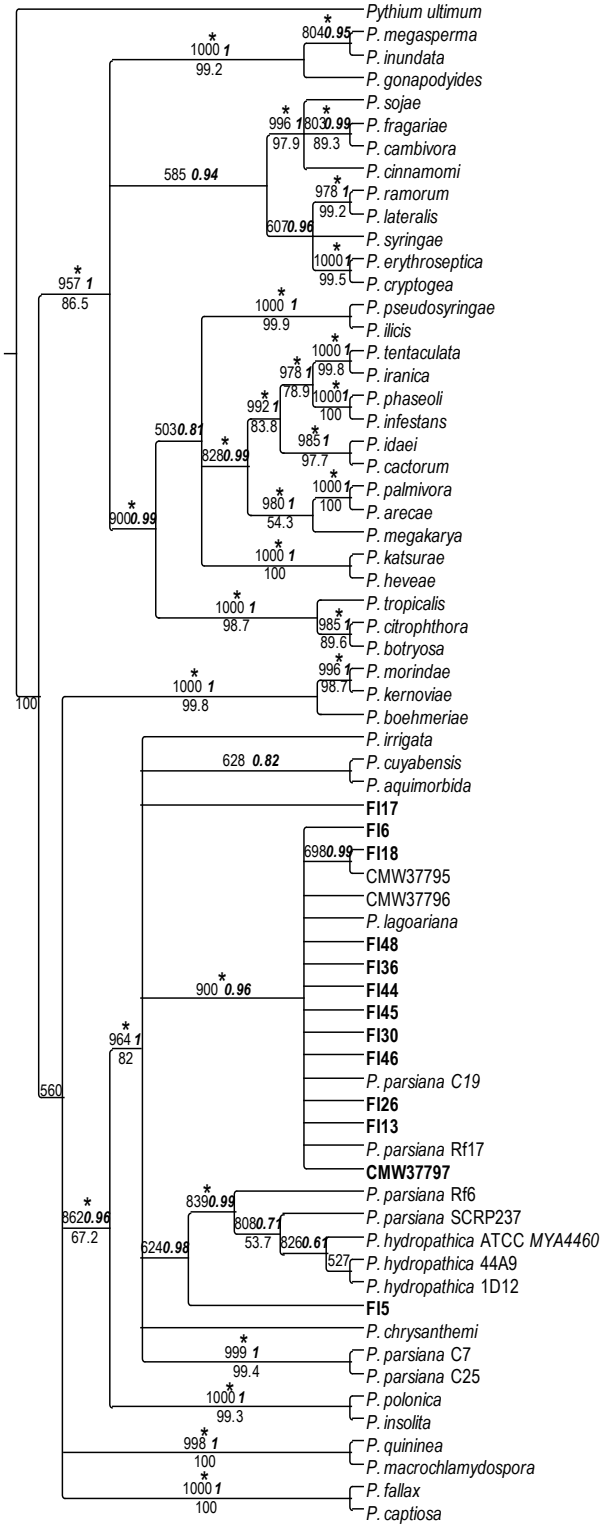


Figure A 2.2 Cladogram derived from maximum likelihood analyses of the ITS region of representatives of *Phytophthora* spp. from all ten clades. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

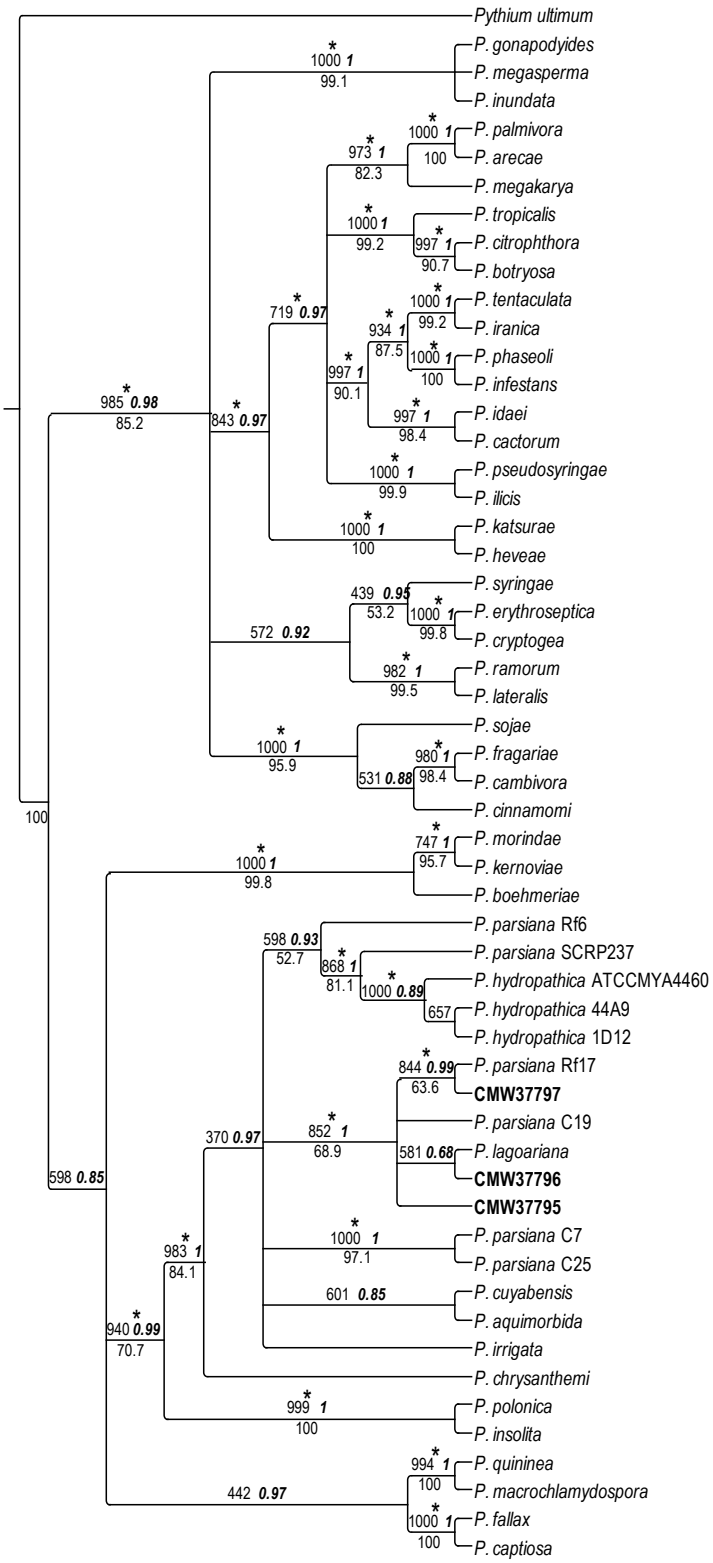


Figure A 2.3 Cladogram derived from maximum likelihood analyses of the ITS region of representatives of *Phytophthora* spp. from all ten clades. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

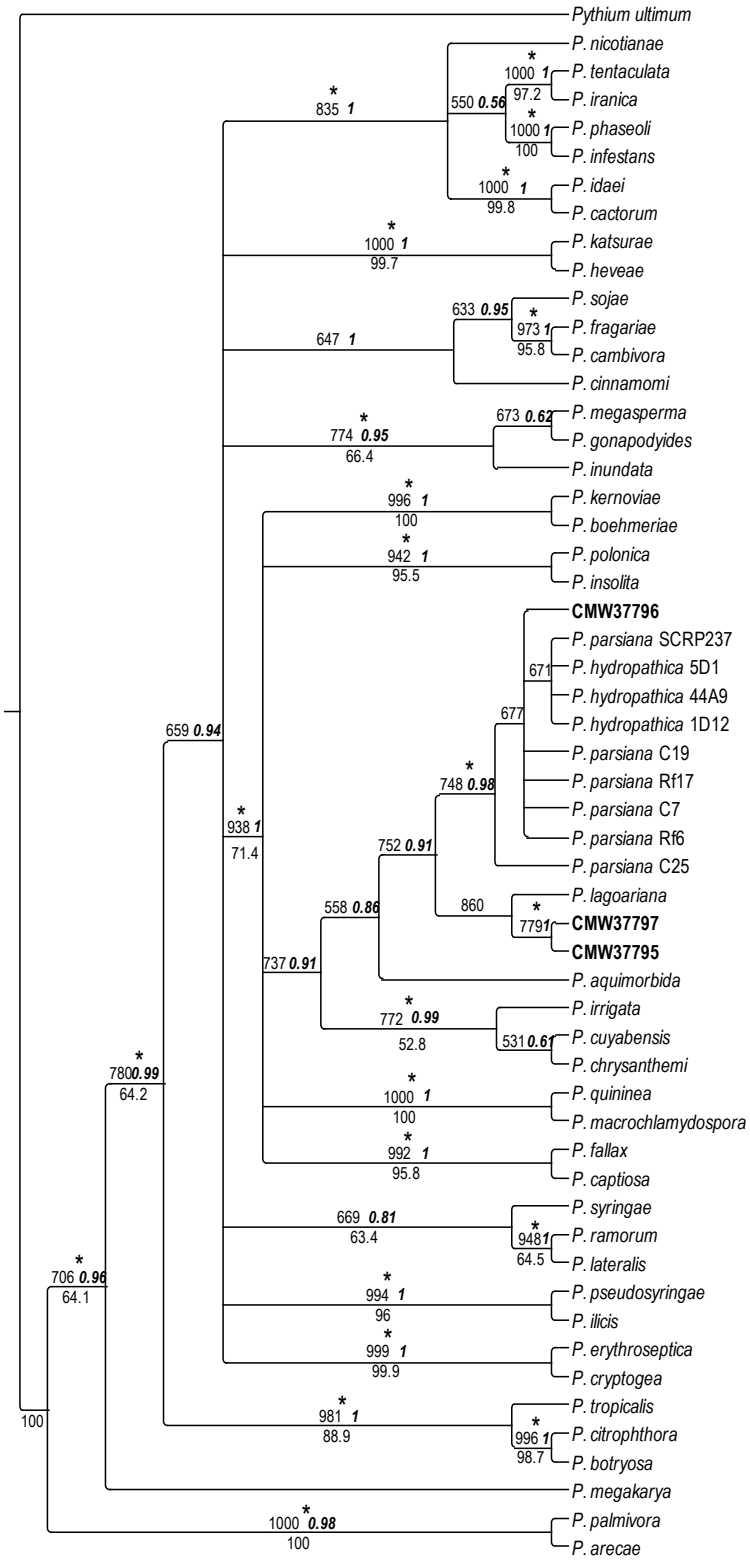


Figure A 2.4 Cladogram derived from maximum likelihood analyses of the β-*tub* region of representatives of *Phytophthora* spp. from all ten clades. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

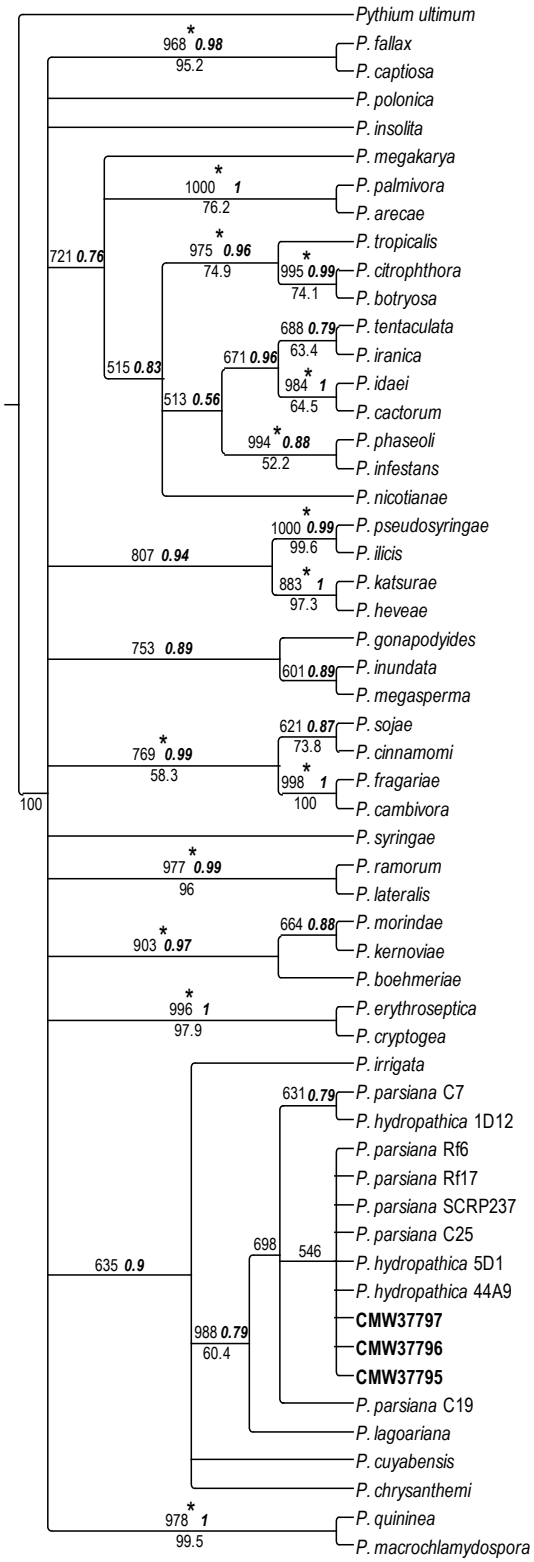


Figure A 2.5 Cladogram derived from maximum likelihood analyses of the *EF*-1α region of representatives of *Phytophthora* spp. from all ten clades. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

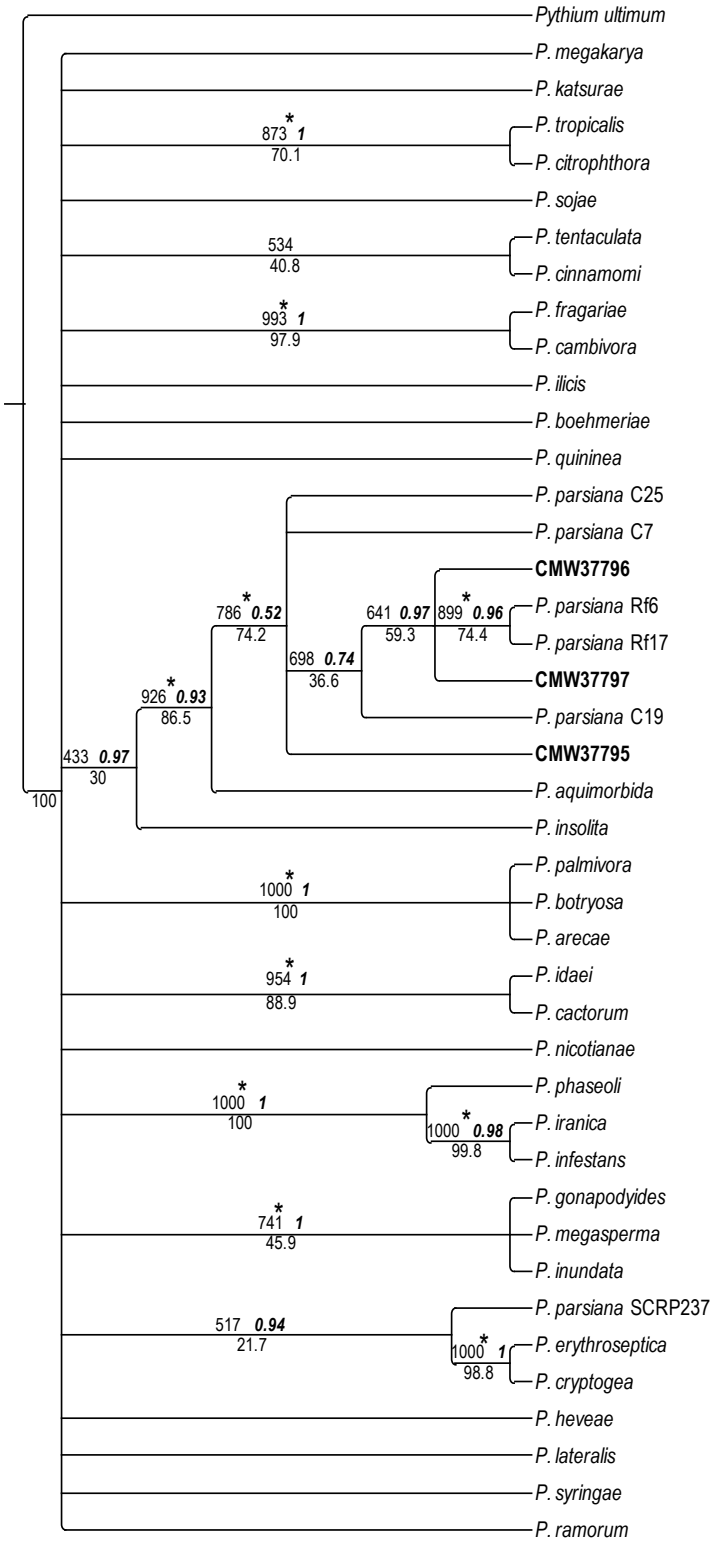


Figure A 2.6 Cladogram derived from maximum likelihood analyses of the *cox*I region of representatives of *Phytophthora* spp. from all ten clades. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

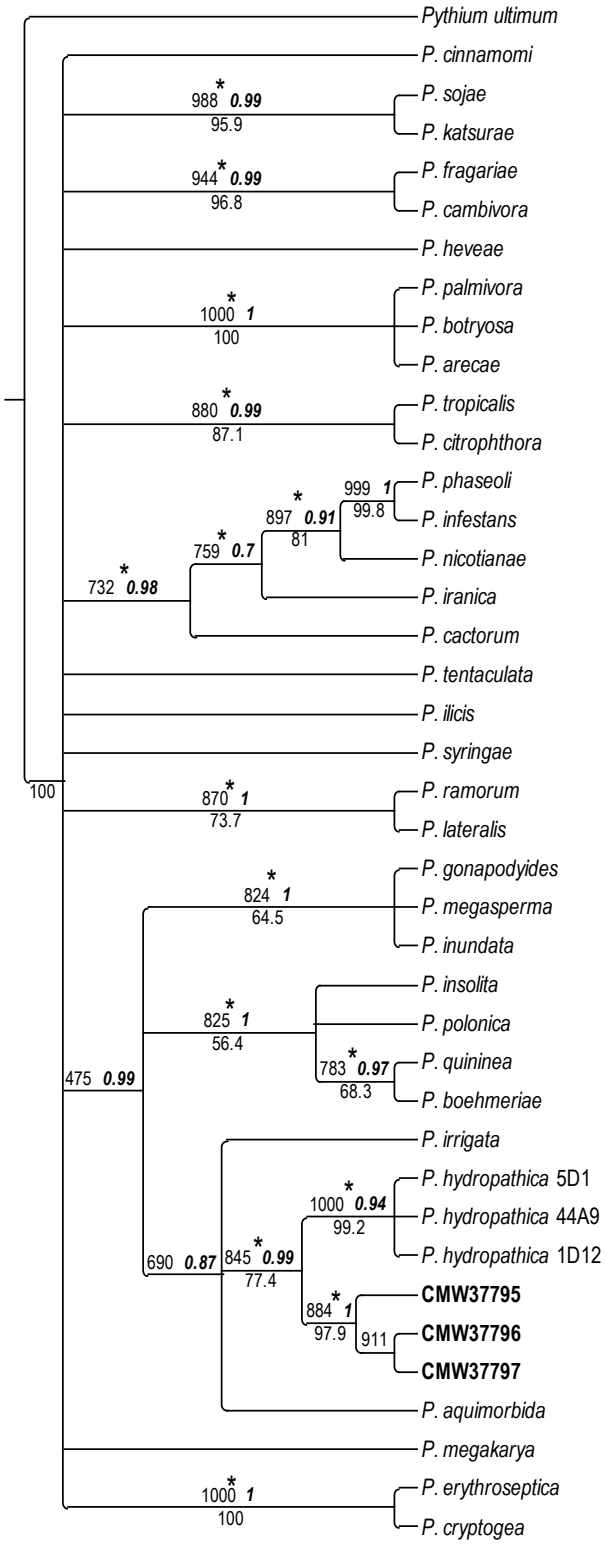


Figure A 2.7 Cladogram derived from maximum likelihood analyses of the *nadh*1 region of representatives of *Phytophthora* spp. from all ten clades. Maximum likelihood and parsimony derived bootstrap values are depicted above and below the branches respectively, and posterior probability values obtained from bayesian inference analyses are indicated above branches in bold and italics. Nodes with two or more support values above the cut-off threshold (>70% bootstrap or 0.95 posterior probability) are marked with *.

Appendix D: Models used for maximum likelihood and Bayesian inference

The models used in PhyML and MrBayes for the various gene regions are indicated for both datasets. Models for PhyML were calculated using JModelTest, and models for MrBayes were calculated using MrModelTest2.

Table A 2.1 JModelTest output for PhyML for all *Phytophthora* clades dataset.

ITS	ITS all isolates	*EF*-1α	β-*tub*	*nadh*1	*cox*I
Model = TPM2uf+G partition = 010212 -lnL = 9828.0303 K = 114 freqA = 0.1477 freqC = 0.1978 freqG = 0.3068 freqT = 0.3477 R(a) [AC] = 1.6882 R(b) [AG] = 3.7807 R(c) [AT] = 1.6882 R(d) [CG] = 1.0000 R(e) [CT] = 3.7807 R(f) [GT] = 1.0000 gamma shape = 0.3820	Model = GTR+G partition = 012345 -lnL = 10463.3536 K = 141 freqA = 0.1616 freqC = 0.1960 freqG = 0.3107 freqT = 0.3317 R(a) [AC] = 1.2140 R(b) [AG] = 2.3702 R(c) [AT] = 1.3413 R(d) [CG] = 0.6457 R(e) [CT] = 3.1948 R(f) [GT] = 1.0000 gamma shape = 0.4010	Model = GTR+I+G partition = 012345 -lnL = 5169.4104 K = 118 freqA = 0.2030 freqC = 0.3144 freqG = 0.3022 freqT = 0.1804 R(a) [AC] = 0.2621 R(b) [AG] = 0.3559 R(c) [AT] = 0.5692 R(d) [CG] = 1.5163 R(e) [CT] = 4.2829 R(f) [GT] = 1.0000 p-inv = 0.4680 gamma shape = 0.2840	Model = TIM2+I+G partition = 010232 -lnL = 5623.5669 K = 116 freqA = 0.2100 freqC = 0.2993 freqG = 0.2696 freqT = 0.2211 R(a) [AC] = 0.4066 R(b) [AG] = 1.5111 R(c) [AT] = 0.4066 R(d) [CG] = 1.0000 R(e) [CT] = 5.7424 R(f) [GT] = 1.0000 p-inv = 0.6260 gamma shape = 1.1050	Model = TIM2+I+G partition = 010232 -lnL = 4016.1125 K = 86 freqA = 0.2736 freqC = 0.1000 freqG = 0.1492 freqT = 0.4772 R(a) [AC] = 4.8452 R(b) [AG] = 5.2690 R(c) [AT] = 4.8452 R(d) [CG] = 1.0000 R(e) [CT] = 11.5224 R(f) [GT] = 1.0000 p-inv = 0.4100 gamma shape = 0.3710	Model = GTR+I+G partition = 012345 -lnL = 3872.5201 K = 92 freqA = 0.2699 freqC = 0.0814 freqG = 0.1830 freqT = 0.4657 R(a) [AC] = 9.1254 R(b) [AG] = 13.2240 R(c) [AT] = 12.3441 R(d) [CG] = 6.6892 R(e) [CT] = 34.2941 R(f) [GT] = 1.0000 p-inv = 0.6250 gamma shape = 0.7910

Table A 2.2 JModelTest output for PhyML for clade 9 *Phytophthora* dataset.

ITS	ITS all isolates	*EF*-1α	β-*tub*	*nadh*1	*cox*I
Model = TrN+G partition = 010020 -lnL = 4919.5730 K = 88 freqA = 0.1663 freqC = 0.2050 freqG = 0.3102 freqT = 0.3185 R(a) [AC] = 1.0000 R(b) [AG] = 1.4235 R(c) [AT] = 1.0000 R(d) [CG] = 1.0000 R(e) [CT] = 3.2456 R(f) [GT] = 1.0000 gamma shape = 0.3020	Model = TIM1+G partition = 012230 -lnL = 5245.3460 K = 113 freqA = 0.1703 freqC = 0.2085 freqG = 0.3037 freqT = 0.3176 R(a) [AC] = 1.0000 R(b) [AG] = 1.4749 R(c) [AT] = 0.7495 R(d) [CG] = 0.7495 R(e) [CT] = 2.9325 R(f) [GT] = 1.0000 gamma shape = 0.3430	Model = TIM2+G partition = 010232 -lnL = 2168.3275 K = 69 freqA = 0.1958 freqC = 0.3168 freqG = 0.3113 freqT = 0.1762 R(a) [AC] = 0.2710 R(b) [AG] = 0.2047 R(c) [AT] = 0.2710 R(d) [CG] = 1.0000 R(e) [CT] = 4.2731 R(f) [GT] = 1.0000 gamma shape = 0.0460	Model selected: Model = GTR+I+G partition = 012345 -lnL = 2460.1978 K = 70 freqA = 0.2021 freqC = 0.3048 freqG = 0.2799 freqT = 0.2132 R(a) [AC] = 0.2380 R(b) [AG] = 1.5137 R(c) [AT] = 0.5605 R(d) [CG] = 2.1703 R(e) [CT] = 7.8182 R(f) [GT] = 1.0000 p-inv = 0.5440 gamma shape = 0.6380	Model = TIM2+I partition = 010232 -lnL = 1658.9462 K = 35 freqA = 0.2682 freqC = 0.0980 freqG = 0.1565 freqT = 0.4773 R(a) [AC] = 5.3591 R(b) [AG] = 2.9282 R(c) [AT] = 5.3591 R(d) [CG] = 1.0000 R(e) [CT] = 5.7790 R(f) [GT] = 1.0000 p-inv = 0.7940	Model selected: Model = GTR+I+G partition = 012345 -lnL = 1648.8879 K = 38 freqA = 0.2513 freqC = 0.1027 freqG = 0.1891 freqT = 0.4570 R(a) [AC] = 0.0000 R(b) [AG] = 4.1539 R(c) [AT] = 10.2797 R(d) [CG] = 1.9562 R(e) [CT] = 19.2479 R(f) [GT] = 1.0000 p-inv = 0.6850 gamma shape = 0.7510

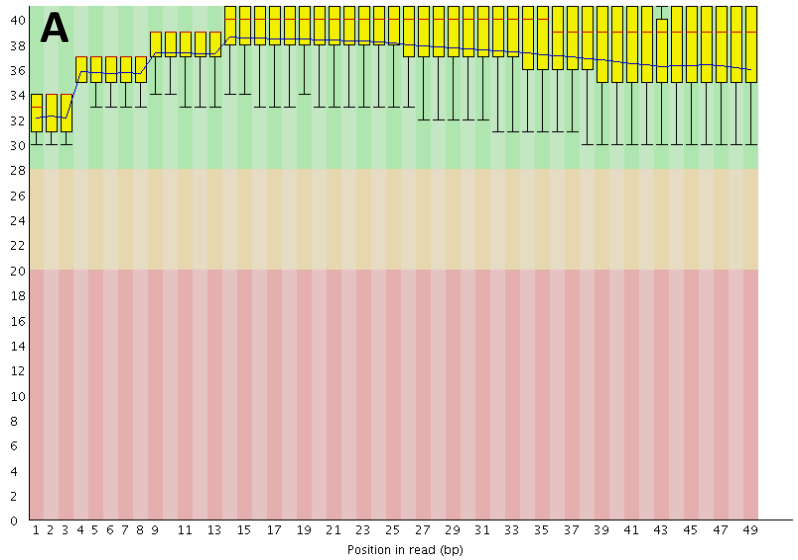
MrModelTest2 suggested the following model for all the different gene regions of both datasets:

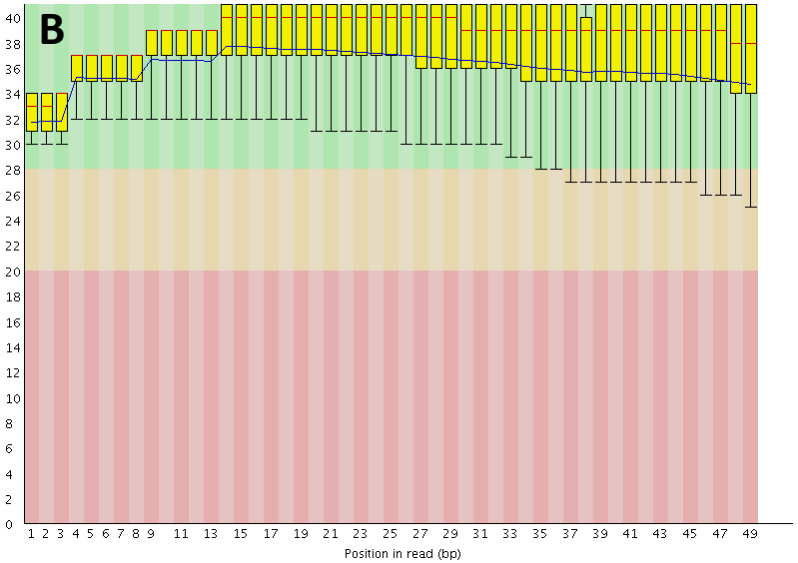
Prset statefreqpr=dirichlet(1,1,1,1);

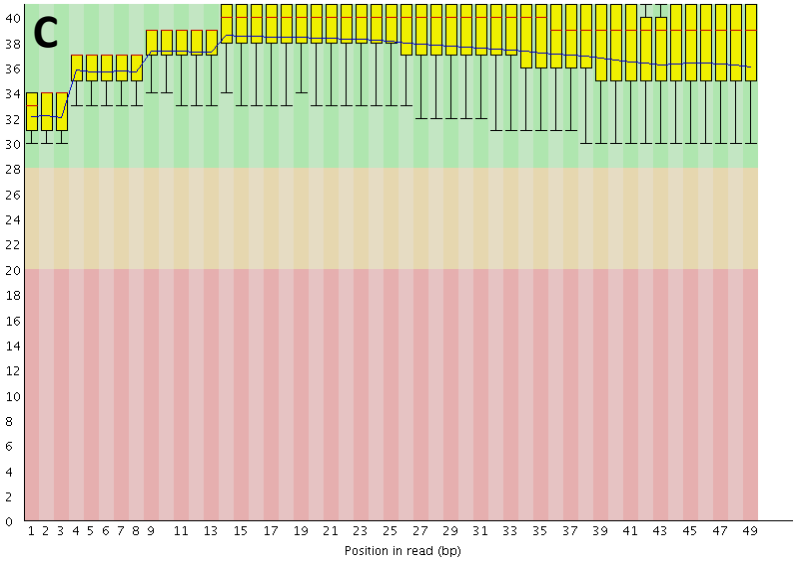
Lset nst=6 rates=invgamma;

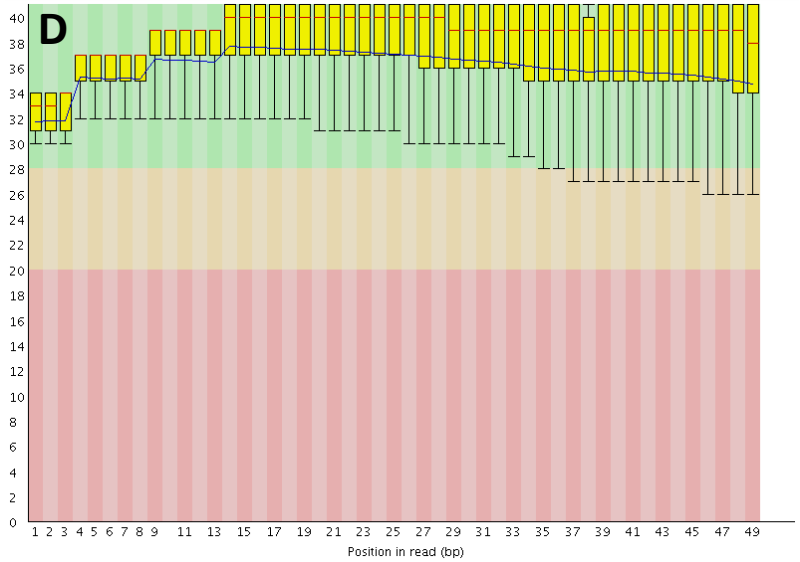
The only exception was the clade 9 dataset for the *nadh1* region where “invgamma” was replaced by “propinv”.

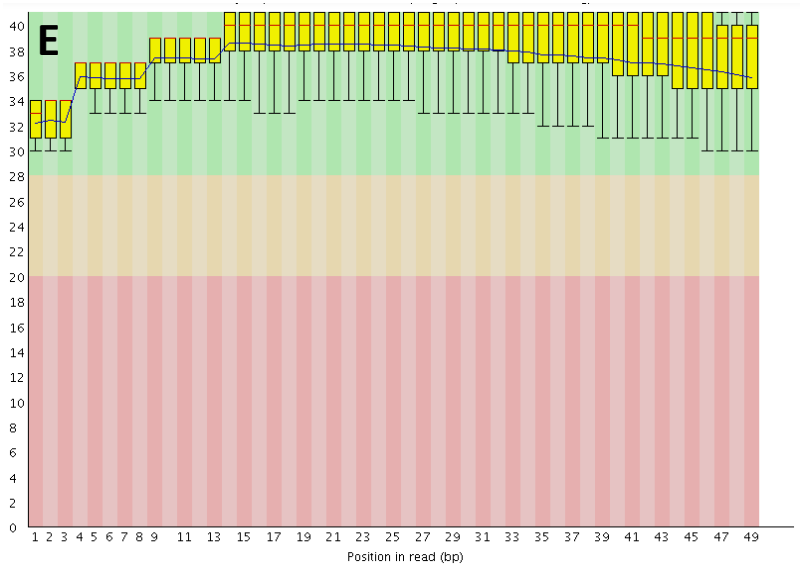
Appendix E: Read quality of RNA-seq data

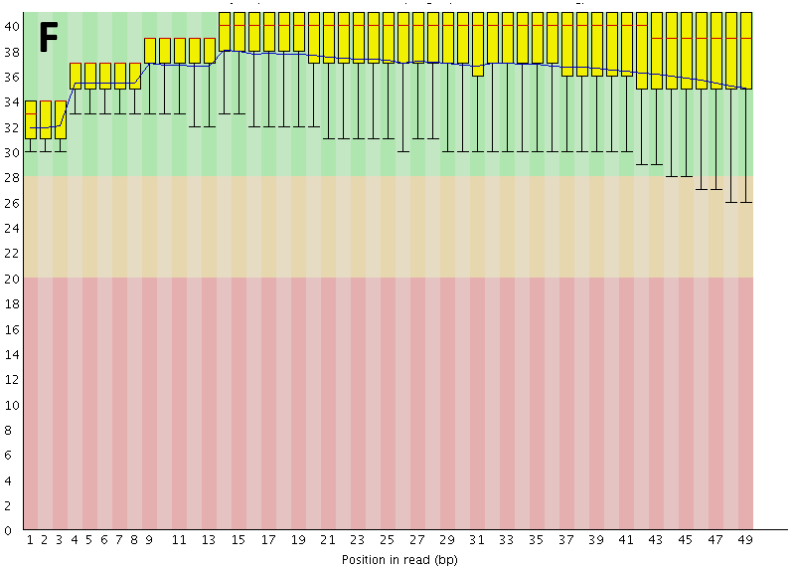


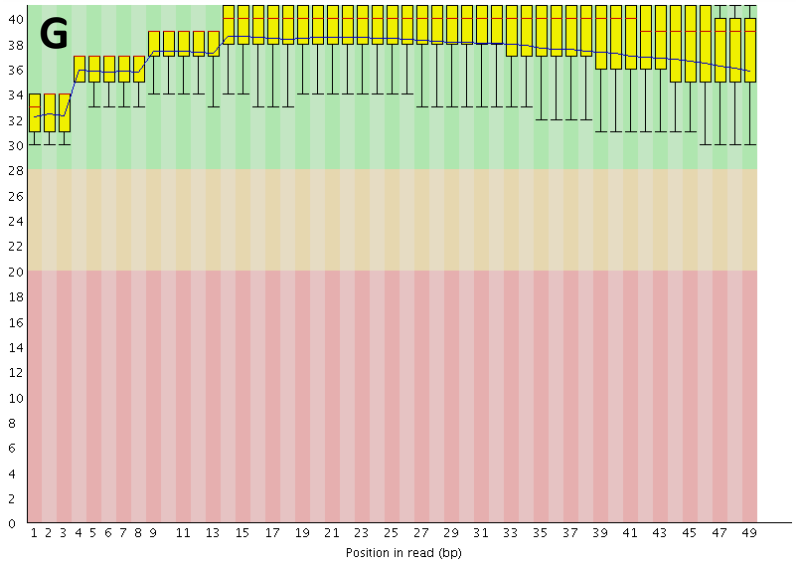


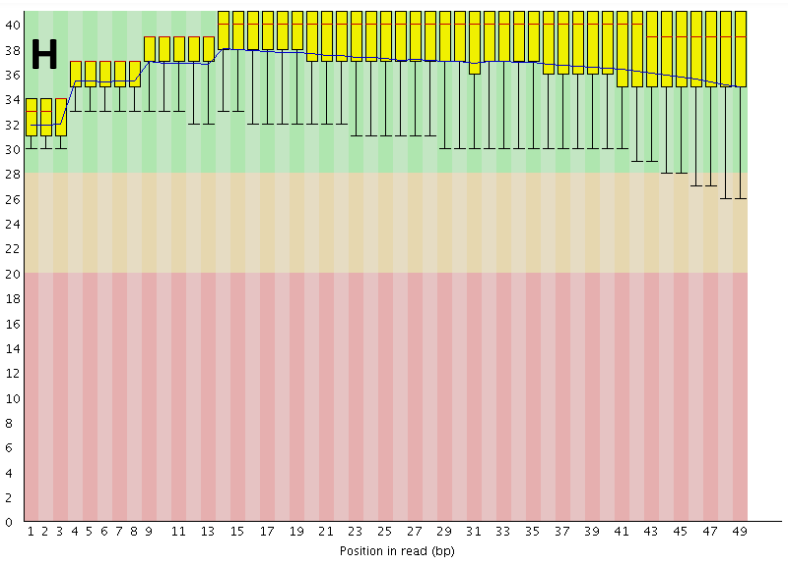


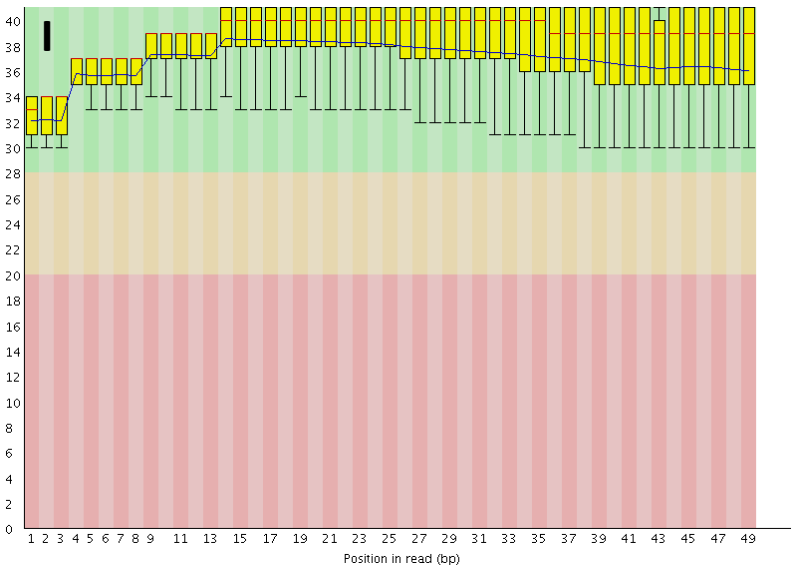












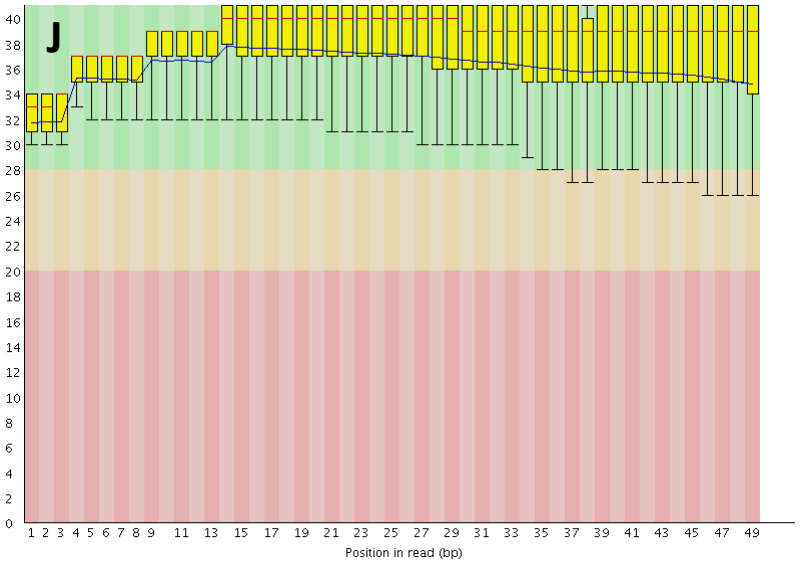


Figure A 3.1 FastQC boxplots depicting the quality scores of RNA-seq read data for (A) Control biological replicate 2 forward (B) reverse reads (C) Control biological replicate 3 forward (D) reverse reads (E) Infected biological replicate 1 forward (F) reverse reads (G) Infected biological replicate 2 forward (H) reverse reads (I) Infected biological replicate 3 forward (J) reverse reads

Appendix F: Annotated Cufflinks and Cuffdiff files

Microsoft Excel files containing the output files from Cufflinks (CD\File A2_Cufflinks gene expression values.xlsx) and Cuffdiff (CD\File A3_Cuffdiff differential genes.xlsx) are saved on the attached CD. The *Eucalyptus* gene models from the files have been matched to their putative *A. thaliana* orthologs based on a reciprocal BLAST, as well as matching PFAM, KEGG and KOG annotations.

Appendix G: Annotated differentially expressed genes

An annotated list of significantly differentially expressed genes and transcript models from Cuffdiff is provided on the attached CD (CD\File A4_Significantly differential genes and transcripts_annotated.xlsx).

Appendix H: FPKM histogram

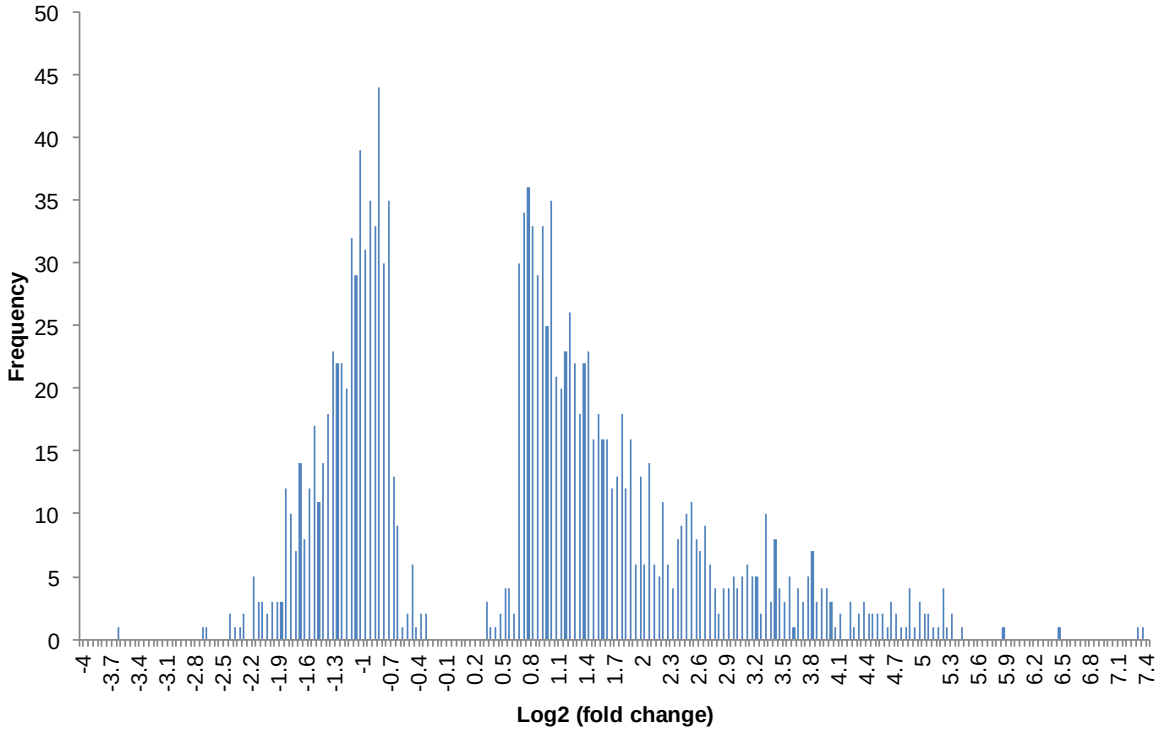


Figure A 3.2 Frequency of the distribution of log2 (fold change) values of significantly differentially expressed genes, arranged in bins of 0.05 units each. Low frequencies close to log2 (fold change) of 0 are present since genes with such low differential expression weren't statistically significant.

Appendix I: BiNGO output files

The data tables for BiNGO output for the up – and down-regulated dataset are provided on the attached CD (CD\File A5_BiNGO result tables.xlsx).

Appendix J: Secondary metabolite table with FPKM values

The data table with average FPKM values for putative orthologs of TAIR10 IDs annotated on the MapMan secondary metabolism pathway (adapted from Table 3.5) is provided on the attached CD (CD\File A6_Table 3.5 with FPKM.xlsx).

Appendix K: *PR* gene table with FPKM values

The data table with average FPKM values for putative *PR* genes from *E. nitens* (adapted from Table 3.6) is provided on the attached CD (CD\File A7_Table 3.6 with FPKM.xlsx).

Appendix L: Mapman pathway overviews

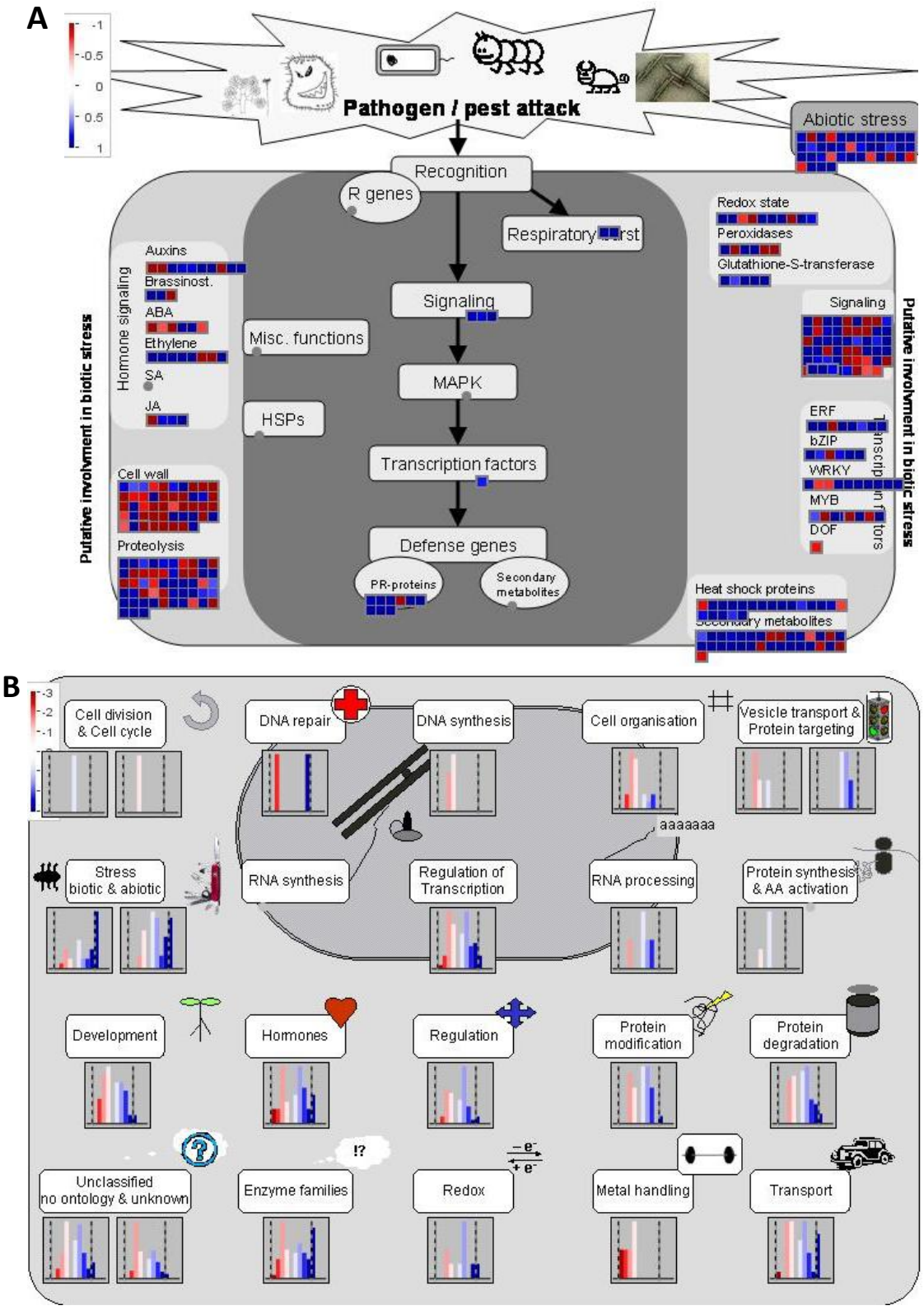


Figure A 3.3 TAIR ID's that are putative orthologs of *E. nitens* genes from this dataset mapped to different bins in MapMan. A putative overview of (A) biotic stress and (B) cell functions in this interaction can be viewed according to which genes were differentially expressed.