THE PHOSPHITE RESPONSIVE TRANSCRIPTOME OF

PHYTOPHTHORA CINNAMOMI

by

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This thesis is presented for the degree of Doctor of Philosophy

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Declaration

The work described in this thesis was undertaken while I was an enrolled student for the degree of Doctor of Philosophy at Murdoch University, Western Australia. I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution. To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged.

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Michaela King

October 2007
Abstract

Phosphite has been used to effectively control the soil-borne plant pathogen *Phytophthora cinnamomi* in many horticultural crops, forest trees and natural ecosystems. However, the molecular mechanisms behind phosphite action on this pathogen are poorly understood.

Several studies have shown that phosphite inhibits growth and zoospore production of *P. cinnamomi* and in addition induces significant physiological and metabolic changes in the mycelium. As an approach to understanding the mechanisms and relevance of these changes in the pathogen, the effect of phosphite on gene expression was investigated using microarray analysis. To construct the microarray, RNA was extracted from phosphite-treated (40 µg/ml) mycelium of *P. cinnamomi* isolate MP 80. The chosen phosphite concentration inhibited the mycelial growth by 70 % but provided sufficient mycelium for RNA extractions after 4 days growth at 25 °C.

The mRNA was reverse transcribed into cDNA and cloned into lambda to construct a library consisting of 2 million pfu of which 80 % were recombinant phage. The inserts were sequenced for a random selection of clones from the library. The nucleotide sequences generated revealed a range of different *P. cinnamomi* genes being expressed and demonstrated that the cDNA library provided a good representation of the transcripts expressed in *P. cinnamomi*. The types of genes found to be expressed in the mycelium of *P. cinnamomi* included genes encoding GTP-binding proteins involved in vesicle transport, structural proteins involved in maintaining cell membrane integrity, elicitors, phosphatases and ribosomal proteins.
Over nine thousand cDNA transcripts were randomly selected from the cDNA library and prepared by PCR amplification and purification for microarray construction. Custom made cDNA arrays containing 9216 cDNA transcripts were constructed and probed with RNA from untreated mycelium and mycelium grown in medium with 40 µg/ml phosphite.

Two genes, EF-1 alpha and cinnamomin gene, identified by qRT-PCR as being constitutively expressed were also positioned on the arrays as positive controls. In the process of identifying constitutively expressed genes, qRT-PCR revealed that phosphite down-regulated a gene encoding ubiquitin-conjugating enzyme, a component of the ubiquitin/proteasome pathway involved in the removal of abnormal and short lived-regulatory proteins and rate limiting enzymes.

From the arrays a further seventy-two transcripts with altered patterns in gene expression (fold change ≥ 2) were identified. The majority of the cDNA transcripts spotted on the array were down-regulated with changes in gene expression ranging from 2- to 3.5-fold. Thirty-two cDNA transcripts were up-regulated with changes in gene expression ranging from 2- to 16-fold. Characterisation by sequencing revealed that the most highly induced transcripts coded for ADP-ribosylation factors, an ABC cassette transporter and a glycosyl transferase. A transcript encoding a vitamin B6 biosynthesis protein was also identified as up-regulated by 2.9-fold. In contrast, the down-regulated transcripts coded for cellulose synthase I, annexin, glutamine synthetase, metallothionein and an alternative oxidase. The results are discussed in terms of possible roles and mechanism(s) of phosphite action within the mycelium of *P. cinnamomi*. 
This work is the first comprehensive screen for phosphite regulated-gene expression in *P. cinnamomi* and represents a significant step towards an understanding of the mode of action of phosphite on this organism. This thesis provides valuable information on the molecular interaction between phosphite and *P. cinnamomi*, which in future studies may stimulate the discovery of novel methods and cellular targets for the control of plant pathogenic Oomycetes.
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<td>amino acid</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>bp</td>
<td>base pair</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>ΔCT</td>
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<tr>
<td>ID</td>
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<tr>
<td>&quot;Hg</td>
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<td>MES</td>
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<td>pfu</td>
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<td>reverse transcriptase</td>
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<td>SSC</td>
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<td>SE</td>
<td>standard error</td>
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<td>transfer RNA</td>
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<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
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Chapter 1

Literature Review

1.1 General Introduction

The global destructiveness of the diseases caused by over 60 phytopathogenic species of *Phytophthora* (Erwin and Ribeiro 1996) has made it the most economically important group of plant pathogens in the world. It is thus not surprising that *Phytophthora* is the most extensively studied genus of Oomycetes. Devastating *Phytophthora* species include *P. infestans* - the potato and tomato late blight agent, *P. sojae* - the cause of soybean rot, *P. palmivora* and *P. megakarya* - the cause of black pod of cocoa and *P. ramorum* - the cause of sudden oak death in California (Fry and Goodwin 1997; Garbelotto 2001; Moy et al. 2004). Other *Phytophthora* species that affect horticultural and ornamental plants as well as natural ecosystems include *P. cambivora*, *P. cryptogea*, *P. fragaria*, *P. nicotianae* (syn. *P. parasitica*) and *P. vignae* (Irwin et al. 1995).

*Phytophthora cinnamomi* is one of the most pathogenic *Phytophthora* species with a host range exceeding 3000 plant species (Hardham 2005), causing significant losses in agriculture, horticulture and forestry. Shearer et al. (2004) reported that of the 5710 described plant species of south-western Australia, 2284 are susceptible and 800 are highly susceptible to *P. cinnamomi*. The pathogen is particularly known for the disease it causes in the *Eucalyptus marginata* forest and the biodiverse sandplain floras in Western Australia (Shearer and Tippett 1989). Infection of these areas results in
a decline in species richness and plant abundance resulting in degradation of faunal
habitat and a greater occurrence of soil erosion (Newhook and Podger 1972; Podger
1975; Shearer and Tippett 1989).

The best ways of containing and preventing the spread of _P. cinnamomi_ from infested
areas to healthy environments are quarantine, sanitary procedures, manipulation of
understorey composition and drainage (D'Souza _et al._ 2004; O'Gara _et al._ 2005).
Control methods also involve developing _Phytophthora_ resistant plants (Stukely _et al._
2001), biological control using arbuscular mycorrhizal fungi (Trotta _et al._ 1996;
Norman and Hooker 2000), and the discovery of chemical and biological compounds
that target specific sites both in plants and the pathogen (Alexander _et al._ 1978;
Downer _et al._ 2001; Kuhajek _et al._ 2003). The most effective way of controlling _P. cinnamomi_
in native communities of Western Australia is use of the fungicide phosphite
(phosphonate) due to its fungistatic properties, low toxicity and high mobility within
plants (Hardy _et al._ 2001).

Phosphite belongs to the group of phosphonate fungicides (aluminium ethyl phosphite –
Fosetyl-Al) introduced in the mid-1970’s, alongside the phenylamides (metalaxyl)
(Guest and Grant 1991), but it was not until 1989 that phosphite was discovered to be
effective in controlling _P. cinnamomi_ in _B. grandis_ (Shearer and Tippett 1989). Despite
its importance, the complex mode of action of phosphite remains unclear and has been
a subject of controversy for many years. This literature review will focus on identifying
the gaps in our knowledge of phosphite action with particular emphasis on recent
molecular developments and genomic resources available to identify the mode of
phosphite action in the pathogen, and on the _Phytophthora_-host interaction.
1.2 Molecular taxonomy of *Phytophthora*

Structural, molecular and phylogenetic studies have confirmed that Oomycetes are not true fungi but belong to the Stramenopiles lineage that also includes heterokont algae (Kamoun 2003; van West *et al.* 2003; Hardham 2005). *Phytophthora* species have been divided into 8 clades based on the sequences in internal transcribed spacer (ITS) regions of rDNA (Cooke *et al.* 2000) and in the mitochondrial and nuclear genes (Kroon *et al.* 2004). The analysis of molecular phylogenetic studies has ranked *P. cinnamomi* with *P. sojae*, *P. cambivora*, *P. fragariae*, *P. sinensis*, and *P. vignae* (Cooke *et al.* 2000; Kroon *et al.* 2004). *P. cinnamomi*, as well as other *Phytophthora* species such as *P. infestans*, exhibits a large phenotypic and pathogenic variation in the field which also contributes to the complexity of its management (Huberli 2001).

1.3 A brief introduction to the biology of *P. cinnamomi*

*Phytophthora cinnamomi* is a soil-borne plant pathogen that depends on moist conditions for survival, sporulation and dispersal (Shearer and Tippett 1989). Somatic hyphae that form the body of the pathogen produce asexual multinucleate sporangia, which differentiate to produce 20-30 uninucleate biflagellate zoospores. The generally short lived (hours) zoospores are the main infective agent of *P. cinnamomi*. They are attracted to the root tips of plants and quickly differentiate into adhesive cysts on the root surface (Figure 1.1).
The cysts produce germ tubes, which penetrate the root (Vidhyasekaran 1997), enabling the mycelium to grow through the root tissue, causing death of cells and rotting of tissues. Zoospores are capable of distinguishing living roots from dead ones using electrotaxis, which could be responsible for selection of infection sites on the root surface (Tyler 2002). The pathogen also recognises the host by chemical and physical properties of the host tissue such as amino acids (aspartate, glutamate, asparagine, glutamine, arginine, and methionine) diffusing from the plant tissue surface. Most *Phytophthora* species, however, show a relatively non-specific attraction to amino acids, which may account for the large host range of some *Phytophthora* species.
Root infection spreads rapidly through the plant and causes a sudden wilt and death. *P. cinnamomi* has the ability to kill tall trees by girdling the large sinker roots of the tree, preventing transport of water. *P. cinnamomi* also produces asexual spores called chlamydomospores in response to adverse environmental conditions. Chlamydomospores are long lived propagules [for a review, see McCarren *et al.* (2005)] that enable the pathogen to persist in soil or infected plant material until they encounter favourable conditions to cause new infection (Vidyasekaran 1997; Hardham 2005). The ability of *P. cinnamomi* to survive as dormant structures for many years (Erwin and Ribeiro 1996) makes its control in natural environments difficult to achieve. Sexual reproduction between the A1 and A2 mating types leads to the production of oospores. While oospores are part of the life cycle of *P. cinnamomi*, it is unlikely that sexual reproduction is happening in Australian environments as A1 isolates are rarely found in Australia (Dobrowolski, 2003).

1.4 A brief introduction to phosphite

Phosphite is the active constituent of Fosetyl-Al. Once inside the plant, Fosetyl-Al is hydrolysed to phosphorous acid (H$_3$PO$_3$), which is then ionised to phosphite (HPO$_3$$^{2-}$) (Figure 1.2).
In vitro, phosphite was found to be much more active than Fosetyl-Al against mycelial growth of several *Phytophthora* species (Fenn and Coffey 1984) leading to the use of phosphite rather than the proprietary fungicide Fosetyl-Al. Phosphite is a systemic fungicide that inhibits but does not eradicate the pathogen. The value of phosphite as a fungicide is that it can be taken up passively or actively through roots, stems, leaves, or flowers and is translocated in both the phloem and the xylem (Ouimette and Coffey 1990; Hardy *et al.* 2001). It has persistent protective and therapeutic functions in plants challenged with pathogens and is most effective as a preventative measure (Hardy *et al.* 2001). In Western Australia, phosphite is applied either as a foliar spray (5 g/L) every 6 - 24 months depending on the plant species, as an autumn aerial ultra-low volume mist (100 - 400 g/L) in plant communities of high conservation value, or as a trunk injection (50 - 200 g/L) in spring to individual trees in small areas (Fairbanks *et al.* 2000; Hardy *et al.* 2001). The trunk injections are generally longer lasting (at least 4 years) than foliar sprays (5-24 months) (Hardy *et al.* 2001).
1.5 Development of resistance to phosphite

Although phosphite remains effective against *Phytophthora*, there were reports of *P. cinnamomi* insensitivity to Fosetyl-Al in the ornamental *Chamaecyparis lawsoniana* in France (Vegh et al. 1985). *P. cinnamomi* isolates from avocado trees treated with phosphonates for 13 years also expressed lowered inhibition by Fosetyl-Al and phosphite *in vitro*, when compared to isolates from untreated trees (Duvenhage 1994).

In the laboratory, chemical mutagenesis of several *Phytophthora* species such as *P. capsici* (Bower and Coffey 1985; Fenn and Coffey 1989), *P. palmivora* (Dolan and Coffey 1988) and *P. parasitica var. nicotianae* (Fenn and Coffey 1989) produced isolates of reduced sensitivity to phosphite. The mutants of *P. palmivora* were tested in plants treated with 1.6 g/L Fosetyl-Al. Reduced phosphite sensitive mutants of *P. palmivora* caused 90% of infected plants to die (Dolan and Coffey 1988). However, the genes affected by mutation were not characterised.

Recent reports of the failure of Fosetyl–Al and foliar supplements containing phosphite to control the lettuce downy mildew pathogen, *Bremia lactucae*, have sparked fresh fears of the development of resistance to phosphonates (Brown 2004). Seedling tests demonstrated widespread occurrence of insensitivity in half of the 134 isolates screened, and unimpaired, or at least moderate sporulation at twice the normal field dose of Fosetyl-Al. Similar results were obtained in seedling assays with phosphite (Brown 2004), however it cannot be confidently extrapolated that this was the result of resistance. Nevertheless, the risk of development of insensitivity emphasises the importance of careful monitoring of phosphite application to control Oomycete pathogens, and the need for better understanding of its mode of action so that alternative methods of control may be developed.
The accumulation of phosphite in the mycelium of *Phytophthora* is only temporary (Grant *et al.* 1990). The anion was shown to reach maximum concentrations in *P. palmivora* 5 days after its application and completely disappeared from the mycelium by day 9. Metabolic studies have shown that phosphite altered the synthesis of lipids and their physical association within the mycelium of *P. palmivora*, such as a reduction in the amounts of C$_{20}$ and C$_{22}$ unsaturated fatty acids, triacylglycerol as well as an increase in the concentration of C$_{20}$ saturated fatty acids and a decrease in the total pool of adenylate (Grant *et al.* 1990; Griffith *et al.* 1990). To the naked eye, the most obvious effect phosphite has on *P. cinnamomi* in vitro is the significant reduction in growth of the mycelium. As little as 5 µg/ml of phosphite had a significant inhibitory effect on the growth of 12 isolates of *P. cinnamomi* from avocado (Coffey and Bower 1984).

Cytological studies of *P. palmivora* have shown hyphal distortions and reduction of sporangial production in phosphite-treated seedlings of *Arabidopsis thaliana* resulting in the restriction of mycelial growth to outer cortical cells (Daniel and Guest 2006). Guest (1986) reported similar findings of hyphal abnormalities and vacuolation in Fosetyl-Al treated *P. nicotianae*. Phosphite treatment of *E. marginata* and *B. grandis* seedlings challenged with *P. cinnamomi* resulted in a significant reduction of zoospore production (Wilkinson *et al.* 2001a), suggesting the efficacy of phosphite may lie in its ability to reduce the capacity of *Phytophthora* to infect or further colonise the host tissue. The study of molecular interaction between *Phytophthora* and phosphite would aid our understanding of how phosphite, for example, causes a reduction of viable infective propagules and the reduction of mycelial growth.
1.7 Phosphite-phosphate antagonism

The variability of efficiency of phosphite to control *Phytophthora* in plants is mainly attributed to its uptake mechanism, for which it competes with phosphate (Griffith *et al.* 1989a; Guest and Grant 1991). Oomycetes in common with many other microorganisms and plants, have at least two phosphate uptake systems. One is a low-affinity system (high $K_m$), active over a wide range of phosphate concentrations. The other is a high-affinity system (low $K_m$) induced only under phosphate limiting conditions (Barchietto *et al.* 1989; Griffith *et al.* 1989a). It was observed that phosphate starvation of mycelium growing on agar had a great impact on growth of the pathogen. A ten-fold decrease in phosphate concentration was more inhibitory on mycelium growth than the addition of an equimolar concentration of phosphite (Darakis *et al.* 1997). The presence of phosphite increased phosphate deficiency in the mycelium of *P. capsici*, therefore, it was suggested that phosphorus metabolism may be one of the sites of phosphite action (Darakis *et al.* 1997; Martin *et al.* 1998).

Phosphite also caused an increase in pyrophosphate levels and it was proposed that the site of phosphite action is the metabolism of pyrophosphate by inhibiting its breakdown (Niere *et al.* 1994). Pyrophosphate is maintained at low levels by inorganic pyrophosphatase. Martin *et al.* (1998) showed that phosphite inhibited pyrophosphatase activity in *P. palmivora* and *P. melonis in vitro* but concluded that the inhibition, when compared to other organisms such as *Saccharomyces cerevisae*, was not sufficient to account for the specificity of phosphite action in *Phytophthora* species.

Four genes encoding the pyrophosphate-utilizing glycolytic/gluconeogenic enzyme pyruvate phosphate dikinase (PPDK) have been isolated and characterised in
*P. cinnamomi* (Marshall *et al.* 2001). Rapid increase in the mRNA levels of *Pdk* was observed in vegetative mycelium when asexual sporulation was induced by transfer of the cultures to nutrient free media, however, the levels of PPDK protein remained constant under the same conditions (Marshall *et al.* 2001). Phosphite (0.4-200 mM) had no effect on PPDK activity in *P. cinnamomi* extracts, therefore it was suggested that phosphite may act on some other pyrophosphate utilising enzymes, such as the pyrophosphate dependent phosphofructokinase or H\(^+\) - translocating pyrophosphatase (Marshall *et al.* 2001).

### 1.8 The role of phosphite in plant-pathogen interactions

Research on the role of phosphite in plant-pathogen interactions has demonstrated that phosphite stimulates plant defence responses crucial for disease resistance (Smith *et al.* 1997; Jackson *et al.* 2000; Pilbeam 2003; Daniel *et al.* 2005; Daniel and Guest 2006), however, further investigation is required to form a complete picture of how phosphite actually achieves this favourable outcome. To build a comprehensive picture, it is necessary to integrate the effect of phosphite on both the pathogen and the host.

Phosphite persists in plants without being metabolised (Guest and Grant 1991) and provides protection from disease for at least 4 years when applied as a trunk injection (Guest and Grant 1991; Shearer 1994). The uptake of phosphite in a plant, which ultimately relates to its capacity to protect itself against pathogen invasion, is largely influenced by the surrounding environment and the life cycle stage of the plant. The intracellular content of phosphate varies in different plants, between 5-20 mM (Bieleski and Ferguson 1983). It was therefore suggested that the high levels of phosphate in plants will prevent sufficient uptake of phosphite to account for...
the mycelial growth arrest in planta (Barchietto et al. 1989; Darakis et al. 1997), which is consistent with the indirect mode of action of phosphite, which involves the stimulation of host defence mechanisms. It remains to be determined whether the stimulation of plant responses is a result of the direct effect of phosphite on the host plant or the indirect effect resulting from phosphite induced stress upon the pathogen.

However, it has been shown that phosphite inhibited the expression of several phosphate starvation induced genes including \textit{LePT1, LePT2, AtPT1}, and \textit{AtPT2} (high-affinity Pi transporters), \textit{LePS2, PAPI} (acid phosphatases) and \textit{LePS3 and TPSI1} (novel genes) in phosphate limited \textit{Arabidopsis} and tomato seedlings. This provided evidence that phosphite interferes with the signal transduction of the phosphate starvation responses in plants at the transcriptional level (Varadarajan et al. 2002).

Apart from inhibiting the mycelial growth, the mode of phosphite action also involves the production of phytoalexins in plants and stimulation of elicitor production in the cell walls of \textit{Phytophthora} (Fenn and Coffey 1984; Saindrenan et al. 1988; Perez et al. 1995). This may induce a hypersensitive response (HR) in the host plant cells. The defence response in \textit{Arabidopsis} plants infected with \textit{P. infestans} was characterised by penetration of epidermal cells, granulated cell cytoplasm, thickened cell walls and condensed nuclei of host cells near the penetration site (Huitema et al. 2003). Prior to the launch of the HR in phosphite-treated \textit{Arabidopsis} challenged with \textit{P. palmivora}, there is a release of reactive oxygen species (ROS) such as the superoxide radical (O$_2^-$), which is converted into the less toxic form (H$_2$O$_2$) through the action of superoxide dismutase (SOD) (Daniel and Guest 2006). This response was absent in untreated seedlings suggesting that the superoxide plays an important role in the induction of the
HR that leads to the death of the cells at the site of infection, restricting further spread of the pathogen.

The development of the HR in plants is followed by the induction of a systemic acquired resistance (SAR) response, where the whole plant becomes resistant to further pathogen infection and is mediated by hormones such as salicylic acid, ethylene, and jasmonic acid (Thatcher et al. 2005). There is evidence of extensive interaction between the signalling pathways and that the signal transduction cascade leads to the expression of pathogenesis related proteins (PR) that help to counter future attacks (Glazebrook 2005; Thatcher et al. 2005). The molecular processes that lead to the activation of plant defence responses are very intricate and are sometimes based on the production of pathogen virulence effectors recognised by corresponding plant resistance genes, which ascribes why certain plants may be resistant to specific pathogens while others are susceptible (Gachomo et al. 2003; Huijtema et al. 2003; Glazebrook 2005; Thatcher et al. 2005).

*Arabidopsis* has been used as a model plant to study plant-pathogen interactions as it has a small diploid genome, is relatively easy to transform and there are available a variety of mutants deficient in specific signalling pathways. These attributes have made it a powerful system for identifying genes involved in plant defence responses (Thatcher et al. 2005). Recently, Robinson and Cahill (2003) proposed a *P. cinnamomi - A. thaliana* pathosystem, but the studies in our laboratory that tested 20 ecotypes of *A. thaliana* with 30 *P. cinnamomi* isolates showed that *Arabidopsis* is not susceptible to *P. cinnamomi* (Jardine pers. comm.). Indeed, if such a pathosystem for *P. cinnamomi* was developed, it would be an invaluable tool to study the genes involved in phosphite-induced plant defence responses.
Understanding the mechanism of how phosphite induces resistance in host plants is crucial for developing effective management strategies such as selection of appropriate species for disease site revegetation, development of more effective phosphite application strategies, monitoring the spread of phosphite resistance genes within populations of *P. cinnamomi*, and developing alternatives to phosphite.

### 1.9 Molecular studies on the pathogenicity of *P. cinnamomi*

Investigations into the molecular aspects of *P. cinnamomi* pathogenicity have identified molecules involved in zoospore adhesion, enzymes responsible for degrading plant cell walls and energy storage resources.

Adhesion of zoospores to the root surface is a key aspect of disease establishment and is achieved through adhesive molecules such as the ventral surface vesicle protein (*PcVsv1*), which was proposed to be at least one of the components of the adhesive material in *P. cinnamomi*. Homologs of *PcVsv1* were found in *P. nicotianae*, *P. infestans*, *P. sojae* and *P. ramorum* (Robold and Hardham 2005). Additionally, the *PcVsv1* protein was found to contain repeats of a domain that is homologous to thrombospondin type 1 repeats (TSR1s), found in adhesive extracellular matrix proteins in animals (Adams and Tucker 2000). Recently, Wong (2006) reported that phosphite up-regulated the expression level of a gene required for the production of proteophosphoglycan in the mycelium of *P. cinnamomi*, and suggested that this compound may function as a hyphal adhesive.
The main pathogenicity factors of *Phytophthora* are considered to be the cell wall degrading enzymes. These include the pectin-degrading enzymes polygalacturonases (PGs). *P. cinnamomi* was found to contain at least 17 different PG genes, suggesting a specific role in host penetration (Gotesson *et al.* 2002), which may also account for the large host range of *P. cinnamomi*. These genes exhibit a higher similarity to fungal rather than plant polygalacturonases (Hardham 2005).

1.10 Generation of *Phytophthora* genomic resources

The generation of *Phytophthora* genomic resources (i.e. cDNA libraries) in recent years has produced thousands of Expressed Sequence Tags (ESTs) from various physiological stages of different *Phytophthora* species (Kamoun *et al.* 1999; Qutob *et al.* 2000; Kim and Judelson 2003; Panabieres *et al.* 2005; Randall *et al.* 2005). The size of the Oomycete genome is variable, ranging from 18-250 Mb, and is marked by an abundance of repetitive sequences grouped into small gene families that are randomly repeated through the genome, as demonstrated by studies in *P. sojae* (Mao and Tyler 1996), *P. infestans* (Judelson and Randall 1998), *P. parasitica* (syn. *P. nicotianae*) (Rump and Karlovsky 1991) and *P. cryptogea* (Panabieres and Berre 1999). Whole genome sequences of *P. sojae* and *P. ramorum* and the availability of large sets of ESTs of other *Phytophthora* species such as *P. infestans* have enabled functional genomic analyses (the study of gene functions) in *Phytophthora*. Resources have been specifically constructed for comparison of *Phytophthora* sequences and are available through the *Phytophthora* Functional Genomics Database (PFGD; [http://www.pfgd.org/pfgd](http://www.pfgd.org/pfgd)), *Phytophthora* Genome Consortium (PGC) ([http://www.xgi.negr.org/pgc](http://www.xgi.negr.org/pgc)) and Joint Genome Institute (JGI [http://www.jgi.doe.gov](http://www.jgi.doe.gov)).
The latter institute contains the whole genome sequences of *P. sojae* and *P. ramorum*. With the availability of these resources, it is possible to obtain meaningful data from gene expression studies focusing on identifying the underlying mechanisms of phosphite action at a molecular level. A fully sequenced genome of *P. cinnamomi* is highly desirable as it would greatly aid in this process, however, one is not available.

**1.10.1 Gene expression approach**

Skalamera *et al.* (2004) have identified several genes that were expressed in zoospores at higher levels than in the mycelium of *P. nicotianae*. A large proportion of these represented genes with core cellular roles as well as genes involved in zoospore development, plant colonisation and cyst germination. Shan *et al.* (2004) identified over 100 putative genes expressed in the germinating cysts of *P. nicotianae*, most of which were up-regulated. The genes encoded proteins involved in protein synthesis, metabolism, signalling, transcription regulation and cell wall biogenesis (Shan *et al.* 2004).

An investigation to compare the content of a library constructed from the mycelium of *P. parasitica* (syn. *P. nicotianae*) to the cDNAs expressed in zoospores (Skalamera *et al.* 2004) and germinating cysts (Shan *et al.* 2004) showed that the mycelium library contained 81% of ESTs characterised in the germinating cysts, but contained only 36% of ESTs classified in the zoospores (Panabieres *et al.* 2005). This provided evidence that a significant number of genes (64%) are expressed specifically in the zoospore stage in contrast to the mycelium.
The functional classification and analysis of ESTs [reflecting the expression of genes during the filamentous growth of *P. parasitica* (syn. *P. nicotianae*) mycelium] showed that 25 % of the genes in the library were required for energy and metabolism, 22 % for protein synthesis and fate, 3.84 % for fatty acid synthesis and 2.1 % for the ubiquitin/proteasome pathway (Panabieres *et al.* 2005). The most conspicuous sequences in the *P. parasitica* (syn. *P. nicotianae*) library were encoding the product actin, which was shown to be of critical importance for the initiation and maintenance of hyphal tip growth (Heath 2000) as well as being involved in the formation and expansion of cleavage membranes during zoosporogenesis of *P. cinnamomi* (Jackson and Hardham 1998). Elicitin-related sequences were overrepresented in the library. Other genes that were represented in the vegetative mycelium library of *P. parasitica* (syn. *P. nicotianae*) include genes that counteract plant defence responses, extracellular proteases and cell wall degrading enzymes, genes coding for surface binding molecules and genes involved in protection against active oxygen species (Panabieres *et al.* 2005). Many of the ESTs from large sequencing projects often have ‘no match’ or ‘unknown function category’ in the database when blasted against the ESTs of other *Phytophthora* species, which brings to light the importance of extending the database collection and developing appropriate genomic resources (Shan *et al.* 2004; Panabieres *et al.* 2005).

*Phytophthora* ESTs are characterised by a higher GC content (58 %) than plant ESTs (46 %), making it possible to distinguish between the two when sequences are generated from *Phytophthora* infected plant tissue (Birch and Kamoun 2000; Qutob *et al.* 2000; Moy *et al.* 2004). It remains to be determined whether this is applicable to other *Phytophthora* pathosystems.
1.10.2 Proteomic approach

The study of how the pathogen is recognised by the host plant has focused on the identification of genes of the pathogen responsible for the production of extracellular proteins (elicitors) that trigger defense response(s) in the host plant (Tyler 2002). The genomic approach in combination with proteomic studies greatly advanced the identification and diversity comparison of genes and proteins involved in the *Phytophthora* pathogen-plant interaction (van West *et al.* 2003). An algorithm called PexFinder V1.0 (*Phytophthora* extracellular protein) was developed (Torto *et al.* 2001) to search through the increasing EST data to identify putative genes coding for extracellular proteins.

1.10.3 Elicitins

The elicitors of fungal pathogens are usually found in the cell walls. Several *Phytophthora* species exhibit a novel family of protein elicitors called elicints (Ricci *et al.* 1992; Ponchet *et al.* 1999). They are holoproteins free of side-chain modification with molecular masses of 10 kDa. They are usually composed of 98 amino acids and have been classified as either acidic (α) elicints or basic (β) elicints (Pernollet *et al.* 1993; Ponchet *et al.* 1999). About 40 *Phytophthora* species were screened for elicitin production and all possessed genes encoding α-elicints, whereas β-elicints (triggering necrosis) encoding genes were restricted to specific species. *P. cinnamomi* produces several isoforms of both classes (α-cinnamomin, β-cinnamomin, cinnamomin-ha1, cinnamomin-ha2) (Nespoulous *et al.* 1992; Duclos *et al.* 1998; Ponchet *et al.* 1999; Rodrigues *et al.* 2002). All elicints are able to bind to the same sites, suggesting that elicints are recognised by the same type of receptor, which was proposed to be
a ligand-dependent calcium channel. The recent discovery of sterol-binding activity of elicitins suggests that elicitins share a common function and molecular characteristics [see Ponchet et al. (1999)].

Phosphite increased the yield of elicitins by a factor of 5.5 in *P. capsici* (capsicein) and a factor of 8 in *P. cryptogea* (cryptogein) *in vitro*, as shown by analytical reverse-phase high performance liquid chromatography. These experiments have also demonstrated that the increase in elicitins was proportional to the reduction in the growth of the mycelium (Perez et al. 1995). Application of cryptogein on tobacco leaves stimulated the production of ethylene and anti-microbial compounds such as phytoalexin (capsidiol) (Milat et al. 1991a; Milat et al. 1991b). Elicitins have also been shown to trigger transcription of genes in tobacco involved in SAR. These genes code for the components of the ubiquitin proteasome pathway such as proteasomes (Etienne et al. 2000; Dahan et al. 2001). The ubiquitin/proteasome pathway plays as much of a role in regulating the plant cell functions as transcription and protein phosphorylation and acts to remove abnormal and short lived-regulatory proteins and rate limiting enzymes (Smalle and Vierstra 2004). The ubiquitin proteasome pathway will be discussed in greater detail in Chapter 4.

### 1.11 Brief description of the molecular tools used to study gene expression in *P. cinnamomi* in the present study

In general, there is a quantitative correspondence between the amount of mRNA (messenger RNA) produced and the amount of protein produced. Thus, the amount of mRNA, which specifies the order of amino acids in a protein and therefore its structure, produced from various genes can be usually translated into gene expression levels
(Draghici 2003). The advantage of a cDNA library (complementary cDNA) is that it contains only the coding sequences of the genes present in a given tissue type under specific experimental conditions (Duggan et al. 1999). Because RNA molecules are exceptionally labile and difficult to amplify in their natural form, the information encoded in mRNA is converted into a stable DNA duplex, which is then ligated to a self-replicating vector and cloned in competent cells. A cDNA library is a population of recombinant molecules each containing a cDNA insert cloned into a vector backbone that may be used to cover the genome (Lodish et al. 2004; Watson et al. 2004; Wu et al. 2004). The construction of a quality cDNA library is critical for downstream gene manipulation. A whole-genome examination of gene expression would allow the formation of a complete picture of the molecular events that take place in *P. cinnamomi* when treated with phosphite.

The large scale examination of gene expression may be performed with a microarray, a powerful tool that enables the screening of thousands of genes at a time (Draghici 2003). The cDNAs (PCR products or oligonucleotides) are first printed on arraying slides and then bound to the slide by UV cross linking. Probes, prepared from RNA extracted from the reference and experimental samples, are labelled with fluorescent dyes (Cy3 and Cy5) and hybridised to the slide. The goal is to achieve high specificity with minimum background fluorescence. Differential gene expression is assessed by scanning the hybridised array using a laser scanner set at an appropriate wavelength. Measuring fluorescence intensities allows the determination of the relative expression levels of all the genes represented in the array (Draghici 2003; Amaratunga and Cabrera 2004). However, differences arising from the experimental variations rather than variations in transcriptional levels must be normalised prior to determination of gene expression levels (Hegde et al. 2000). The source of variability may be attributed to
differences in the amount of starting material (DNA spotted on arrays and RNA preparation), labelling efficiencies and hybridisation specificity, dye bias, saturation effects and background fluorescence (Amaratunga and Cabrera 2004). Data normalisation can be performed using an invariant gene set (e.g. constitutively expressed genes or all the genes on the array) (Amaratunga and Cabrera 2004) and fluorescence intensity-dependent software packages (Yang and Speed 2003).

A more accurate and faster method for determination of gene expression requiring minimal amount of RNA is quantitative real-time PCR (qRT-PCR), a fluorescence-based reverse transcription polymerase chain reaction (Bustin 2000). However, qRT-PCR is restricted to examining only a few genes (of known sequence) at a time. This method involves the reverse transcription of mRNA into first stranded cDNA, which is then amplified by PCR. The amplification of the product is monitored using either a fluorescent dye (SYBR Green I), which binds to double stranded DNA, or gene specific fluorescent probes (Taqman) (Pfaffl 2003). Two strategies can be performed in qRT-PCR to quantify gene expression: an absolute or relative quantification of gene expression. The easier and more common method of relative gene expression requires an internal reference standard whose transcription level should remain unaffected under the experimental conditions being studied (Pfaffl 2003; Radonic et al. 2004). The expression level of a gene of interest is adjusted according to the internal reference standard to account for differences in the amount and quality of starting material, and differences in RNA preparation and cDNA synthesis efficiency (Pfaffl 2003; Radonic et al. 2004).

Quantitative RT-PCR is becoming one of the most common methods to confirm the expression levels of genes detected in microarrays. The high throughput capacity of
microarrays and the specificity of qRT-PCR therefore represent a powerful combination in gene expression studies (Pfaffl 2003).

1.12 Thesis aims

Phosphite elicits plant defence responses to *P. cinnamomi* in many plant species that are otherwise susceptible to the pathogen. The mechanism of phosphite action, however, is not clear and it has been found that not all isolates of the pathogen are responsive to the treatment. This study contributes to an understanding of the mechanism(s) of phosphite action including the phosphite-pathogen interaction, phosphite-host interaction and the combined effect of all three.

The main objective of this project was to identify genes of *P. cinnamomi* that are up- or down-regulated when mycelium is treated with phosphite and elucidate the mode of action of phosphite on pathogenicity (ability to cause disease). This in turn may relate to how phosphite stimulates defence responses in host plants.

Specifically, the aims of this thesis were to:

1) Investigate the degree to which a range of phosphite concentrations inhibit the growth of the vegetative mycelium of several *P. cinnamomi* isolates *in vitro* (Chapter 2),

2) Construct a cDNA library from phosphite-treated mycelium of a phosphite-sensitive isolate, and prepare recombinant cDNA clones for microarray construction (Chapter 3),
3) Identify constitutively expressed genes in phosphite-treated mycelium to use as standard controls in microarray expression analysis and qRT-PCR (Chapter 4),

4) Construct custom made cDNA microarrays (Chapter 5),

5) Characterise the differentially expressed genes through sequence analysis and functional category enrichment (assign putative function), and confirm the differential gene expression by qRT-PCR (Chapter 6),

6) Discuss the relevance of differentially expressed genes in the mycelium of phosphite treated *P. cinnamomi* (Chapters 6 and 7).
Chapter 2

In vitro sensitivity of Phytophthora cinnamomi isolates to phosphite.

2.1 Introduction

Striking differences have been reported in phosphite sensitivity within and between Phytophthora species (Coffey and Bower 1984; Fenn and Coffey 1984; Wilkinson et al. 2001b). The variations in growth inhibition between the isolates of Phytophthora have been linked to the amount of phosphite within the mycelium (Griffith et al. 1993; Darakis et al. 1997). It is well established that the rate at which phosphite accumulates in the mycelium of Phytophthora can be affected by the level of phosphate in the growth medium (Bompeix and Saindrenan 1984; Griffith et al. 1989b). This is believed to be due to uptake competition between the two anions (Barchietto et al. 1989). Phytophthora possesses at least two phosphate uptake mechanisms; a high affinity system induced in phosphate limiting conditions and a low affinity system that functions when extracellular phosphate is sufficient (Barchietto et al. 1989; Griffith et al. 1989a). Griffith et al. (1993) reported that the growth of phosphite-sensitive isolates of P. palmivora was inhibited by phosphite at a range of phosphate concentrations, while tolerant isolates were inhibited only when phosphate was limiting. Increasing the phosphate concentration from 0.084 mM (‘low’) to 8.4 mM (‘high’) in a medium containing 0.57 mM (47 µg/ml) phosphite caused a small but significant decrease in the percentage growth inhibition of P. cinnamomi from 67 % to 52 % (Fenn and Coffey 1984).
The content of phosphate in plants can also affect the efficacy of phosphite in planta. It was observed that the effectiveness of phosphite is much higher and long lasting in native plant species (0.5-2 years) (Shearer and Fairman 2007) compared to that in agricultural plant species (0.06-0.9 years) (Lim et al. 1990; Wicks and Hall 1990). This was attributed to different levels of available phosphate in soils (Shearer and Fairman 2007). In contrast to the Australian native plants growing on low phosphate soils, agricultural plants are exposed to much higher levels of phosphate due to the use of fertilisers. The concentrations of phosphite necessary to reduce the mycelial growth of several Phytophthora species in vitro are generally higher than those required in vivo (Saindrenan et al. 1988). It is therefore thought that the hyphae in vivo absorb less phosphite (Barchietto et al. 1989). However, the phosphite content in plant tissue could account for direct reduction in mycelial growth in Phytophthora species that are more sensitive to phosphite in vitro (Bompeix and Saindrenan 1984; Guest and Grant 1991). Ouimette and Coffey (1989) reported 213 µg/g of phosphite content in the roots of avocado plants, indicating that phosphite could have a direct anti-fungal effect.

Therefore, experiments attempting to elucidate the mechanism(s) of phosphite action may benefit from using an isolate which is sensitive to phosphite in vitro, in the presence of phosphate levels similar to those in planta. The aim of this study was to identify an isolate of P. cinnamomi highly responsive to phosphite in vitro even when grown in the presence of a ‘high’ level of phosphate (10 mM) (Fenn and Coffey, 1984) and to determine the concentrations of phosphite sufficient to cause a significant reduction of hyphal growth, yet still providing sufficient mycelium for RNA extractions for later experiments.
2.2 Materials and methods

2.2.1 Source of phosphite

Phosphorous acid (H₃PO₃) crystals 99% (Aldrich, CAS 13598-36-2) were dissolved in deionised water and neutralised with KOH to make up a phosphite stock solution of 108 g/L. An aliquot of the solution analysed by Murdoch Environmental Science Analytical Laboratories confirmed the concentration of the stock solution. Stock solution was filtered through a sterile 0.22 μm filter (Millipore) and stored in the dark at 4 °C.

2.2.2 General growth conditions

*Phytophthora cinnamomi* isolates were obtained from the Centre for *Phytophthora* Science and Management (CPSM) Culture Collection and were maintained on Ribeiro’s agar at 25 °C (Table 2.1).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mating Type</th>
<th>Host</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP 125</td>
<td>A2</td>
<td><em>Eucalyptus marginata</em></td>
<td>Huntley, WA</td>
</tr>
<tr>
<td>MP 32</td>
<td>*</td>
<td><em>Banksia spp.</em></td>
<td>Jarrahdale, WA</td>
</tr>
<tr>
<td>MP 97</td>
<td>A2</td>
<td><em>E. marginata</em></td>
<td>Jarrahdale, WA</td>
</tr>
<tr>
<td>MP 94-03</td>
<td>A2</td>
<td><em>E. marginata</em></td>
<td>Willowdale, WA</td>
</tr>
<tr>
<td>MP 62</td>
<td>A2</td>
<td><em>E. marginata</em></td>
<td>Jarrahdale, WA</td>
</tr>
<tr>
<td>MP 80</td>
<td>A2</td>
<td><em>Corymbia calophylla</em></td>
<td>Jarrahdale, WA</td>
</tr>
</tbody>
</table>

*details not known

The isolates were passaged through Granny Smith apples to rejuvenate their pathogenicity prior to the experiments and to bring all isolates to a similar physiological status. A small inoculum plug was inserted into the apple, which was then sealed in
a plastic bag, and incubated at 20 °C for 9 d. The infected tissue was transferred to a *Phytophthora* selective agar NARPH (Appendix 1) and segments of the mycelium were subcultured on plates containing 15 ml of modified Ribeiro’s agar (Appendix 1). Plugs (8 mm) from actively growing colonies of 4-d old isolates were used to inoculate plates containing 20 ml of Ribeiro’s liquid medium (Appendix 1) with 10 mM KH₂PO₄ as a source of phosphate. All six isolates were tested at seven phosphite concentrations ranging from 0 - 160 µg/ml phosphite. The cultures were incubated at 25 °C for 7 d and the dry weight was measured on the final day. The mycelium was lifted out of the liquid, blotted dry with paper towels and dried in an oven at 40 °C overnight. There were four replicates per treatment. The experiment was repeated twice. No significant (P > 0.05) differences were observed between the two experiments for the isolate MP 80.

Based on this experiment, the most phosphite responsive and fast growing isolate, MP 80, was then used in a time series experiment to determine the most appropriate stage to harvest the mycelium treated with phosphite (40 µg/ml) for RNA extractions using the growth conditions described above. Dry weight was measured every day and there were three replicates per treatment.

Data analysis was performed using 2-way factorial ANOVA (Statistica 5). The data satisfied all the assumptions for Analysis of Variance and no transformation of the data was necessary. The data were standardised as a percentage of the growth of the control isolate to account for some isolates growing faster than others (dry weight phosphite treated mycelium/dry weight non-treated mycelium x 100). The EC₅₀ values were calculated from quadratic regression equations generated in Minitab 13.31 (Appendix 2). Data from the growth curve were heteroscedastic and this could not be
corrected with logarithmic transformation. Therefore, the untransformed data were analysed with a factorial design and P-values were set at 0.01.

2.3 Results

In vitro, the mycelial growth of all six *P. cinnamomi* isolates was significantly (P < 0.01) inhibited by phosphite (Figure 2.1). Although most of the isolates followed a similar trend of reduction in growth as phosphite concentrations increased, there were significant (P < 0.01) variations in the rate and the extent of growth reduction between the isolates.

![Figure 2.1: Growth responses of mycelium of six *Phytophthora cinnamomi* isolates, MP 94-03 (□), MP 62 (■), MP 32 (○), MP 97 (▲), MP 80 (◊), MP 125 (●), to different concentrations of phosphite in liquid Ribeiro’s medium amended with 0-160 µg/ml phosphite. Percentage growth was determined by dry weight standardized over the control (0 µg/ml phosphite). Each point represents a mean of four replicates (SE of means < 0.1; too small to be displayed).](image)

The isolate MP 94-03 was less responsive to phosphite at all concentrations compared to the other isolates with an EC\textsubscript{50} > 160 µg/ml phosphite (Table 2.2). At 5 µg/ml
the growth actually increased marginally for both MP 94-03 and MP 32 under the conditions of this study. Among the most sensitive isolates were MP 125 and MP 80 with EC$_{50}$ values of 37.7 and 39.5 µg/ml, respectively, followed by MP 97 and MP 32 with EC$_{50}$ values of 42.6 and 55.6 µg/ml, respectively. The isolate MP 62 was the second most tolerant isolate with an EC$_{50}$ value corresponding to 155.9 µg/ml phosphite.

Table 2.2: The EC$_{50}$ values for inhibition of mycelial growth (dry weight) by phosphite of six *P. cinnamomi* isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Inhibition of growth by phosphite EC$_{50}$(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP 125</td>
<td>37.7</td>
</tr>
<tr>
<td>MP 80</td>
<td>39.5</td>
</tr>
<tr>
<td>MP 97</td>
<td>42.6</td>
</tr>
<tr>
<td>MP 32</td>
<td>55.6</td>
</tr>
<tr>
<td>MP 62</td>
<td>155.9</td>
</tr>
<tr>
<td>MP 94-03</td>
<td>&gt;160</td>
</tr>
</tbody>
</table>

During the growth experiments, it was observed that some isolates grew more slowly (MP 125) than others (MP 80). The isolate MP 80 was selected for further study due to its fast growth and high susceptibility to phosphite. A phosphite concentration of 40 µg/ml resulted in 78 % growth inhibition by day 7 (Figure 2.2).
A growth response curve was generated for MP 80 in the presence (40 µg/ml) and absence of phosphite (Figure 2.3).

Figure 2.2: Mycelial growth of *Phytophthora cinnamomi* isolate MP 80 in aqueous Ribeiro’s medium containing 10 mM phosphate after 7 d in the absence (A) and presence (B) of 40 µg/ml phosphite. The plates were inoculated with a single inoculum plug. Budding of mycelium occurred in the liquid medium (A). Bar-scale = 1 cm.

Figure 2.3: Mycelial growth response curve of *Phytophthora cinnamomi* isolate MP 80 in Ribeiro’s medium in the absence (□) and presence (■) of phosphite (40 µg/ml). Dry weight was measured every day for 7 d in triplicate. Bars represent standard error (SE) of the means.
After the initial lag phase (first 48 h) the mycelium entered the exponential rate of growth. After day 6, no further increase in biomass occurred. In contrast, growth inhibition of phosphite-treated (40 µg/ml) mycelium was evident from day 2. The mycelium entered the exponential growth after 48 h and displayed a much slower rate. During this stage, phosphite had an adverse effect on the hyphal morphology (Figure 2.4) and inhibited the mycelial growth by 68.6 % on day 4 (P < 0.01). Subsequently, day 4 was chosen as the best time to harvest the mycelium for RNA extraction. The mycelium grown in the presence of 40 µg/ml phosphite exhibited distortions and swelling of hyphae and the production of short and stunted branches, in comparison to the mycelium grown without phosphite (Figure 2.4).
**Figure 2.4:** Light microscopy of the hyphal morphology of *Phytophthora cinnamomi* isolate MP 80 in liquid Ribeiro’s medium (day 4). Normally developed hyphae in medium with no phosphite (A, B), distorted hyphae with coralloid growth, abundant hyphal swellings and short, stunted branches after treatment with phosphite (40 µg/ml) (C, D).
2.4 Discussion

The extent of the growth inhibition response to phosphite application varied significantly (P < 0.01) among the six *P. cinnamomi* isolates tested, with EC$_{50}$ values ranging from 37.7 to > 160 µg/ml phosphite. The isolate MP 80 was chosen as the model isolate for the further studies in this project (Chapter 3-6). The optimum harvest time of the mycelium treated with 40 µg/ml phosphite, which caused a 68.6 % arrest of hyphal growth (P < 0.01) in the presence of 10 mM phosphate, was found to be 4 days. The results also indicated that at least *in vitro*, *Phytophthora* is capable of absorbing a sufficient amount of phosphite, which directly reduces its growth in the presence of 10 mM phosphate. Further work is required to measure the amount of phosphite and phosphate transported into the mycelium.

Although the mycelium of *P. cinnamomi* is typically coralloid with abundant hyphal swellings and swollen vesicles (Erwin and Ribeiro 1996), this pattern was less developed in the aqueous Ribeiro’s medium. In contrast, the hyphae grown in phosphite amended medium produced many short, distorted branches and swollen hyphae. Similar observations were made by Wong (2006) in *P. cinnamomi*, *P. nicotianae*, *P. palmivora* and *P. capsici*. She also noted lysis of spores and hyphal walls *in vitro* as a response to phosphite (5-100 µg/ml).

The higher phosphate levels used in the present study contributed to the differences in inhibition observed when compared to other *in vitro* experiments that used lower phosphate concentrations. Griffith *et al.* (1989b) demonstrated that the amount of phosphite accumulated by *P. palmivora* depends considerably on the external concentration of phosphate. In their study containing 10 mM phosphate in liquid
medium, no phosphite was detected in the mycelium grown in 0.01 mM phosphite, a low amount (1.8 nmol/mg dry weight) in the mycelium grown in 0.1 mM phosphite and a higher amount (12.1 nmol/mg dry weight) was detected in the mycelium grown in 10 mM phosphite. In contrast, in a low phosphate medium (0.1 mM), phosphite was detected at high levels (14.6 nmol/mg dry weight) at the lowest phosphite concentration tested (0.01 mM).

Fenn and Coffey (1984) reported a 52 % growth inhibition of *P. cinnamomi* isolates in liquid Ribeiro’s medium containing 47 µg/ml phosphite and 8.4 mM phosphate. Wong (2006) examined the effect of different phosphate concentrations (0-10 mM) on the *in vitro* growth of five species of *Phytophthora*, and found that an addition of 0.1-10 mM of phosphate had a positive effect on the growth of *P. cinnamomi*. Recently, Wilkinson *et al.* (2001b) examined seventy-one isolates of *P. cinnamomi*, including those tested in the present study, for phosphite sensitivity on a solid medium containing 7.35 mM phosphate and found a large variation within the isolates, with EC50 values ranging from 4-148 µg/ml phosphite. The response of isolates to 5 µg/ml phosphite in the medium ranged from a 4 % promotion of radial growth to 63 % inhibition. The concentrations required for 50 % inhibition were lower than those observed in the present study. For example, the EC50 value of MP 80 was 5 µg/ml compared to 39.5 µg/ml in the present study. However, it is difficult to compare the results of Wilkinson *et al.* (2001b) with those obtained in the present study due to differences in phosphate concentrations, the nature of the medium used (solid versus liquid) and the method of growth assessment. While Wilkinson *et al.* (2001b) measured the colony diameters to assess the growth on agar, and while this is relatively simple, it does not allow the determination of how much of the mycelium has grown above or into the agar, and does not account for some isolates having a more dense growth form than others. A more accurate
method is to measure the mycelial dry weight, when grown in liquid medium (Davison and Tay 1986; Guest and Grant 1991). Additionally, Grant et al. (1990) have found that isolates grown on solid medium were generally inhibited at lower concentrations of phosphite than isolates grown in liquid culture.

The current study clearly shows that phosphite has a direct inhibitory effect on the in vitro growth of *P. cinnamomi* and that this sensitivity to phosphite varies significantly between isolates. Differences to phosphite sensitivity between species and within isolates of *Phytophthora* have been suggested to be due to differences in the ability to exclude phosphite (Griffith et al. 1993) or to remove phosphite from their hypha (Dunstan et al. 1990) and a difference in the ability to activate a second transport system which has a higher affinity for phosphite when phosphate is limited (Griffith et al. 1989b).

In conclusion, a phosphite sensitive isolate (MP 80) was selected for experiments on the molecular effects of phosphite on *P. cinnamomi* mycelium described in Chapters 3 to 6. The culture protocol selected is growth for four days in Ribeiro’s medium containing 10 mM phosphate with or without 40 µg/ml phosphite.
Chapter 3

Construction and characterisation of a cDNA library from phosphite-treated mycelium of *Phytophthora cinnamomi* and preparation of cDNA inserts for microarray construction.

3.1 Introduction

It has been suggested that a combination of both a direct and indirect mode of phosphite action are involved to achieve effective control of the disease caused by *Phytophthora* (Smillie *et al.* 1989; Guest and Bompeix 1990). Several studies have shown that phosphite has a direct inhibitory effect on the growth of *Phytophthora* (Bompeix and Saindrenan 1984; Coffey and Bower 1984; Fenn and Coffey 1984; Fenn and Coffey 1985) and therefore the primary effect of phosphite is believed to be on the growth inhibition of the pathogen. Other studies have revealed that phosphite can elicit defence responses in many plant species by altering the metabolism of the pathogen (i.e. stimulating the release of elicitors or decreasing the amount of suppressors of host defence mechanisms) (Dunstan *et al.* 1990; Grant *et al.* 1990; Saindrenan *et al.* 1990; Ali *et al.* 1993; Perez *et al.* 1995). After years of research the precise details of the action of phosphite in the pathogen remain unknown.

One way to investigate the effect of phosphite on *Phytophthora cinnamomi* would be to examine the effect on the gene expression profile. The abundance of a particular mRNA transcript can be used to provide an indicative measure of the level of gene expression (Watson *et al.* 2004). By detecting differences in mRNA levels, genes whose transcriptional patterns are changed by phosphite can be identified.
One approach to examine the abundance of a particular mRNA is to probe an arrayed set of intragenic DNA fragments (microarray), representing the genome of the organism, with labelled cDNA synthesised from RNA extracted from cells grown in the conditions of interest. Differences in the level of a particular transcript can be detected by a direct comparison of the hybridisation signal intensity between conditions (Draghici 2003).

If the genome of the organism has been sequenced, DNA sequences representing putative genes can be synthesised and then spotted onto a slide to construct the array (Amaratunga and Cabrera 2004). However, if the genome sequence is unavailable (as is the case with *P. cinnamomi*) DNA fragments representing expressed genes can be obtained for the microarray from inserts in a cDNA library of the organism. This approach has previously been used to construct arrays to identify genes involved in sporangium development in *P. infestans* (Kim and Judelson 2003) and zoospore development in *P. nicotianae* (Skalamera *et al.* 2004).

This chapter reports on the construction of a cDNA library of *P. cinnamomi* to enable the subsequent PCR amplification of cDNA transcripts from thousands of clones in preparation for the construction of a microarray (Chapter 5).
3.2 Materials and methods

All plastic and glassware used in RNA and mRNA extractions were soaked overnight in 0.1 % diethylpyrocarbonate (DEPC) (Sigma) and autoclaved for 30 min at 121 °C to destroy RNAase activity.

3.2.1 Extraction of total RNA from \textit{P. cinnamomi}

Total RNA was extracted from \textit{P. cinnamomi} isolate MP 80 as described by Logemann \textit{et al.} (1987). Approximately, 0.8 g (dry weight) of mycelium treated with 40 µg/ml phosphite was harvested from liquid Ribeiro’s medium on day 4 as described in Chapter 2 (section 2.2.2). The mycelium was snap frozen with liquid nitrogen and ground to a fine powder using a mortar and pestle. The cell lysate was stabilised in 2 volumes of guanidine hydrochloride buffer (8 M guanidine hydrochloride, 20 mM MES, 20 mM EDTA, pH 7) mixed with 0.34 % (v/v) mercaptoethanol and then centrifuged at 23 708 g (Beckman Avanti J-25 I) at 4 °C for 20 min. The supernatant was filtered through autoclaved Miracloth (Calbiochem, Australia) and homogenised with 0.5 volume of phenol/chloroform/isoamyl alcohol (25:24:1) (v/v). The phases were separated by centrifugation at 23 708 g at room temperature for 45 min. The supernatant was transferred to a fresh tube with 0.2 volume of 1 M sodium acetate (pH 5.2) and 0.7 volume of cold -20 °C 100 % (v/v) ethanol and left overnight at -80 °C to precipitate the RNA. The precipitate was collected by centrifugation for 10 min at 23 708 g. The RNA pellet was washed twice with 3 M sodium acetate (pH 5.2), dissolved in 100 µl of sterile water and stored at -80 °C.
3.2.2 mRNA isolation

Isolation of mRNA was performed using a Qiagen Oligotex mRNA Mini Kit with minor changes. Briefly, 230 µg of total RNA was mixed with 250 µl of OBB buffer and 15 µl of Oligotex suspension. The secondary structure of RNA was disrupted by incubating at 72 °C for 6 min. The oligo dT$_{30}$ was hybridized to the poly-A tail of the mRNA at 37 °C for 3 min with temperature gradually decreasing to 25 °C over the next 3 min. The mRNA complex was left standing for 15 min at room temperature. The sample was then centrifuged at 18 000 g (Eppendorf Centrifuge 5417C) for 2 min, the supernatant was discarded and the pellet resuspended in 400 µl of OW2 buffer and transferred to a spin column. The spin column was centrifuged at 18 000 g for 1 min, transferred to a new collection tube and re-washed with 400 µl of OW2 buffer for 1 min. The flow-through was discarded. Using a new collection tube, the resin was resuspended in 50 µl of hot 70 °C OEB buffer and spun down for 1 min at 18 000 g and the elution step repeated with 20 µl of OEB buffer.

3.2.3 Quality assessment of RNA and mRNA preparations

The quality of RNA was determined by electrophoresing a sample on a 1 % agarose/formaldehyde gel run at 80 V for approximately 2 h. Each RNA sample (3.5 µl) was mixed with 3.5 µl of formaldehyde, 2 µl of 5x MOPS buffer (0.1 M 3-(N-morpholino)propanesulfonic acid, 40 mM sodium acetate, 5 mM EDTA, pH 7), 1 µl of 10 mg/ml ethidium bromide and 10 µl of formamide. The mixture was placed in a 65 °C water bath for 10 min and then cooled on ice. An aliquot of 2 µl of loading buffer (Fermentas) was then added to each sample.
The gel was photographed under UV light. Quantification of all RNA and mRNA samples was determined at 260 nm and 280 nm ensuring a ratio of 260/280 in the region of 1.8-2.3 on a QuantaGene II spectrophotometer (model 80-2105-98, Pharmacia Biotech).

### 3.2.4 Construction of a cDNA library

A cDNA library was created using Stratagene’s Zap-cDNA® Gigapack® III Gold Cloning Kit (Figure 3.1). The Uni Zap XR vector system consists of a Lambda Zap II vector and a pBluescript SK (-) phagemid contained within the vector (Appendix 3). The phagemid and DNA fragment cloned in Lambda Zap can be excised by f1 or M13 helper phage eliminating the need to subclone DNA inserts from the lambda phage into a plasmid by restriction digestion and ligation. The plasmid DNA is 3.0 kb long and displays ampicillin resistance, has a ColE1 origin of replication, a pBluescript SK polylinker, and the alpha portion of the lacZ gene and T7 and T3 RNA polymerase promoters (Short et al. 1988).
Chapter 3: Construction of a cDNA library of *P. cinnamomi*

**Figure 3.1**: cDNA library construction: 1) The poly (dT) region of the linker-primer binds to the poly (A) tail of the mRNA template. The 1st strand cDNA is reverse transcribed by Stratascript RT and hemimethylated by 5-methyl dCTP to protect it from being cleaved by *Xho*I in the subsequent cloning steps. 2) RNase H cuts the RNA bound to the 1st strand cDNA to produce a multitude of fragments, which serve as primers for the 2nd strand synthesis by DNA polymerase I. 3) The ends of the double stranded cDNA are filled in with cloned *Pfu* DNA polymerase and *Eco*RI adaptors are ligated to the blunt ends using DNA ligase. 4) The restriction enzyme *Xho*I cuts unmethylated restriction site within the linker primer to release the adaptor and residual linker primer - these are separated from the rest of the cDNA on a chromatography column. 5) Lambda Zap vector arms pre-digested with *Eco*RI and *Xho*I are ligated to the inserts, long concatamers form connected by COS sites. 6) DNA is packaged into lambda capsids in a packaging reaction containing all the protein components needed to create viable phage particles, each containing a unique cDNA inserted in its genome. These infect the host *Escherichia coli* (XL1-Blue MRF’), which undergo the lytic cycle resulting in multiplication of phage and lysis of the host cell (visible as plaques on agar plates) (Stratagene 2003).

3.2.4.1  cDNA synthesis

Several changes were made to the cDNA synthesis protocol (Stratagene 2003) and no radioactive isotope was used during the process. Approximately, 5.7 µg (37.5 µl) of mRNA from phosphite-treated mycelium was primed with the oligo(dT) linker-primer in a reaction containing 5 µl of 10x first-strand buffer, 3 µl of first-strand methyl nucleotide mixture, 2 µl of linker-primer (1.4 µg/µl) and 1 µl of RNase Block Ribonuclease Inhibitor (40 U/µl). The primer was allowed to anneal to the template for 15 min at room temperature (22 °C) and the first strand was synthesised using 1.5 µl of StrataScript Reverse Transcriptase (50 U/µl). The reaction was gently mixed and the contents spun down in a microcentrifuge and incubated at 42 °C for 1 h. Following the first strand cDNA synthesis, 2 µl of RNase H (1.5 U/µl) was used to nick the RNA into a multitude of fragments to serve as primers for DNA polymerase I (11 µl, 9.0 U/µl) during the second strand DNA synthesis in a reaction containing 20 µl of 10x second-strand buffer, 6 µl of second-strand dNTP mixture and 114 µl of sterile distilled water. The contents of the tube were gently mixed, centrifuged and incubated for 2.5 h at 16 °C.

3.2.4.2  Quantification of double stranded cDNA using an ethidium bromide assay

A 10 ml volume of 0.8 % (w/v) agarose in Tris-acetate (TAE) buffer and 0.01 % (v/v) ethidium bromide solution (10 mg/ml) was poured into 9 mm Petri plates and allowed to harden. Serial dilutions of calf thymus DNA were made in 100 mM EDTA (5 ng, 10 ng, 20 ng, 50 ng, 100 ng) as standards for the assay. An aliquot of 0.5 µl of cDNA and each standard was spotted on an ethidium bromide plate and allowed to absorb into
the agarose for 15 min. The plate was visualised under UV light and the concentration of synthesised cDNA (ng/µl) was estimated with reference to the DNA standards.

3.2.4.3 Ligation of EcoRI adapters to cDNA

Prior to EcoRI ligation, the ends of the cDNA were blunted by adding 2 µl of cloned Pfu DNA polymerase (2.5 U/µl) and 23 µl of blunting dNTP mix (Stratagene) and incubated at 72 °C for exactly 30 min. A volume of 200 µl of phenol–chloroform 1:1 (v/v), equilibrated to pH 8 was added to the mixture and vortexed. The reaction was centrifuged for 2 min at 20 800 g at room temperature and the upper aqueous layer containing the cDNA transferred to a new tube. An equal volume of chloroform was added and the phase separating step repeated. Then the cDNA was precipitated by adding 20 µl of 3 M sodium acetate (pH 5.2) and 400 µl of 100 % (v/v) ethanol in an overnight incubation at -20 °C. The precipitated DNA was collected by centrifugation at 20 800 g for 60 min at 4 °C. The supernatant was carefully removed, and the pellet gently washed with 500 µl of 70 % (v/v) ethanol and then lyophilised at room temperature. The cDNA was dissolved in 9 µl of EcoRI adapters (0.4 µg/µl) at 4 °C for 60 min. Then 1 µl of 10x ligase buffer, 1 µl of 10 mM rATP and 1 µl of T4 DNA ligase (4 U/µl) were added to the reaction, the mix briefly centrifuged and then incubated at 4 °C for 2 d. The ligase was heat-inactivated at 70 °C for 30 min and the 14-mer adapter oligonucleotide was phosphorylated in a reaction containing 1 µl of 10x ligase buffer, 2 µl of 10 mM rATP, 5 µl of water and 2 µl of T4 polynucleotide kinase (5 U/ul) at 37 °C for 30 min. The kinase was heat inactivated at 70 °C for 30 min.
3.2.4.4 Digestion of cDNA strands with XhoI

The cDNA was digested by adding 3 µl of XhoI (40 U/µl) restriction endonuclease and 28 µl of XhoI buffer supplement at 37 °C. After 1.5 h the enzyme was heat inactivated at 65 °C for 10 min.

3.2.4.5 Size-fractionation of the cDNA

The residual adapters, linker-primer and fragments smaller than 400 bp were separated from high molecular weight cDNA by chromatography on a sepharose (CL-4B) column (SizeSep 400 Spun Columns, Amersham Pharmacia Biotech) equilibrated to pH 7.5 in 1x STE buffer (0.1 M NaCl, 20 mM Tris-HCl pH 7.5, 10 mM EDTA). The column was placed in a 50 ml Falcon tube and the resin compacted by centrifugation for 2 min at 400 g. The cDNA was diluted to a final concentration of 1x STE buffer, and gently applied to the centre of the gel. The column was placed back in the 50 ml conical tube with the tip of the column inside a small 1.5 ml centrifuge tube to collect the eluent. The whole apparatus was centrifuged for 2 min at 400 g. DNA in the eluate was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold (-20 °C) 100 % (v/v) ethanol and incubating at -80 °C for 3 h. The precipitated cDNA was collected by centrifugation at 20 800 g for 60 min at 4 °C and the supernatant discarded. The pellet was carefully washed with 200 µl of 80 % (v/v) ethanol and dried in a vacuum. The cDNA was resuspended in 5 µl of sterile water.
3.2.4.6 Ligation of inserts to the Uni-Zap XR vector arms

The size-fractionated cDNA was quantified using the ethidium bromide plate assay. A total of 4 ligations containing insert and vector arms were performed. Three ligations contained 100 ng of cDNA and 1 ligation contained 150 ng of cDNA. In addition, each ligation contained 1.0 µl of the pre-digested Uni-Zap XR vector (1 µg) (Figure 3.2), 0.5 µl of 10x ligase buffer, 0.5 µl of 10 mM rATP (pH 7.5), 0.9 µl of water and 0.5 µl of T4 DNA ligase (4 U/µl)). A control ligation reaction was set up using the test insert supplied by Stratagene. The reactions were incubated for 2 d at 4 ºC.

**Figure 3.2:** The Uni-Zap XR insertion vector (40.8 kb long) is pre-digested with *Eco*RI and *Xho*I for directional cloning and can accommodate inserts ranging between 0-10 kb. An important property of the vector is the presence of part of the β-galactosidase gene (*lacZ* gene) (a total of 131 amino acid coding sequence interrupted by the large polylinker), which enables screening of recombinants with the blue/white colour selection. The vector is also capable of expression of fusion proteins under control of the *lacZ* promoter (Stratagene 2003).
3.2.4.7 Packaging reactions

A total of 8 packaging reactions were performed using 1 µl, 2 µl or 3 µl of ligation mixture containing vector and cDNA or a test insert. The packaging reactions were spun down for 5 s and incubated at room temperature (22 °C) for 2 h. SM buffer (500 µl) and chloroform (20 µl) were added to the contents of the tube, and then briefly spun to sediment the debris. The supernatant containing the phage was transferred to a fresh tube and stored at 4 °C.

3.2.4.8 Preparation of the host bacteria

XL1-Blue MRF’ cells were streaked on an LB agar (1 % (w/v) NaCl, 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 2 % (w/v) agar, pH 7) plate with tetracycline (15 µg/ml) and incubated overnight at 37 °C. A 50 ml culture of XL1-Blue MRF’ cells was prepared in LB broth with supplements (1 % (w/v) NaCl, 1 % (w/v) tryptone, 0.5 % (w/v) yeast, pH 7, filter sterilized 0.01 M MgSO₄, 6 mM maltose) using a single colony from the LB plate and incubated with shaking at 37 °C until the cells reached an OD₆₀₀ₙₘ of 0.8. The bacteria were pelleted at 1000 g for 10 min (Megafuge 1.0, Heraeus Sepatech), the supernatant was discarded and the cells gently resuspended in 25 ml of sterile 10 mM MgSO₄.

3.2.4.9 Titering protocol

The titre of each packaged reaction was determined prior to library amplification using two dilutions. An aliquot (1 µl) of the undiluted and a 1/10 dilution of each packaging reaction were combined with 200 µl of XL1-Blue MRF’ cells at an OD₆₀₀ₙₘ of 0.8.
The phage and the bacteria were incubated at 37 °C for 15 min to allow the phage to attach to the cells. Three ml of NZY top agar (0.5 % (w/v) NaCl, 0.2 % (w/v) MgSO₄·7H₂O, 0.5 % (w/v) yeast extract, 1 % (w/v) NZ amine – casein hydrolysate, 0.7 % (w/v) agarose) melted and cooled to 48 °C was added to the mixture, which was then poured evenly onto dry, pre-warmed NZY agar (0.5 % (w/v) NaCl, 0.2 % MgSO₄·7H₂O, 0.5 % (w/v) yeast extract, 1 % (w/v) NZ amine – casein hydrolysate, 1.5 % (w/v) agar, pH 7.5) plates. The plates were allowed to set for 10 min and then inverted and incubated at 37 °C. The plaques were visible 12 h after incubation and the titre determined in plaque-forming units per microliter (pfu/µl) using the following formula:

\[
\text{Phage Titre} = \frac{\text{No. of plaques (pfu) } \times \text{ dilution factor}}{\text{Volume plated (µl)}}
\]

In a separate experiment, the percentage of recombinant plaques versus non recombinant plaques was determined by the colour selection (non coloured versus blue) by adding 15 µl of 0.5 M IPTG (isopropyl-β-D-thiogalactopyranoside) and 50 µl of X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) (250 mg/ml in DMF) to the 3 ml of NZY top agar.

### 3.2.5 Amplifying the library

Fresh host bacteria were prepared for the amplification of the primary library. Aliquots of the library suspension containing 5 × 10⁴ pfu of bacteriophage were combined with 600 µl of XL1-Blue MRF’ cells grown to an OD₆₀₀nm of 0.8 and incubated for 15 min at 37 °C. Each aliquot of phage infected *E. coli* was mixed in 6.5 ml of NZY top agar and spread evenly onto freshly poured 150 mm NZY agar plates. The plates were incubated
at 37 °C for 8-12 h until the plaques were between 1-2 mm in size. Ten ml of SM buffer (100 mM NaCl, 8 mM MgSO₄.7H₂O, 50 mM Tris-HCl pH 7.5, 0.01 % (w/v) gelatin) was poured over each plate and stored at 4 °C overnight with gentle rocking to diffuse the phage into the SM buffer. The bacteriophage suspension was recovered from each plate and pooled into 50 ml Falcon tubes. The plates were rinsed with an additional 2 ml of SM buffer and then pooled together. Chloroform was added to a 5 % (v/v) final concentration. The suspension was well mixed and incubated for 15 min at room temperature. The cell debris was removed by centrifugation for 10 min at 500 g. The supernatant was recovered and chloroform was added to a 0.3 % (v/v) final concentration. Aliquots of the amplified library were stored in sterile cryogenic tubes in 7 % (v/v) DMSO at -80 °C. For identification of recombinant plaques 30 µl of 0.5 M IPTG and 100 µl of X-gal (250 mg/ml in DMF) were added to the molten top agar.

3.2.6 Selection of recombinant plaques

Twenty thousand plaques were manually picked from 90 NZY agar plates (150 mm) with approximately 200-250 plaques selected from each plate. Using sterile toothpicks, and working in a laminar flow to minimise contamination, the plaques were transferred to 384-well microtitre plates (Packard BioSciences), containing 50 µl of SM buffer per well, so that each plaque was in a separate well. A final concentration of 7 % (v/v) DMSO and 3 % (v/v) chloroform was added to a plate for long term storage at -80 °C. Only non-coloured plaques were picked from 1-2 d old plates. To enhance discrimination between recombinant (non coloured) and non recombinant (blue) plaques, several changes to the medium and picking techniques were tested. S-gal (300 mg/L), an alternative of X-gal, was tested with ferric ammonium citrate (500 mg/L) (Sigma) to make the non-recombinant plaques appear black instead of blue. Attempts to
pick the plaques with a GeneTAC™ G³ workstation for picking colonies and plaques (Genomic Solution, Perkin-Elmer) were unsuccessful due to the limitations of the picking application software. The best discrimination was obtained by adding a green food dye (Icing Colours-Juniper green, Wilton Industries) to the top agar prior to autoclaving for sharper contrast of plaques against the background of the agar.

3.2.7 PCR amplification of DNA inserts

Fifteen thousand PCR reactions were manually set up with a multichannel pipette in 96- and 384-well plates. The inserts were amplified directly from phage in SM buffer in 27 µl reactions containing 1x PCR reaction buffer (Invitrogen), 0.25 mM dNTP’s, 2 mM MgCl₂, 0.62 µM of each primer and 0.1 unit of Taq DNA polymerase (Invitrogen). The optimized cycling conditions for all reactions consisted of 94 °C for 1 min, 59 °C for 1 min, 72 °C for 4 min and 15 °C hold. Two universal primers (Geneworks) were used in the PCR reactions: M13-20 5’-TTgTAAaACgACggCCAgTg-3’ and M13 Reverse 5’-ggAAACAgCTATgACCATg-3’. The priming sites are illustrated in Figure 3.3 on the polylinker sequence of the vector.

Figure 3.3: Multiple cloning site region of the pBluescript SK (-) phagemid with 6 different priming sites spanning 225 bp in the sequence of the vector (acc. no. X52324) (Stratagene 2003). M13-20 and M13 Reverse primer binding sites were used for PCR amplification and DNA sequencing.
A volume of 3 µl of each PCR reaction was diluted with 20 µl of water and loaded on 96-well E-gels (Invitrogen) with a low range quantitative DNA ladder (Invitrogen) to check for the presence and the size range of the cDNA inserts. Each gel was allowed to run for 12 min and then visualized under UV light.

3.2.8 Purification of PCR products using 96-well and 384-well PCR clean up plates

The purification of 9216 PCR products was manually performed in 96- and 384-well MultiScreen PCR clean up plates (Millipore). The volume of each PCR reaction was adjusted to 100 µl with sterile water and then loaded in the purification plate placed on a vacuum manifold. A vacuum pressure at 18” Hg was applied to the 96-well plates for 4.5-7 min until all wells were dry and the droplets at the bottom of the plates on the outside were removed with absorbent paper towels. The samples in 96-well plates were resuspended in 25 µl of sterile water on a microtitre plate shaker (Eppendorf Thermomixer Comfort) at 1200 rpm for 10 min. A pressure at 10” Hg for 13 min was applied to the 384-well plates. An aliquot of 30 µl of water was added to each well in 384-well plates which were then shaken at 1200 rpm for 20 min. The purified PCR products were finally transferred to 384-well arraying microtitre plates (Genetix). A set of 20 randomly chosen samples from each 96- and 384-well plate were analyzed on Nanodrop (ND-1000 Spectrophotometer) at 260 and 280 nm to determine an estimate of the quantity of the purified product. All purified PCR products were air-dried by placing the plates, covered with a Kimwipe (Kimberly-Clark), in a laminar flow for 24-48 h.
3.2.9 Sequencing of cDNA inserts from the library

In preparation for DNA sequencing of the amplified inserts, MinElute PCR clean up columns (Qiagen) were used to clean up 9 PCR reactions. The concentrations of the PCR products were then measured on the Nanodrop Spectrophotometer at 260/280 nm. Half sequencing reactions were prepared in 0.2 ml tubes (4 µl Dye terminator, 3.2 pmoles primers, 10 ng DNA for PCR products between 200-500 bp, and 20 ng DNA for PCR products between 500-2000 bp). Sequencing of the DNA inserts was performed using both the M13-20 Forward primer and M13 Reverse primer. After thermal cycling (96 °C for 2 min, 96 °C for 10 s, 59 °C for 5 s, 60 °C for 4 min at 25 cycles) the reactions were purified using ethanol precipitation in 0.5 ml tubes (25 µl of 100 % (v/v) ethanol, 1 µl of 3 M sodium acetate (pH 5.2), 1 µl 125 mM EDTA, 10 µl PCR product). They were then left to precipitate at room temperature for 20 min and then centrifuged (20 800 g) for 30 min. The supernatant was discarded, the DNA pellet rinsed with 80 % (v/v) alcohol and dried in a desiccator for 15 min. The samples were sequenced in the State Agricultural Biotechnology Centre (Western Australia) and Royal Perth Hospital (Western Australia) depending on availability. Alignment and comparison of the cDNA sequences was performed through the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) with the basic local alignment search tool (BLAST) algorithm (Altschul et al. 1997) using BLASTN and BLASTX (Table 3.1). The sequences were also compared to P. sojae and P. ramorum sequences in the Joint Genome Institute database (JGI, http://www.jgi.doe.gov/).
3.3 Results

3.3.1 Analysis of RNA

The quality assessment of RNA preparations extracted from *P. cinnamomi* was performed by gel electrophoresis (Figure 3.4). The gel images revealed the presence of the expected two ribosomal bands (28S and 18S) with the intensity of the 28S ribosomal band approximately twice that of the 18S band.

![Figure 3.4](image)

**Figure 3.4:** RNA from phosphite-treated mycelium of *Phytophthora cinnamomi* isolate MP 80 (Lane 1, 2) electrophoresed on a denaturing agarose/formaldehyde gel (1.5 %).

The RNA preparations were also assessed by spectrophotometric analysis prior to and after enrichment for mRNA (Table 3.1). The results showed that the extracted RNA and mRNA were intact and of high purity.
Table 3.1: Spectrophotometric analysis of RNA and mRNA sample from phosphite-treated mycelium of *Phytophthora cinnamomi*.

<table>
<thead>
<tr>
<th></th>
<th>Total RNA</th>
<th>mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD 260</td>
<td>2.167</td>
<td>0.382</td>
</tr>
<tr>
<td>OD 280</td>
<td>1.039</td>
<td>0.188</td>
</tr>
<tr>
<td>Purity (260/280 nm)</td>
<td>2.086</td>
<td>2.03</td>
</tr>
<tr>
<td>Concentration</td>
<td>1386.88 µg/100 µl</td>
<td>10.7 µg/70 µl</td>
</tr>
</tbody>
</table>

* Pure RNA has a ratio of 260/280 nm of 1.8-2.3

### 3.3.2 cDNA synthesis for library construction

Approximately, 20.3 µg of double stranded cDNA was synthesised (100 ng/µl) from the mRNA isolated from phosphite-treated mycelium of *P. cinnamomi*. After purification by size fractionation, approximately 500 ng (100 ng/µl) of cDNA was recovered (Figure 3.5).

**Figure 3.5:** Progressive quantification of cDNA from *Phytophthora cinnamomi* phosphite-treated mycelium during the construction of the library on an ethidium bromide plate assay using DNA samples of known concentration as comparative standards ranging from 5-200 ng/0.5 µl: cDNA after the second strand synthesis – sample 1 (50 ng/0.5 µl) was used for library construction (A); cDNA with EcoRI adaptors > 100 ng/0.5 µl (the presence of dNTPs exaggerates the amount of cDNA in the reaction) (B); size-fractionated cDNA used for ligation to the vector (50 ng/0.5 µl) (C).
The large loss of cDNA that was encountered after size fractionation (up to 45% of cDNA) was overcome by omitting an ethanol precipitation and phenol-chloroform extraction. An experiment testing the efficiency of the size-fractionation column with a 100 bp marker revealed that cDNA of the desired size (bigger than 400 bp) was retained in the column after elution contributing to the observed loss of cDNA (Figure 3.6).

![Figure 3.6: A 100 bp marker (Promega) (Lane 1) was used to test the efficiency of the size-exclusion column from Amersham. The first elution from the column contained cDNA of 400 bp and above (Lane 2). Following a 2nd elution from the column, cDNA fragments smaller than 400 bp passed through the column (Lane 3). This revealed that cDNA of the desired size (> 400 bp) was retained in the column contributing to the observed decrease in yield.](image)

3.3.3 Ligation and packaging

The final yield was sufficient to ligate size fractionated cDNA to vector arms. Following ligations, the cDNA was packaged using 8 reactions. The titre of the 8 packaging reactions varied between 330 ± 125 and 1000 ± 150 pfu/µl and enabled a primary cDNA library to be constructed consisting of 2.15 x 10^6 pfu. The percentage of non recombinant background plaques was determined on NZY agar plates containing
IPTG and X-gal and determining the ratio of blue (non-recombinant) to non-coloured (recombinant) plaques. Under these conditions the cDNA library contained ~ 80% recombinant phage.

### 3.3.4 Characterisation of the cDNA library

Several cDNA inserts were analysed on agarose gels and by sequencing, before thousands of cDNA inserts were prepared for the construction of a microarray. Random non-coloured and blue plaques were picked with sterile toothpicks into SM buffer, and 2 µl used as a template in PCR reactions containing M13-20 Forward and M13 Reverse primer. While characterizing the amplified inserts on gels it was observed that some inserts from non-coloured plaques were almost the same size as inserts from blue plaques, 220 bp in length, yet they had appeared non-coloured on the plates (Figure 3.7 A lane 9; B lane 7; C lane 13).

![Figure 3.7: Agarose gel (2 %) analysis of cDNA clones amplified by PCR: Lane 1: 100 bp marker, Lane 3: control (no cDNA), Lane 4: insert from blue plaque, Lanes 5-9: inserts from non-coloured plaques (A); Lane 1: 100 bp marker, Lane 2: control (no cDNA), Lanes 3-7: inserts from non-coloured plaques, Lane 8: control (E. coli cells) (B); Lane 1: control (no cDNA), Lane 2: control (SM buffer), Lane 3: control (E. coli cells), Lane 5: insert from blue plaque, Lanes 6-7: inserts from non-coloured plaques, Lanes 9-10: inserts from non-coloured plaques, Lanes 12-13: inserts from non-coloured plaques, Lane 14: 100 bp marker (C).](image-url)
Substrates other than X-gal were used to potentially improve the colour based discrimination between blue and non-coloured plaques, including the use of S-gal. The latter substrate, however, was abandoned since no improvement in distinguishing the recombinant plaques (non-coloured) from the non-recombinant ones (blue) was achieved.

3.3.4.1 Analysis of recombinant phage cDNA inserts by sequencing

A set of 9 randomly chosen phage inserts from non-coloured plaques were sequenced after amplification with the two primers. Each insert was amplified directly from a plaque picked by sterile toothpick from a NZY agar plate and resuspended in PCR reaction cocktail. The DNA sequence was established using M13 Reverse primer. Similarities were searched against the NCBI and JGI database using BLASTN and BLASTX (Table 3.2). The results confirmed that the cDNA library was from *P. cinnamomi*, and the nature of some of the clones (PC01 – PC08) represented within the library was revealed. For one clone (PC09) out of the 9, small DNA fragments had been cloned into the Multiple Cloning Site (MCS) of the vector which were sufficient to disrupt the expression of functional β-galactosidase.
Table 3.2: List of homologous sequences of nine sequenced clones from the cDNA library.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Closest putative homologue/function</th>
<th>Accession No. of homologue</th>
<th>Extent of homology</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC01</td>
<td><em>P. infestans</em> ras related protein piYpt 1 gene (gene involved in vesicle transport)</td>
<td>U30474.1</td>
<td>306/332</td>
<td>8e-124</td>
</tr>
<tr>
<td>PC02</td>
<td><em>P. infestans</em> mRNA for RIC1 protein (membrane spanning protein, <em>ric 1</em> gene, stress induced gene)</td>
<td>AJ133023.1</td>
<td>189/210</td>
<td>2e-60</td>
</tr>
<tr>
<td>PC03</td>
<td><em>P. infestans</em> clone MY-10-H-01 thioredoxin peroxidase mRNA (extracellular elicitor protein)</td>
<td>AF424662.1</td>
<td>144/166</td>
<td>3e-36</td>
</tr>
<tr>
<td>PC04</td>
<td><em>P. polymorphica</em> 5.8S ribosomal RNA gene</td>
<td>AY598669.1</td>
<td>107/107</td>
<td>5e-52</td>
</tr>
<tr>
<td>PC05</td>
<td><em>P. megasperma</em> gene for 28S ribosomal RNA</td>
<td>X75631.1</td>
<td>801/814</td>
<td>0.0</td>
</tr>
<tr>
<td>PC06</td>
<td><em>P. sojae</em> estExt_fgenesh_pg.C_90206</td>
<td>129929</td>
<td>27/28</td>
<td>6e-12</td>
</tr>
<tr>
<td>PC07</td>
<td><em>P. sojae</em> estExt_fgenesh_pg.C_970031 (protein phosphatase)</td>
<td>141343</td>
<td>104/113</td>
<td>8e-69</td>
</tr>
<tr>
<td>PC08</td>
<td><em>P. sojae</em> estExt_fgenesh1_pg.C_840054</td>
<td>140327</td>
<td>85/89</td>
<td>4e-53</td>
</tr>
<tr>
<td>PC09</td>
<td>pBluescript SK(-) vector DNA (nucleotides preset)</td>
<td>X52324.1</td>
<td>125/128</td>
<td>3e-53</td>
</tr>
</tbody>
</table>

3.3.5 Plating and picking of recombinant plaques

To circumvent the tedious nature of manually picking the plaques, an automated plaque picker (GeneTac™ G3 robotic work station) was trialled. However, the sensitivity limit of the robot for plaque visualisation did not offer sufficient distinction of potential recombinants from undesired non-recombinants. Therefore, twenty thousand non-coloured plaques were manually picked into 384-well plates. The picking of plaques was greatly improved by enhancing the visibility of non-coloured plaques by adding a green food dye to the top agar (Figure 3.8).
Figure 3.8: Comparison of the visibility of plaques in the lawn of *E. coli* cells on NZY agar plates. Green food dye was added to the top agar to improve the visibility of plaques (A). No X-gal and IPTG were added to the medium, therefore only non-coloured plaques can be seen. In the second NZY agar plate, X-gal and IPTG were added for selection of recombinant (non-coloured) and non recombinant (blue) plaques (B). The blue plaques stand out against the background of the agar, but the non-coloured plaques targeted for picking are barely visible.

3.3.6 **PCR amplification of DNA inserts**

The vector multiple cloning site region was amplified from DNA extracted from 15 000 recombinant phage particles using the M13-20 and M13 Reverse primer. Single band amplification success rate was approximately 95 % in 96-well plates. Fewer than 5 % of reactions failed to amplify and about 0.5 % contained multiple bands as a result of cross contamination or merged plaques being picked. The reactions performed in 96-well plates were consistently better than the ones in 384-well plates. For the latter plates, evaporation from insufficient sealing of the PCR film during the PCR run was evident.
The size of the average insert ranged between 400-800 bp, the largest inserts were 2000 bp in size. It was found that 20-25 % of amplified cDNA containing clones had small inserts or nucleotides that had ligated to the vector.

After the removal of contaminating dNTP’s and primers with a spin column (Qiagen) of some of the PCR products, it was estimated that the total yield of DNA was 1 µg per reaction. The average concentration of PCR products was also quantified visually from the E-gels with respect to the quantitative DNA ladder used (Figure 3.9).

**Figure 3.9:** Electrophoresis of the amplified MCS region of phage clones on a 1 % E-gel (Invitrogen) with a low range quantitative ladder (Invitrogen). The concentrations of the bands in the ladder (10 µl) are as follows: 100 ng (2000 bp), 40 ng (800 bp), 20 ng (400 bp), 10 ng (200 bp) and 5 ng (100 bp) of DNA, respectively.
3.3.7 Purification of PCR products

The major factor contributing to the amount of recovered DNA from the PCR clean up plates seemed to be the filtration time. Over drying of DNA on the filtration membrane by as little as 1 min was detrimental to the recovery of the final concentration of the purified product. Good agitation of samples during resuspension was a critical component aiding the PCR product recovery. The vacuuming times varied from plate to plate and had to be carefully monitored. For 96-well plates, this ranged from 5-7 min, while for the 384-well plates it varied from 13-16 min. It was also found that the recovery from the 96-well plates was higher and more consistent across the plate, as most of the wells filtered at approximately the same time. The filtration of products in the 384-well plates varied greatly and occasionally the outer wells did not filter at all. The average DNA concentrations of inserts recovered from the plates varied between 350 and 650 ng (Table 3.3). All 9216 purified PCR products were transferred to 24 x 384-well plates (Genetix) compatible with a microarray spotter. Each plate was labelled and a corresponding database of all the clones was constructed (Appendix 4).
**Table 3.3:** A summary of DNA concentrations recovered after PCR purification as measured on a Nanodrop Spectrophotometer at 260/280 nm.

<table>
<thead>
<tr>
<th>Plate ID</th>
<th>Highest concentration (ng)</th>
<th>Lowest concentration (ng)</th>
<th>Average (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1/2/6-2)</td>
<td>851</td>
<td>264</td>
<td>462</td>
</tr>
<tr>
<td>2 (1/2/6-1)</td>
<td>832</td>
<td>231</td>
<td>408</td>
</tr>
<tr>
<td>3 (3/2/6-0)</td>
<td>1016</td>
<td>202</td>
<td>517</td>
</tr>
<tr>
<td>4 (3/2/6-1)</td>
<td>1069</td>
<td>248.6</td>
<td>572</td>
</tr>
<tr>
<td>5 (3/2/6-3)</td>
<td>906</td>
<td>216</td>
<td>400</td>
</tr>
<tr>
<td>6 (7/2/6-1)</td>
<td>645</td>
<td>303</td>
<td>445</td>
</tr>
<tr>
<td>7 (7/2/6-2)</td>
<td>895</td>
<td>222</td>
<td>400</td>
</tr>
<tr>
<td>8 (9/2/6-1)</td>
<td>702</td>
<td>178</td>
<td>350</td>
</tr>
<tr>
<td>9 (9/2/6-2)</td>
<td>558</td>
<td>282</td>
<td>400</td>
</tr>
<tr>
<td>10 (9/2/6-3)</td>
<td>1032</td>
<td>326</td>
<td>506</td>
</tr>
<tr>
<td>11 (28/2/6-2)</td>
<td>670</td>
<td>205</td>
<td>400</td>
</tr>
<tr>
<td>12 (15/2/6-*)</td>
<td>1230</td>
<td>260</td>
<td>450</td>
</tr>
<tr>
<td>13 (15/2/6-8)</td>
<td>683</td>
<td>205</td>
<td>405</td>
</tr>
<tr>
<td>14 (21/2/6-1)</td>
<td>1316</td>
<td>360</td>
<td>650</td>
</tr>
<tr>
<td>15 (21/2/6-2)</td>
<td>665</td>
<td>174</td>
<td>418</td>
</tr>
<tr>
<td>16 (21/2/6-3)</td>
<td>1012</td>
<td>170</td>
<td>468</td>
</tr>
<tr>
<td>17 (21/2/6-4)</td>
<td>808</td>
<td>178</td>
<td>405</td>
</tr>
<tr>
<td>18 (22/2/6-1)</td>
<td>476</td>
<td>174</td>
<td>350</td>
</tr>
<tr>
<td>19 (22/2/6-2)</td>
<td>388</td>
<td>228</td>
<td>350</td>
</tr>
<tr>
<td>20 (23/2/6-1)</td>
<td>814</td>
<td>264</td>
<td>449</td>
</tr>
<tr>
<td>21 (24/2/6-1)</td>
<td>873</td>
<td>264</td>
<td>425</td>
</tr>
<tr>
<td>22 (24/2/6-2)</td>
<td>823</td>
<td>260</td>
<td>528</td>
</tr>
<tr>
<td>23 (28/2/6-1)</td>
<td>1030</td>
<td>222</td>
<td>506</td>
</tr>
<tr>
<td>24 (2/3/6-1)</td>
<td>702</td>
<td>195</td>
<td>425</td>
</tr>
</tbody>
</table>

*Highest concentration (ng)/Lowest concentration (ng) – represents the concentration of 1 purified insert DNA with the highest/lowest recovery (ng) out of 20 randomly selected DNA inserts
Average (ng) – represents the mean of DNA concentrations (ng) of 20 inserts measured in a plate
3.4 Discussion

A representative cDNA library was successfully constructed from the mycelium of four day-old *P. cinnamomi* treated with 40 µg/ml of phosphite. Although other cDNA libraries have been constructed from different *Phytophthora* species (Weerakoon et al. 1998; Qutob *et al.* 2000; Kim and Judelson 2003; Shan *et al.* 2004; Skalamera *et al.* 2004), this is the first reported library which can be used for screening phosphite induced gene expression and function analysis in *P. cinnamomi*.

Since the cDNA library cannot be better than the mRNA from which it is derived, it was essential to start with good quality mRNA before it was used as a template for the synthesis of cDNA. Additionally, the identities and amounts of transcribed mRNA are in constant flux within cells. Therefore, the tissue was carefully chosen with respect to age (while the mycelium was still actively growing) and function (while the effect of phosphite was significant on the mycelial growth) prior to cDNA production. Preliminary experiments conducted on the isolate MP 80 (Chapter 2) identified that 4 d post phosphite treatment restricted growth of the pathogen yet provided sufficient mycelium for RNA extractions. Although the phosphite concentration in the mycelium was not measured at this time, Grant *et al.* (1990) have revealed with metabolic profiling that the highest concentration of phosphite occurs in the mycelium of *P. palmivora* on day 5 after phosphite treatment.

The transcripts in the constructed cDNA library were cloned from the population of mRNAs isolated from vegetative mycelium on day 4. At this time genes would be expected to be transcribed at different rates as required by the needs of the hyphae. mRNAs transcribed at lower rates, representing less than 0.5 % of the total mRNA
population of the cell are classified as low abundance or rare mRNAs (< 14 copies/cell) (Sambrook et al. 1989; Farrell 2005). The probability of isolating cDNA clones from mRNA of this type depends on the cDNA library constructed, whose size is sufficient to ensure that even the rare mRNAs have a good chance of being represented (Sambrook et al. 1989). The primary titre of the library constructed in the present study was $2.15 \times 10^6$ pfu. Additionally, the library was amplified only once to prevent the transcripts from less abundant mRNAs from being diluted out of the library.

The characterization of cDNA inserts by gel analysis revealed that the average clone insert was 650 bp in length with inserts ranging up to 2000 bp. Fractionation of double stranded cDNA was performed to enrich the library with full-length clones, which is a more practical alternative to mRNA fractionation used in the past (Sambrook et al. 1989), as DNA is less susceptible to degradation by contaminating nucleases. It was estimated that approximately 25% of the inserts within the library were due to the incorporation of small DNA fragments into the vector MCS. These DNA fragments must have passed through the size-fractionation column and been present in the ligation mix.

To verify that the cDNA library contained cDNA synthesized from *P. cinnamomi* mRNA, nine randomly selected inserts were characterized by sequencing. Some of the inserts sequenced matched the sequences deposited in the NCBI database, having similarity with *P. infestans ras* related GTP binding-protein encoded by the *piYpt 1* gene, proposed to be involved in vesicle transport (Chen and Roxby 1996). It is believed that the presence of large numbers of vesicles at the hyphal tips of *Phytophthora* (Hemmes 1983) is essential for the transport of materials required for the growth of hyphae (Chen and Roxby 1996). A homologue to *P. infestans* clone coding for RIC 1
protein, which is involved in maintaining membrane integrity especially in stress inducing conditions (van West et al. 1999a) was isolated from the cDNA library. The expression of the gene ric 1 was found predominantly in the mycelial stage and during *in planta* growth of *P. infestans* (van West et al. 1999a). A member of the extracellular elicitor proteins was also identified with similarity to *P. infestans* MY-10-H-01 thioredoxin peroxidase. Elicitors are known to stimulate plant defence mechanisms in plant-pathogen interactions (Benhamou 1996; Kamoun et al. 1997; Rodrigues et al. 2006). Other cDNA transcripts corresponded to the ribosomal RNA genes of different *Phytophthora* species, these transcripts were actually found to encode mitochondrial proteins of unknown function and were produced within the 25s rRNA gene. Some of the insert sequences aligned with proteins of unknown function from *P. sojae*.

Almost 10 000 cDNA clones were prepared for profiling changes in mRNA transcription by microarrays. Ideally, cDNA clones are selected to represent as many unique transcripts as possible (Hegde et al. 2000), but as the library had not been screened before, the 20 000 recombinant clones were picked based only on the colour selection (blue versus no colour). As all the high-throughput processing of these samples was performed manually with a multi-channel pipette, the final number of cDNA clones to be screened on microarrays was restricted by physical limitations and time restrictions. Initially, it was planned to use robotics (GeneTac G3) to pick recombinant clones. However, the software proved to be inadequate, hence limiting the number of cDNA clones picked.

Fifteen thousand cDNA inserts were amplified by PCR. A single product was obtained from approximately 95% of clones, whilst the rest of the reactions contained no product or multiple bands. Finally, approximately 10 000 PCR reactions were purified for
downstream applications by removing unincorporated nucleotides, primers and salt by filtration in PCR purification plates. This was essential to ensure efficient binding of the transcripts to the microarray slide and improve hybridization specificity (Hegde et al. 2000; Holloway 2003). Initially, the high-throughput PCR clean up was performed in 384-well plates but because of inconsistent results, 96-well plates were used with better results. Most of the PCR reactions and purifications were preferentially performed in 96-well plates, which produced more consistent and reproducible results compared to the 384-well plates. The average recovery of purified PCR products was 450 ng of DNA. This concentration was sufficient for printing on microarray slides, the minimum requirement being 400 ng (Mark van Der Hoek pers. comm.). Finally, 9216 cDNA purified PCR inserts were transferred to 384-well arraying plates and a database containing the identification and position of each clone in the plates was constructed.

In summary, a cDNA library from axenically grown phosphite-treated mycelium of 
*P. cinnamomi* was constructed. This cDNA library, consisting of over 2 million pfu, is a new resource that can be used to study the molecular interaction between phosphite and *Phytophthora* and aid in our understanding of how phosphite can control Oomycetes. This in turn may lead to the development of novel control strategies. The construction of the cDNA library has laid solid foundations for finding phosphite relevant genes and investigating their function. Over nine thousand cDNA inserts were selected from the cDNA library, amplified by PCR and purified by filtration in preparation for microarray analysis to profile gene expression patterns in the presence of phosphite, which will pave the way for the detection of large scale changes in pathogen expression that underlie the mechanisms of virulence and pathogenicity of *P. cinnamomi*. The library is a resource that can be used to generate ESTs (Expressed
Sequence Tags), which can be compared with ESTs of other *Phytophthora* species to assess the similarity of *P. cinnamomi* sequences to existing EST data and enrich the public database with novel ESTs unique to *P. cinnamomi*. 
Chapter 4

The selection of constitutively expressed genes for microarray expression normalisation and evidence of phosphite repressed gene expression in the mycelium of Phytophthora cinnamomi using quantitative real-time PCR.

4.1 Introduction

Gene expression is a multi-step process that begins with transcription of DNA into mRNA (Cooper and Hausman 2004). The expression of genes may be assessed with RNA quantification methods such as microarrays, northern blotting and real-time PCR. Gene expression using microarrays is measured by the binding of probes onto cDNA transcripts (for cDNA microarrays) arrayed on a glass slide (Hegde et al. 2000; Amaratunga and Cabrera 2004).

Due to the complex nature of the procedure of preparing and using the microarray slides (Chapter 1), systematic variations that affect the measurement of gene expression can occur at any step of the process from printing of slides and hybridisation of probes to data extraction (Amaratunga and Cabrera 2004). In order to make valid comparisons across microarrays, the effects of systematic variations need to be normalised across the whole experiment so that the data from different slides are assessed on a common scale (Amaratunga and Cabrera 2004). One way to achieve this is to integrate into the microarray a number of genes, which are constitutively expressed under the conditions being tested (Holloway 2003). Analysis of the hybridisation data obtained from these controls provides information on the dynamic range, sensitivity and
specificity of the microarray process, and enables statistical analysis of the reproducibility of the observed expression ratios to be performed. By spotting the constitutively expressed genes at different locations in the array, systematic spatial variation and bias in the strength of the Cy3 and Cy5 signals can be measured and corrected (Draghici 2003; Yang and Speed 2003).

Many studies in the literature have used different genes as internal controls, such as those encoding actin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal RNA, cyclophilin and elongation factors (Sturzenbaum and Kille 2001; Bezier et al. 2002; Dean et al. 2002). However, one inherent problem is that a good standard for one study may not be suitable for another study when different conditions are being examined. Candidate constitutively expressed genes may be assessed using quantitative real-time PCR (qRT-PCR), which allows simultaneous quantification and amplification of cDNA reverse transcribed from mRNA, and therefore enables the assessment of relative gene expression at a particular time or between treatments (Bustin 2000; Radonic et al. 2004). The qRT-PCR may be performed with a fluorescent intercalating double strand DNA binding dye, SYBR Green I, to monitor the amplicons during each cycle of the PCR (Pfaffl, 2003).

In Chapter 3, cDNA transcripts were prepared from mRNA extracted from phosphite-treated (40 µg/ml) mycelium of Phytophthora cinnamomi to establish a microarray. The aim of this study was to identify appropriate constitutively expressed genes that could be used as positive controls in the microarray. For this, RNA transcription levels of four candidate genes expressing glyceraldehyde-3-phosphate dehydrogenase (GAPDH), translation elongation factor 1 α (EF-1 alpha), ubiquitin-conjugating enzyme (Ubc) and beta-tubulin in mycelium growing with or without the presence of phosphite were
examined using qRT-PCR. Since phosphite has previously been shown to increase the secretion of necrosis-inducing elicitin proteins in *Phytophthora* (Perez *et al.* 1995), the effect of phosphite on the expression of the gene encoding cinnamomin was also assessed in this chapter.

### 4.2 Materials and Methods

#### 4.2.1 Primer design

Sequences of *GAPDH*, *EF-1 alpha*, beta-tubulin, Ubc and cinnamomin genes were obtained from the GenBank database (NCBI, [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). Primer pairs were designed from either aligned or single sequences of *P. cinnamomi* and/or other *Phytophthora* species (90-150 bp length of amplicon, primer GC content 40-70 %, optimum primer length 15-25 bp, with all primers ending with G or C at the 3’ end) using Primer Express 1.5 software (Table 4.1).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession No.</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Amplicon Size (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>GAPDH</em></td>
<td>AY292378.1</td>
<td>GAPHD_F1</td>
<td>CCATCCgTgTgTTCAACgAgTgAAggCAACCgTggAC</td>
<td>90</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAPDH_R1</td>
<td></td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Ubc gene</td>
<td>AF424641.1</td>
<td>Ubc_F1</td>
<td>CTgAACATCTACTTCCCCggCC</td>
<td>90</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubc_R1</td>
<td>CgTTggCATTgATTTgCAg</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>beta-tubulin</td>
<td>AY766221.1</td>
<td>Tub_F1</td>
<td>TgACCCAgCAgCAgTTgCG</td>
<td>116</td>
<td>58</td>
</tr>
<tr>
<td>gene</td>
<td></td>
<td>Tub_R1</td>
<td>CATgACCTCTCTCTgTTgCTC</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td><em>EF-1 alpha</em></td>
<td>AY564110.1</td>
<td>efl_F1</td>
<td>CgAgAAgTgCggCAACATgC</td>
<td>89</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>efl_R1</td>
<td>TTgTCAACCgCG</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>cinnamomin</td>
<td>AJ000071.1</td>
<td>Cinn_F1</td>
<td>AgTgCTCCCAAgACTCgg</td>
<td>105</td>
<td>55</td>
</tr>
<tr>
<td>gene</td>
<td></td>
<td>Cinn_R1</td>
<td>TTgATCATgTTgCAgC</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

* Tm, melting temperature
4.2.2 RNA extractions

Four day-old mycelium of *P. cinnamomi* isolate MP 80 that was phosphite-treated (40 µg/ml) or untreated was harvested (Chapter 2, section 2.2.2) and the RNA extracted using the procedure described in Chapter 3, section 3.2.1 (Logemann *et al*. 1987). The high sensitivity of the qRT-PCR means that even small amounts of DNA could interfere with the specificity of the amplification reaction. To remove the genomic DNA, the RNA samples were treated with RQ1 RNase free DNase (Promega) at 37 ºC for 30 min in 20 µl reactions. Reaction 1 (RNA from untreated mycelium): 6.5 µl of RNA (933.2 ng), 1 µl of DNase (1U), 1 µl of 10x buffer, 1.5 µl of H2O; Reaction 2 (RNA from phosphite-treated mycelium): 6.5 µl of RNA (1880 ng), 2 µl of DNase (2U), 1 µl of 10x buffer, 0.5 µl of H2O. The reactions were terminated by adding 1 µl of RQ1 DNase stop solution and incubating at 65 ºC for 10 min to inactivate the DNase. The quantity and quality of RNA samples were measured in duplicates on a Nanodrop ND-1000 Spectrophotometer at 260/280 nm. The RNA concentrations of each sample was normalised to 47 ng/µl prior to the synthesis of single stranded cDNA.

4.2.3 First-strand cDNA synthesis

Reverse transcription was performed using the SuperScript III Platinum Two-step qRT-PCR kit with SYBR Green (Invitrogen) in 20 µl reactions containing 10 µl of 2x RT Reaction mix, 2 µl of RT Enzyme Mix and 8 µl of RNA (376 ng). The tubes were incubated in a PCR thermal cycler (Corbett Palm Cycler) at 25 ºC for 10 min and then for 50 min at 42 ºC. The reactions were terminated at 85 ºC for 5 min and then chilled on ice. Two units of *E. coli* RNase H were added to the cDNA samples, which were then incubated at 37 ºC for 20 min and then stored at -20 ºC.
4.2.4 Evaluation of candidate constitutively expressed genes by qRT-PCR

The RNase treated first strand cDNA from both treatments was diluted with water to 1:10 and 1:100 prior to amplification. Quantitative RT-PCR was performed in the iCycler (My-iQ Bio Rad) in 25 µl reactions containing 12.5 µl of Platinum SYBR Green qPCR SuperMix-UDG, 1 µl of forward primer (5 µM), 1 µl of reverse primer (5 µM), 2.5 µl of cDNA and 8 µl of H2O. PCR conditions were: 50 °C for 2 min, 95 °C for 2 min, 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The melting curve was performed at 0.5 °C intervals from 95 °C to 55 °C following 30 s incubation. The experiment was repeated twice and the mean C_T values averaged across the experiments. Three control reactions were performed (-RNA, -cDNA, -RT).

4.2.5 Relative gene expression quantification by the 2^{ΔΔC_T} method

Quantitative real-time PCR revealed that expression of the Ubc gene was down-regulated in phosphite-treated mycelium. This was further investigated in correlation with the elicitin gene cinnamomin using the comparative 2^{ΔΔC_T} method (Livak and Schmittgen 2001), with EF-1 alpha as a reference gene. For a reliable comparison between samples, the method of relative quantification requires the efficiencies of amplification of the target and reference genes to be within ± 10 % of each other (Livak and Schmittgen 2001). To assess that all three products were amplified with the same efficiency, standard curves consisting of 6-point 4-fold dilution series were constructed using the cDNA synthesised from RNA extracted from the untreated mycelium (MyiQ).
Quantification of relative gene expression was then determined by amplifying the target genes and the reference gene in triplicate/per treatment, along with no template control (−cDNA). Changes in expression levels were determined with respect to the reference gene.

### 4.3 Results

The integrity of the RNA samples used in RT-PCR reactions was assessed on a 1.5 % agarose/formaldehyde gel, which showed the presence of two intact RNA ribosomal bands in both samples (Figure 4.1). RNA extracted from the untreated mycelium had a purity of 2.3 (260/280 nm) and contained 47 ng/µl of material compared to the RNA from phosphite-treated mycelium, which had a purity of 1.93 (260/280 nm) and contained 54 ng/µl of material. The amount of RNA in the latter sample was diluted to 47 ng/µl prior to the reverse transcription.

![Figure 4.1](image) **Figure 4.1** Formaldehyde agarose gel electrophoresis of RNA extracted from untreated *Phytophthora cinnamomi* mycelium (Lane 1) and phosphite-treated (40 µg/ml) *P. cinnamomi* mycelium (Lane 2).
4.3.1 Evaluation of candidate constitutively expressed genes by qRT-PCR

RNA transcription levels of four genes, *EF-1 alpha*, *GAPDH*, Ubc and beta-tubulin genes were analysed by qRT-PCR to evaluate their consistency of expression in phosphite-treated and untreated mycelium. The average C_T values, defined as the number of cycles needed for the fluorescence signal to reach a statistically significant threshold level of detection, were compared between treatments for each gene (Table 4.2). The difference in transcription levels (ΔC_T) of the genes was calculated by subtracting the C_T values of phosphite-treated sample C_T (+P) from the untreated sample C_T (-P). As there were no significant differences between the experiments, the C_T values were averaged from both experiments for each dilution (1:10 and 1:100) (Appendix 5).

Table 4.2: The summary of gene expression assessment of four genes from *Phytophthora cinnamomi* mycelium in the presence and absence of phosphite.

<table>
<thead>
<tr>
<th>Genes</th>
<th>ΔC_T (1:10)</th>
<th>ΔC_T (1:100)</th>
<th>ΔC_T (average)</th>
<th>Cycle difference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EF-1 alpha</em></td>
<td>-0.10</td>
<td>-0.05</td>
<td>-0.075 ± 0.018</td>
<td>3.5</td>
</tr>
<tr>
<td>beta-tubulin</td>
<td>-0.19</td>
<td>-0.56</td>
<td>-0.38 ± 0.13</td>
<td>3.6</td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>-0.86</td>
<td>-1.06</td>
<td>-0.96 ± 0.07</td>
<td>3.4</td>
</tr>
<tr>
<td>Ubc gene</td>
<td>-1.22</td>
<td>-1.42</td>
<td>-1.32 ± 0.07</td>
<td>3.3</td>
</tr>
</tbody>
</table>

ΔC_T = C_T (-P) - C_T (+P); Cycle difference = C_T (-P 1:100) - C_T (-P 1:10) or C_T (+P 1:100) - C_T (+P 1:10)  
Average ΔC_T values and cycle differences represent a mean of four replicates.

The calculations were performed with respect to the amount of nucleic acid template being normalised between the two treatments prior to RT reaction and PCR amplification. The use of the two point dilution (1:10 and 1:100) in each treatment allowed for the reflection of experimental error and biological relevance. The cycle difference between a 10-fold dilution should be 3.3. As indicated in Table 4.2, the experimental error was no larger than 0.3 cycle difference.
The analysis of the $\Delta C_T$ values showed that the best reference gene for these particular experimental conditions was *EF-1 alpha* with $\Delta C_T$ values close to zero, indicating constitutive expression in the presence and absence of phosphite. Expression of the *GAPDH* and the Ubc genes was down-regulated and these genes were thus unsuitable as constitutively expressed standards. The 2.5-fold down-regulation (fold change $= 2^{1.32}$) of the Ubc gene indicates that expression of this gene is in fact responsive to phosphite. Due to this observation, the expression of the Ubc gene and a gene encoding cinnamomin was further analysed using *EF-1 alpha* as a reference gene (Section 4.3.2).

The gene encoding beta-tubulin, which was also unaffected by phosphite, was omitted from the analysis as the melting curve revealed the presence of primer-dimers, which reduced the efficiency of the PCR amplification and caused overestimation of the target gene concentration (since SYBR Green binds to any double stranded DNA). *GAPDH* was not used in further experiments since the fold-change was $< 2$, falling below the accepted threshold of significant change in gene expression adopted in this study ($\geq 2$).

The real-time PCR results of the two genes of interest revealed that *EF-1 alpha* exhibited constitutive expression in both treatments and was the most highly expressed gene amongst those tested from the mycelium of *P. cinnamomi* with a $C_T$ value of $\sim 17.14$ (Figure 4.2). In contrast, the ubiquitin-conjugating enzyme encoding gene was the least expressed of the two genes ($C_T$ value $\sim 22.49$). The control reactions without template DNA or RNA, or containing no RT in a PCR reaction produced no amplification product.
Figure 4.2: qRT-PCR transcription profiles of EF-1 alpha (A) and the Ub encoding gene (B) amplified from phosphite treated (+P) and untreated (-P) Phytophthora cinnamomi using two dilutions (1:10 and 1:100) of cDNA. The C_T values were inversely correlated with the amount of template nucleic acid present in the reaction. The reactions with more template cDNA (1:10) amplified earlier (by ~3.3 cycles) than the reactions with 10x less cDNA (1:100). In general all reactions followed the same trend of amplification, with amplification signals above that of the background level. The C_T threshold was set in the linear amplification phase of the PCR during which the relative changes in the level of transcription of the target gene were extrapolated from the corresponding C_T values. Once the reaction reagents were exhausted at the end of the PCR, the rate of additional product gain eventually reached a plateau.
The amplicons were analysed on a gel to confirm that the primers amplified only one PCR product of the correct size (Figure 4.3).

![Figure 4.3: Visualisation of the amplified RT-PCR product using Ubc (Lane 2) or EF-1 alpha (Lane 3) primers in a RT-PCR reaction containing P. cinnamomi RNA as a template. A Promega 100 bp ladder (Lane 1) was used as a size reference.](image)

The specificity of the primers was further confirmed by the appearance of a single peak in the melting curve analysis following completion of the amplification reaction, with the melting temperatures in accordance with those calculated (Figure 4.4).
Figure 4.4: Melting curve analysis of qRT-PCR generated amplicons of EF-1alpha (A) and Ubc gene (B) confirming the single amplification product of each. The fluorescence of the DNA coupled with SYBR Green I is brightest when the two strands are annealed. As the temperature is raised towards the Tm of the duplex, there is a dramatic reduction in the fluorescence resulting in a peak, which represents the Tm of the DNA determined by plotting the negative first derivative (-dF/dT) of fluorescence versus temperature.

4.3.2 Relative gene expression quantification by the $2^{-\Delta\Delta C_T}$ method

Changes in gene expression levels of the Ubc and cinnamomin encoding genes between the phosphite-treated and untreated mycelium were calculated using the $2^{-\Delta\Delta C_T}$ method, where the variation in transcription between the two treatments ($\Delta\Delta C_T$) was normalised relative to the reference gene, EF-1 alpha, for each target gene. The fold expression was then determined using the formula $2^{\Delta\Delta C_T}$. To satisfy the assumption of the $2^{-\Delta\Delta C_T}$ method, the amplification efficiencies of the reference and target genes were assessed using standard dilution curves (Figure 4.5).
Figure 4.5: Standard curves of qRT-PCR targeting EF-1 alpha (A), cinnamomin (B) and Ubc (C) genes. The $C_T$ values for each dilution were plotted against concentration resulting in a linear graph and the slope of the graph was used to calculate the measure of efficiency (MyiQ). This describes how much of the sequence of interest is being produced with each cycle. 100 % efficiency means that the sequence of interest is increasing by a factor of two with each cycle.
All standard curves had a good linear fit ($R^2 > 0.99$) (Figure 4.5). The performance of the three primer pairs was within $\pm 10\%$ of each other and all had a high amplification efficiency ($E = 99$ to 100.8\%), satisfying the assumption of the $2^{-\Delta\Delta CT}$ method of the normalised gene expression. The relative quantity of the genes encoding Ubc and cinnamomin was calculated using $EF-1\ alpha$ as a reference gene to normalise the expression of the target genes with respect to RNA quantity (Figure 4.6).

![Figure 4.6: Normalised gene expression of the Ubc (green) and cinnamomin (blue) encoding genes. Normalised gene expression ($\Delta\Delta CT$) is the relative quantity of the target gene normalised to the quantities of the reference gene ($EF-1\ alpha$), which accounts for variations in the amount of cDNA represented in each sample and loading differences. Bars represent standard error (SE) of the mean of three replicates.](image)

This experiment revealed that the gene encoding cinnamomin showed no change in transcription between the two treatments, with a fold difference of 1.02. The melting curve and gel analysis confirmed the specificity of the primers. The gene encoding Ubc on the other hand showed 2.1-fold down-regulation in the phosphite-treated mycelium (Table 4.3).
Table 4.3: Differences in transcription of three *Phytophthora cinnamomi* genes in phosphite-treated and untreated mycelium as determined by the 2-\(\Delta\Delta T\) method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>(\Delta C_T) (-P)</th>
<th>(\Delta C_T) (+P)</th>
<th>(\Delta \Delta C_T)</th>
<th>Fold difference</th>
<th>Primer efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF-1 alpha</td>
<td>22.65</td>
<td>22.66</td>
<td>-0.01</td>
<td>1.01</td>
<td>101.1</td>
</tr>
<tr>
<td>cinnamomin gene</td>
<td>22.56</td>
<td>22.54</td>
<td>0.03*</td>
<td>1.02</td>
<td>99</td>
</tr>
<tr>
<td>Ubc gene</td>
<td>24.86</td>
<td>25.93</td>
<td>-1.06*</td>
<td>2.08</td>
<td>100.8</td>
</tr>
</tbody>
</table>

\(\Delta C_T\) values represent a mean of three replicates; \(\Delta \Delta C_T\) values = \(\Delta C_T\) (-P) - \(\Delta C_T\) (+P), Fold difference = 2\(^{-\Delta \Delta C_T}\); *normalised \(\Delta \Delta C_T\) value = [\(\Delta \Delta C_T\) target gene - \(\Delta \Delta C_T\) housekeeping gene]; 100 % efficiency means that the sequence of interest is increasing by a factor of two with each cycle.

### 4.4 Discussion

In this study the *P. cinnamomi* genes, *EF-1 alpha* and cinnamomin, were constitutively expressed, regardless of the presence or absence of phosphite. Both these genes were determined to be good reference genes for the particular conditions of this study, against which the expression levels of other genes could be compared to reveal transcriptional variation in phosphite-treated and untreated *P. cinnamomi* mycelium. The qRT-PCR profiles revealed that the two genes were also highly expressed in the cells of the mycelium (low \(C_T\) values). In contrast, phosphite down-regulated the expression of glyceraldehyde-3-phosphate dehydrogenase and the ubiquitin-conjugating enzyme encoding genes, which were thus unsuitable as reference genes.

Elongation factor 1 \(\alpha\) is an enzyme that mediates peptide chain elongation in eukaryotes (Riis *et al.* 1990), while cinnamomin is a necrosis inducing elicitin protein secreted by *P. cinnamomi* (Pernollet *et al.* 1993; Perez *et al.* 1999; Ponchet *et al.* 1999). The activity of GAPDH, an enzyme of the glycolytic pathway, was previously reported to be inhibited by phosphite (IC\(_{50}\) = 0.74 ± 0.07 mM) in enzyme assays from *P. palmivora* (Stehmann and Grant 2000). This concurs with the finding in the present study that transcriptional levels appeared to be down-regulated for this gene (1.9-fold). However,
it is generally accepted that a change in transcriptional expression needs to be higher than 2-fold to be significant (Hegde et al. 2000; Rajeevan et al. 2001). Interestingly, the transcription of the Ubc encoding gene was reduced by 2.1-fold in phosphite-treated mycelium. The biological significance of Ubc will be discussed below (section 4.4.1). The evaluation of the transcription level of beta-tubulin gene showed that this gene could potentially be used as constitutively expressed gene for these experimental conditions. However, due to the compromised PCR efficiency of the amplification by the primers designed, the expression of this gene was not examined further.

The results in this study highlight the importance of careful selection of internal controls for gene expression analysis and testing the stability of the standards specifically for the type of material used and the particular conditions of a given experiment. Normalisation with genes of variable expression can lead to misinterpretation of results. For example, in the work of Yan and Liou (2006) on *P. parasitica* (syn. *P. nicotianae*), the elongation factors were the most highly expressed genes across different life stages of the pathogen, but ranked poorly as internal controls due to variability in expression. In contrast, they found the expression level of the ubiquitin-conjugating enzyme encoding gene consistent and suitable as a constitutive expressed marker for the life stages of *P. parasitica* (syn. *P. nicotianae*). This is quite the opposite of the findings in the present study.

4.4.1 Biological significance of the ubiquitin-conjugating enzyme

The real-time PCR analysis in the present study revealed that treatment of *P. cinamomomi* mycelium with 40 µg/ml phosphite suppressed the expression of the gene encoding the ubiquitin-conjugating enzyme, a component of the ubiquitin
proteasome pathway (UPP), by over 2-fold. The UPP plays an essential role in the degradation of proteins that are abnormal or no longer required in cells. Furthermore, it is involved in the fine tuning of cellular functions and signalling pathways by regulating the intracellular levels of a wide range of proteins, including those involved in the control of the cell cycle, transcriptional activation, apoptosis, and cell signalling (Glickman and Ciechanover 2002; Smalle and Vierstra 2004).

Proteins targeted for degradation by the UPP are modified with polyubiquitin chains in a three-step, highly regulated enzymatic process involving a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase enzyme (E3) (Figure 4.7).

![Figure 4.7](http://www.hhmi.org/research/investigators/schulman.html, 21/08/07)

**Figure 4.7:** The 3-step cascade of protein ubiquitination. First, the ubiquitin-activating enzyme (E1) activates the C-terminal glycine residue of ubiquitin (Ub) using energy from adenosine triphosphate (ATP), allowing it to react with the lysine residue of the target proteins. The C-terminal glycine residue of Ub forms a thiol ester linkage with the active site cysteine residue of E1. The activated Ub moiety is then transferred to a ubiquitin-conjugating enzyme (E2), which catalyses the attachment of Ub to the substrate protein by forming a second thiolester linkage with the C-terminal glycine and transfer the activated Ub moiety via an additional high energy thiol ester intermediate to the substrate that is specifically bound to ubiquitin ligase (E3). E2 enzymes can function alone or in conjunction with E3 ligases to catalyse the attachment of Ub to acceptor lysine residues of target proteins to form isopeptide bonds. The Ub protein ligases (E3) are responsible for recognising the substrate proteins for ubiquitination (Smalle and Vierstra 2004).
Additional ubiquitin proteins attach to the lysine residue of the first ubiquitin to build up a poly-ubiquitin chain, allowing the tagged protein to be recognized by the proteasome (Figure 4.8).

![26S Proteasome](image)

*(Unno et al. 2002)*

**Figure 4.8:** Recognition of the tagged protein by the proteasome and subsequent protein degradation. The 26S proteasome consists of a 20S catalytic subunit (core) that is bound to two copies of 19S regulatory subunits. The core (CP-core protease) houses the enzymatic functions of the proteasome and is composed from 28 proteins arranged into four stacked rings to form a hollow cylinder: two α rings which perform a structural role (catalytically inactive), and two β rings which comprise the catalytic core of the proteasome. The cap (RP-regulatory particle) is responsible for the recognition of the polyubiquitin chains, unfolding the target proteins, and releasing the ubiquitin tags for recycling. The protein is degraded in the catalytic core to peptides numbering 3 to 22 amino acids in length (Unno et al. 2002).

Components of the ubiquitin/proteasome pathway have been identified in several *Phytophthora* species including *P. nicotianae* (Shan et al. 2004; Skalamera et al. 2004), *P. infestans* (Pieterse et al. 1991) and in the genomes of *P. ramorum* and *P. sojae* (JGI, [http://www.jgi.doe.gov/](http://www.jgi.doe.gov/)). The functional classification and analysis of Expressed Sequence Tags (ESTs) reflecting the expression of genes during the filamentous growth of *P. parasticta* (syn. *P. nicotianae*) mycelium showed that 2.1 % of the genes in the library related to the ubiquitin/proteasome pathway (Panabieres et al. 2005). This suggests that the UPP is just as critical in the regulation of cellular processes in *Phytophthora* species as in other eukaryotic organisms. Down-regulation of the
expression of the Ubc gene occurs in the mycelium of phosphite-treated *P. cinnamomi*, inferring that the gene encoding Ubc may be responsive to altered growth conditions of *P. cinnamomi*. Although the mycelium was grown in the presence of ‘high’ phosphate levels (10 mM), the growth of the phosphite sensitive isolate MP 80 was reduced by 70% when treated with 40 µg/ml phosphite. The change in the transcription of the Ubc gene could be due to phosphite toxicity in the mycelium of *P. cinnamomi* or phosphate starvation induced in the presence of phosphite.

Recently, several studies have revealed that the regulation of inorganic phosphate in plants involves the suppression of a ubiquitin-conjugating enzyme (Ubc; *At2g33770*) by a specific microRNA, miR399 (Chiou et al. 2006). MicroRNAs represent a class of non-coding small RNAs that function as post-transcriptional negative regulators and facilitate cleavage of mRNA (Du and Zamore 2006). Chiou et al. (2006) demonstrated that under phosphate starvation, the miR399 targets multiple sites in the 5’ untranslated region of Ubc mRNA resulting in a five-fold down-regulation of Ubc in the roots of *Arabidopsis thaliana*. They also showed that the changes in Ubc gene expression affected phosphate allocation between roots and shoots, and phosphate remobilization within leaves. Transgenic *Arabidopsis* plants over-expressing miR399 accumulated five to six times the normal phosphate levels in shoots and displayed toxicity symptoms, suggesting that the amount of Ubc in plants is critical for preventing the overloading of phosphate into plants (Fujii *et al.* 2005; Aung *et al.* 2006; Chiou *et al.* 2006). It was later revealed that the Ubc gene (*At2g33770*) encodes a phosphate accumulator PHO2 (Bari *et al.* 2006).

There is evidence that the adaptive responses of plants to phosphate deficiency are regulated by post translational regulation of other components of the UPP pathway.
A small ubiquitin-like modifier (SUMO) E3 ligase, \textit{AtSIZ1}, was established to control phosphate starvation dependent responses in \textit{Arabidopsis} by acting on the transcription factor (PHR1) of phosphate starvation genes (Miura \textit{et al.} 2005). The E3 ligase is involved in a novel post-translational modification system biochemically analogous to, but functionally distinct from ubiquitination, called sumoylation. Sumoylation to components of the transcriptional machinery can both repress and activate transcription (Gill 2005; Miura \textit{et al.} 2005). Subsequently a model was proposed for phosphate signalling in plants where the transcription factor PHR1 is upstream of the phosphate accumulator PHO2 and the miR399 in phosphate signalling (Bari \textit{et al.} 2006) (Figure 4.9).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure49.png}
\caption{Proposed model for phosphate signalling in plants involving PHO2, miR399 and PHR1. The activity of PHR1 might be regulated by the E3 ligase SIZ1. miR399, downstream of PHR1 which might be required for induction of miR399, is responsible for regulating the expression of PHO2 (Ubc), which in turn regulates the expression of a subset of phosphate starvation induced genes. Other signalling pathways may be independent of this model and are illustrated by the dashed arrows (Bari \textit{et al.} 2006).}
\end{figure}
It would therefore be worthwhile examining the role of Ubc (At2g33770) and E3 ligases in experiments investigating the effect of phosphite on phosphate starvation responses in plants. Phosphite has been previously shown to obstruct the phosphate signalling mechanism in *Saccharomyces cerevisiae* (McDonald *et al.* 2001) and *Ulva lactuca* (Lee *et al.* 2005) and caused a down-regulation of phosphate starvation response genes in *Arabidopsis* and tomato seedlings, preventing the plants to respond to phosphate limiting conditions (Varadarajan *et al.* 2002). The mechanism of how phosphite induces suppression of phosphate starvation responses is unclear. Since phosphite affects the transcription of Ubc encoding gene in *P. cinnamomi*, it may also have an effect on the transcription of Ubc in plants. The observations by Chiou *et al.* (2006) and the results in the present study, suggest that phosphite might interfere with the ubiquitin-phosphate signalling pathway in plants. This could then be one of the mechanisms by which phosphite disrupts the phosphate starvation responses in plants.

The down-regulation of the gene encoding Ubc in the present study also suggests that it may play a role in the mechanism of phosphite/phosphate uptake in *P. cinnamomi* and consequently on the growth of the pathogen. It is likely that if the mycelium was grown in the presence of lower levels of phosphate, the transcriptional suppression of the gene encoding Ubc would be greater. Further studies could investigate changes in the mRNA levels of Ubc in response to a phosphate dose curve in *P. cinnamomi*.

The UPP pathway also has a direct association with other interactions between plants and pathogens. Recognition of elicinins by plants leads to hypersensitive response at the site of infection (Ricci *et al.* 1989; Kamoun *et al.* 1993). It has been demonstrated that phosphite alters the composition of mycelial cell wall and causes a release of elicinins (Rouhier *et al.* 1993; Perez *et al.* 1995). Phosphite has been shown to stimulate
the secretion of elicits in *P. capsici* and *P. cryptogea* (Perez *et al.* 1995). However, this study has revealed that the level of mRNA encoding the elicitin cinnamomin is constitutive irrespective of the presence or absence of phosphite. This might suggest then that the increase in elicitin protein levels after phosphite treatment could result from post translational regulation, from enhanced secretion or from enhanced leakage.

The transcription of the acidic elicins remains to be determined. In a preliminary study (data not shown), the precipitation of proteins from the growth medium of both phosphite-treated and untreated mycelium, and characterisation on an SDS-polyacrylamide gel revealed the presence of proteins 10 kD in size. Peptide sequencing, carried out at the Lottery State Biomedical Facility, Proteomics Node, WA, confirmed that the protein was cinnamomin (match to s06671) from *P. cinnamomi*. However, further study is required to assess whether the concentration of *P. cinnamomi* elicins is greater in the phosphite amended medium used in this study with MP 80. Further research is necessary to establish if *Phytophthora* elicins are targeted for post translational modification by the UPP pathway and whether the suppression of this pathway by phosphite correlates with increased secretion of elicins in *P. cinnamomi*.

Although the mechanisms of ubiquitination and subsequent protein degradation are well understood, the process of protein selection for destruction by the proteasome is only just beginning to be elucidated. Some of the mechanisms of protein recognition for degradation/sumoylation by the UPP pathway include phosphorylation of a target protein by a signal transduction cascade, exposure of a hydrophobic protein surface via disaggregation of a protein complex or protein denaturation and specific N-terminal residues of the target protein (Glickman and Ciechanover 2002; Smalle and Vierstra 2004).
While the present study focused on and observed a change in Ubc gene regulation in *P. cinnamomi* in response to phosphite, it is also likely to have a role *in planta*. In host plants, elicitors trigger transcription of genes previously shown to be expressed during the establishment of systemic acquired resistance (SAR). These SAR genes are expressed locally corresponding to necrosis formation, and systematically during induction of resistance (Etienne *et al.* 2000).

A newly identified SAR gene, encoding a β-subunit of proteasome, called tcI7 (tobacco cryptogein induced) was shown to be up-regulated in tobacco plants following an elicitor treatment with cryptogein and parasiticein (Etienne *et al.* 2000). Subsequently, inoculation of the cryptogein-treated plants with *P. parasitica* (syn. *P. nicotianae*) further induced the expression of tcI7, leading to the conclusion that tcI7 could be involved in the early onset of SAR (Etienne *et al.* 2000). In addition, two other subunits alpha 3 and 6 proteasome subunits were up-regulated during the initiation of defence reactions by cryptogein in tobacco. This up-regulation was observed prior to the appearance of HR and correlated with the induction of SAR (Dahan *et al.* 2001). These studies provide evidence that specific proteolysis is involved in defence reactions (Dahan *et al.* 2001).

It was also found that treating plants with pathogen elicitors induces the expression of specific UPP genes, including a ubiquitin conjugating enzyme *OsUBC5b* and a RING E3 ligase EL5 in rice (Takai *et al.* 2002). Similarly, the sequence analysis of an *in planta* induced gene of *P. infestans* showed that the gene codes for ubiquitin (Pieterse *et al.* 1991). These observations and the reports of enhanced host defence responses in phosphite-treated plants (Guest 1986; Daniel and Guest 2006) suggest that phosphite may have the opposite effect on the UPP pathway in plants in comparison to the effect
observed on the pathogen in the present study. Ubiquitination may be a mechanism of recognition of the pathogen on the cell surface of the plant. If this is the case, then proteolysis of elicitors (or other elicitors) by the host plant may be a signal that leads to stimulation of defence responses. The interaction between the UPP pathway and phosphite was subsequently formulated into a hypothesis with links to host interaction (Figure 4.10).

In conclusion, two constitutively expressed genes were identified, *EF-1 alpha* and cinnamomin gene, which were suitable for gene expression studies measuring transcriptional changes in phosphite-treated (40 µg/ml) mycelium of *P. cinnamomi*. The results of this study can be applied in future qRT-PCR experiments using the same experimental conditions. Normalisation with the reference genes will account for both the differences in starting concentrations of RNA as well as differences in cDNA synthesis efficiency, to obtain results with biological significance. Elongation factor 1 α (*EF-1 alpha*) and the gene encoding cinnamomin will be chosen as positive controls in microarray experiments (Chapter 5). While the primary objective of this study was to identify suitable constitutively expressed genes for use in microarray studies, an additional interesting finding was the down-regulation of the Ubc gene in response to phosphite. Subsequent to these observations several lines of evidence highlight the importance of the ubiquitin-proteasome pathway in pathogen-plant interactions and host defence mechanisms as demonstrated by recent research (Ingvardsen and Veierskov 2001; Azevedo *et al.* 2002; Liu *et al.* 2002; Peart *et al.* 2002; Sullivan *et al.* 2003; Vierstra 2003). In the present study, phosphite was shown to suppress the transcription of a gene encoding the ubiquitin-conjugating enzyme in phosphite-treated mycelium of *P. cinnamomi in vitro.*
Figure 4.10: Proposed model for one of the mechanism(s) of phosphite action involving the ubiquitin-conjugating enzyme and the interaction between the pathogen and the host plant: Phosphite induced transcriptional down-regulation of Ubc gene in Phytophthora cinnamomi mycelium, which could result in less degradation of proteins, such as elicits, responsible for the induction of HR in plants. The stimulation of HR, which could occur through the recognition of elicitin by the proteasomes in the host plant, may lead to the development of SAR. The regulation of Ubc by phosphite is likely to play an important role in the regulation of phosphate starvation responses in both the pathogen and the host plant. The structural similarity between phosphite and phosphate allows both anions to be taken up by the same transport system. In the pathogen, this may result in increased uptake of phosphite and less phosphate, hence the reduced mycelial growth. In plants, phosphite disrupts the phosphate starvation response and prevents the upload of phosphate. The hypothesis is that phosphite interferes with the mechanism by which plants perceive and respond to phosphate deprivation by targeting the expression of Ubc in planta.
Further research is necessary to examine the effect of this suppression on the cellular functionality of the pathogen, which could potentially result in the recognition of the pathogen by the host plant. Other studies could focus on the interaction between phosphite and the components of the ubiquitin proteasome pathway such as ubiquitin ligases and their role in the defence mechanisms of plants (JA-signalling and SA-signalling) that eventually lead to the development of systemic resistance as well as the role of Ubc in phosphate homeostasis.
Chapter 5

Construction of cDNA microarrays from the mycelium of phosphite-treated *Phytophthora cinnamomi*.

5.1 Introduction

One of the most powerful tools to study transcriptional profiling is microarray technology which allows the examination of expression of thousands of genes at a time (Hegde *et al.* 2000; Draghici 2003). The most common use of microarrays is to explore patterns of differential gene expression from the mRNA levels in genetically identical cells subjected to different stimuli or at different developmental stages.

To construct microarrays, DNA segments, either cDNA or oligonucleotides, are mechanically spotted at high density on chemically coated microscope slides, and hybridised with fluorescently labelled probes (usually cDNA) prepared from the experimental and reference sample. By measuring the fluorescence intensities of each hybridised probe with a laser scanner at the appropriate wavelength, relative expression levels of the genes represented in the array can be determined (Hegde *et al.* 2000; Amaratunga and Cabrera 2004).

To date, studies aimed at understanding the mechanism(s) of phosphite action against *Phytophthora* have mainly concentrated on cytological and metabolic profiling (Guest 1986; Dunstan *et al.* 1990; Grant *et al.* 1990; Barchietto *et al.* 1992; Daniel and Guest 2006). Over the last decade, the advances in technology and the generation of *Phytophthora* genomic resources have enabled researchers to study the changes in gene expression in both *Phytophthora* and their hosts to identify genes encoding proteins.
involved in pathogenicity and defence signal transduction (Birch et al. 1999; Beyer et al. 2001).

Microarrays have been exploited to monitor gene expression levels in *P. infestans* in potato leaves from cultivars with horizontal resistance (Tian et al. 2006) and for transcriptional profiling of cellular responses to arachidonic acid (a membrane component of *Phytophthora*) capable of triggering systemic acquired resistance (SAR) against *P. infestans* in potato tubers (Bougri et al. 2002). Moy et al. (2004) used the technology to determine patterns of gene expression in soybean and *P. sojae* and proposed that *P. sojae* changed from biotrophy to nectrophy 12-24 hours after infection. The microarrays were constructed from a collection of available cDNAs from ESTs of *P. sojae* and soybean.

Since the genomic sequence of *P. cinnamomi* is not known, the microarrays were constructed by arraying cDNA clones from a cDNA library produced from phosphite-treated mycelium of *P. cinnamomi* (Chapter 3). The aim of this study was to detect transcriptional events that take place in response to treatment of *P. cinnamomi* with phosphite using custom-made cDNA microarrays containing the 9216 cDNA transcripts prepared in the previous study (Chapter 3). Six arrays were hybridised with two different samples from control and phosphite-treated mycelium to identify changes in gene expression induced by phosphite.
5.2 Materials and methods

*P. cinnamomi* mycelium was grown in Ribeiro’s medium with and without phosphite (40 µg/ml) for 4 d at 25 °C (Chapter 2, section 2.2.2). RNA was extracted from the mycelium using a Qiagen RNeasy plant mini kit (Cat. No. 74903) with an on-column DNase digestion (Cat. No. 79254) according to the manufacturer’s instructions. The quality and quantity of RNA was assessed on Agilent 2100 Bioanalyser (Australian Genome Research Facility, Victoria) using the RIN (RNA Integrity Number) software algorithm. In this way the ratio of the ribosomal bands (28S/18S) and RNA concentration could be determined. The quality of RNA based on the entire electrophoretic trace of the sample provided a visualization of the presence or absence of degradation products.

5.2.1 Microarray slides

The microarrays consisted of 9216 cDNAs printed onto amine-coated microarray slides (Corning GAPSII) with a VersArray ChipWriter Pro system (BioRad, USA) using 16 tungsten pins (PointTech, Costa Rica). The constitutively expressed genes encoding *EF-1 alpha* and cinnamomin (Chapter 4) were positioned in arraying plates so that both would be spotted by each of the 16 pins and distributed across the whole array as positive controls. The printing, probe preparation, hybridizations and data analyses were performed by the Adelaide Microarray Centre (South Australia). The construction of the microarray experiment is illustrated in Figure 5.1.
Figure 5.1: Schematic representation of the microarray experiment. The cDNA from 384-well plates was printed on a microarray slide with a print head of 16 pins in a 4 x 4 array. Probes prepared from treated and untreated mycelium were labelled with fluorescent dyes Cy3 and Cy5 and hybridised to the array consisting of 9216 cDNA transcripts. The hybridised slide was scanned simultaneously at 543 nm (Cy3) and 633 nm (Cy5) and the image was analysed to calculate the relative expression levels of each transcript (Yang and Speed 2003).

5.2.2 cDNA synthesis and indirect dye coupling

The cDNA probes were synthesised from 20 µg total RNA (in 20 µl) mixed with anchored polyT (V) N (2 µg/µl) and 0.5 µl random primers (2 µg/µl). The reaction was incubated at 70 °C for 10 min and then chilled on ice. Reverse transcription was performed using 2 µl Superscript II (200 U/µl), 6 µl 5x Superscript II buffer, 2 µl 0.1 M DTT and 0.6 µl aminoallyl (aa) dNTP mix (25 mM dATP, 25 mM dGTP, 25 mM dCTP, 10 mM dTTP and 15 mM aa dUTP) incubated at 42 °C for 2.5 h. Following the cDNA synthesis the RNA was hydrolysed by adding 10 µl of 0.5 M EDTA (pH 8) and
10 µl 0.25 M NaOH, and incubating at 65 °C for 15 min. Then, 15 µl 0.2 M acetic acid was added to neutralise the reaction. Unincorporated nucleotides were removed from the cDNA using a Qiaquick PCR purification kit (Qiagen, Cat. No. 28104). Briefly, the cDNA was mixed with 300 µl of buffer PB, then applied to the column and centrifuged at 6500 g for 1 min. The eluent was re-passed through the column. The column was washed twice with 600 µl buffer PE and the residual buffer was removed by spinning the column at 6500 g for 1 min. The sample was eluted in 90 µl of Milli-Q water. The purified cDNA was dried under reduced pressure and then dissolved in 9 µl 0.1 M NaHCO₃ (pH 9) in preparation for fluorescent dye coupling. Cy dyes (Amersham, Cy3 PA23001, Cy5 PA25001) were dissolved in 45 µl anhydrous DMSO and 4.5 µl aliquots of the dye were distributed into tubes. Each aliquot was dried under reduced pressure, sealed and then stored in a desiccator at -20 °C. The cDNA was added and mixed with the appropriate Cy dye aliquot and then left to incubate at room temperature for 60 min in the dark. Following the dye coupling, the labelled cDNA was mixed with 39 µl of Milli-Q water, 3 µl 0.2 M acetic acid and then purified using a Qiaquick PCR purification kit as described above, with the exception of the cDNA sample being mixed with 250 µl of buffer PB.

5.2.3 Hybridisation and washing

Prior to hybridisation the slides were incubated in a container filled with pre-warmed blocking solution (10 mg/ml BSA, 25 % formamide, 5x SSC, 0.1 % SDS) for 1 h at 42 °C. The slides were rinsed three times in filtered Milli-Q water and dried by centrifugation at 50 g for 5 min. 20 µl of Cot 1 DNA (1 mg/ml), 4 µl of Poly dA (2 mg/ml) and 0.64 µl of Yeast tRNA (25 mg/ml) was added to the labelled cDNA to
reduce non-specific binding. The cDNA probe mix was dried under reduced pressure and resuspended in 14 µl of formamide and 14 µl of 6.25x SSC and then heat denatured at 100 °C for 3 min. Following cooling on ice, 0.2 µl of 10 % SDS was added to the mix prior to the solution being applied to the center of a 50 x 24 mm cover slip. The hybridisation mixture was sandwiched between the array and the cover slip by carefully lowering the slide onto the cover slip, then inverting the slide. The array was incubated at 42 °C for 16 h in a hybridisation chamber (Corning #2551) which was protected from light using aluminium foil. After hybridisation, the slides were removed using forceps and immersed in 0.5x SSC and 0.01 % SDS until the cover slip slid off. The slide was washed in 0.5x SSC and 0.01 % SDS for 1 min, 0.5x SSC for 3 min, 0.06 x SSC for 3 min and briefly rinsed in deionised water. Following the final wash the slide was dried in a centrifuge at 50 g for 5 min and stored in the dark prior to scanning.

5.2.4 **Scanning**

The slides were scanned at 10 µM resolution with a Genepix 4000B Scanner (Molecular Devices, USA). The detector PMT voltages were adjusted individually for each slide so that the total red (Cy5) and green (Cy3) fluorescence signals for each channel were approximately equal. Typical settings were 550V for the Cy3 channel and 700V for the Cy5 channel.

5.2.5 **Computational methods**

Foreground and background mean pixel intensity values were extracted from the scanned images for both channels (Cy3, Cy5) using the Spot v3 plugin (CSIRO, Australia) within the R statistical software package (<http://www.R-project.org>). After
background subtraction, the foreground intensities were log2 transformed and a single ratio (Cy5/Cy3) value was obtained for each probe. The ratio values were normalised within the Limma plugin (WEHI, Australia) for R using the Print tip-Loess normalisation routine (Smyth and Speed 2003). This intensity-based normalisation procedure assumes that a large percentage of the probes are not differentially expressed. The replicate arrays were normalised to each other to give similar ranges of mRNA expression values. For each probe across the arrays a linear model was fitted to determine a final expression value for each mRNA probed and associated statistics (Smyth 2004). These statistics were used to rank the mRNA’s from those most likely to be differentially expressed to the least likely.

5.3 Results

Hybridisation to the microarrays consisting of 9216 cDNA transcripts from the mycelium of phosphite-treated P. cinnamomi was repeated six times to assess the reproducibility of the results with six biological replicates of RNA extracted from the phosphite-treated (40 µg/ml) mycelium and five RNA samples from the untreated mycelium (RNA sample MK2 was used twice) (Table 5.1). Samples with RNA integrity number (RIN) in the region of 8-10 or 260/280 nm ratio above 1.8 were used for probe preparation. RIN score is calculated automatically by the Bioanalyser software, which sometimes fails to give a value.
Table 5.1: The assessment of RNA samples from *Phytophthora cinnamomi* mycelium cultured with (+P) or without (-P) phosphite (40 µg/ml) as determined on an Agilent 2100 Bioanalyser.

<table>
<thead>
<tr>
<th></th>
<th>RNA conc. µg/µl</th>
<th>rRNA ratio 28S/18S</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>-P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK1-0</td>
<td>0.73</td>
<td>1.7</td>
<td>-</td>
</tr>
<tr>
<td>MK2*</td>
<td>1.32</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>MK2-0</td>
<td>3.75</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>MK3</td>
<td>1.06</td>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td>MK4</td>
<td>1.69</td>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>+P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK1-4</td>
<td>0.96</td>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>MK2-4</td>
<td>1.01</td>
<td>2.3</td>
<td>10</td>
</tr>
<tr>
<td>MK5</td>
<td>1.64</td>
<td>2.2</td>
<td>10</td>
</tr>
<tr>
<td>MK6</td>
<td>1.50</td>
<td>2.4</td>
<td>10</td>
</tr>
<tr>
<td>MK7</td>
<td>0.61</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>MK8</td>
<td>2.59</td>
<td>2.0</td>
<td>10</td>
</tr>
</tbody>
</table>

28S/18S > 1.8 is considered high quality RNA; RIN (RNA integrity number) value ranges from 1-10. A value of 10 represents the most intact RNA.

*MK2 was used in two hybridisations.

The RNA with lower rRNA ratio (MK1-0 and MK1-4) was not necessarily of poor quality because the electrophoretic trace showed no degradation products and/or the RIN equalled 10. The most distinct features on the electropherograms of all the RNA samples were the 18S and 28S ribosomal peaks, which run at approximately 43 s and 49 s (Figure 5.2).
Chapter 5: Construction of cDNA microarrays from phosphite-treated *P. cinnamomi*

Figure 5.2: Electrophoretic separation and laser induced fluorescence (FU) detection of a RNA sample used for the probe preparation in the microarray experiments. The lower peak, which ran at 41-45 s, corresponds to 18S RNA and the higher peak at 47-50 s, corresponds to 28S RNA. No degradation products were evident.

All RNA samples were reverse transcribed to single stranded cDNA, purified and labelled with Cy3 and Cy5, respectively. The labelled cDNA samples appeared purple after being dried under reduced pressure, indicating good incorporation of Cy3/Cy5 fluors to the cDNA. Probes synthesised from RNA from phosphite-treated (40 µg/ml) and untreated mycelium were hybridised to each of the six arrays. These were then scanned to detect fluorescence intensities of the dye bound to each cDNA hybridised to a complementary sequence of DNA deposited on the surface of the slide.

A variety of graphical displays were used to represent and evaluate the slide data. These displays assisted in deciding whether the experiment was successful and helped to identify experimental problems and choose the correct analysis tools.

Overlapping the scanned outputs from Cy3 and Cy5 channels offered a quick visualisation of the microarray experiment in regards to uniformity of hybridisation, background fluorescence, colour balance, spot regularity and artifacts such as dust and
scratches (Figure 5.3). Overall, all six hybridised arrays exhibited a uniform hybridisation with a low background fluorescence, and only two of the slides had slight scratches on the array edges. The composite image also provided a rough estimate of the number of transcripts (> 100) that were differentially expressed. Transcripts equally expressed between the two samples were represented by yellow spots in the composite image, or shades of green or red if differentially expressed (Figure 5.3). To filter out false positives the dye assignments were reversed between the two probes for half the slides.

Apart from this, many of the spots exhibited low signal intensity and the presence of ‘doughnut’ shaped spots on the arrays was also revealed. The central intensities of doughnuts are lower than the periphery intensity values, making it harder to extract the correct fluorescence intensities, but the problem can be addressed with segmentation logarithms such as Spot. The Spot v3 plugin for R software package was used to obtain quantitative quality scores and differences in expression. The raw intensities obtained from the image analysis program were transformed and expressed as values to the log base 2 before further analysis. This allowed a more even spread of the values across the range and decreased the variability.
Figure 5.3: A two-colour cDNA microarray. The spots on the slide appear in the form of 16 subarrays (pin groups) of 625 spots each. Yellow spots represent transcripts with equal expression in both treatments, red spots correspond to up-regulated transcripts and green spots correspond to down-regulated transcripts (and vice versa when dyes were exchanged) in phosphite-treated mycelium.
Box plots were used to display a statistical summary consisting of the median, upper and lower quartiles, the range and possibly extreme values of the data distribution for all slides (Figure 5.4). The second slide, mic002 was noticed as having a larger interquartile range (IQR; difference between the upper and lower quartile) compared to the rest of the slides, and the data in slide mic003 and mic006 were compressed (Figure 5.4 A). Based on this initial plot, it was decided to also normalise between the slides. After post array normalisation the profiles showed the spread of the data (M values) more equally distributed, with all six arrays having log ratios with the same centres, at zero, and the same scales (Figure 5.4 B).

**Figure 5.4:** Distribution of the array data represented by M box plots post within-array normalisation (A) and M box plots post between-array normalisation (B). The central box in a plot contains the values between the upper quartile (75th percentile) and lower quartile (25th percentile) and represents the middle 50% of the data. The line in the middle of the box (median) represents a measure of central location of the data. Extreme values were plotted individually. Y-axis: M (log₂ R/G)

Transcripts with potentially strong differential expression were depicted by spots with extreme values (log ratios). The narrow size of the central box (small IQR) in the plot after post array normalisation indicated little background noise in the experiment.
The effectiveness of normalisation was also illustrated by raw data plots (Figure 5.5). These plots showed the red and green channel histograms (RG) for all of the arrays before any normalisation (i.e. the dynamic range). Plot densities graphs focus on the individual red (R) and green (G) intensity values rather than merely on the log intensity. Since it is assumed that most transcripts show no differential expression, the curves are expected to approximately overlap. The red and green profiles for some slides matched as they exhibited the same shape of profile, but the red and green curves were offset when raw. Within array normalisation made the red and green curves overlap, and between array normalisation made each of sets of RG curves overlap.

![RG densities](image1)

**Figure 5.5:** Plot densities graph of Red (R) and green (G) densities (raw data) (A) and RG densities (after normalisation) (B). Without normalisation there was considerable variation between both red and green channels and between arrays. After normalisation of the M-values for each array the red and green distributions became essentially the same.

MA plots (a plot of log-ratio of two expression intensities versus the mean log-expression of the two) were constructed to display the fold-change in gene expression of all the gene transcripts across the 6 slides (Figure 5.6). The transcripts that were not differentially expressed had their M values close to zero across the intensity range.
The differentially expressed transcripts hovered either above, for up-regulated transcripts, or below this range, for down-regulated transcripts.

**Figure 5.6:** MA-plot of an average of all the transcripts in the six experiments. The log ratios of red and green channels are plotted on the y-axis against the log of the mean of the signal strengths (overall intensity) for each spot on the slide. $M = \log_2 (\text{Red/Green})$ (i.e. the log differential expression ratio); $A = \log_2 [(R+G)/2]$ i.e. (the log intensity of the spot, a measure of overall brightness of the spot).

Expression profile of the *EF-1 alpha* (blue) and cinnamomin (red) encoding genes clearly fitted in the zero expression range, while the most differentially expressed transcripts appeared to display a change in expression up to 16-fold for up-regulation and to almost 4-fold for down-regulation. Linear modelling was used to statistically
analyse the normalised data to assess the fold-change in expression of all the transcripts on the array, and ranked the transcripts from the most likely differentially expressed to the least. Each transcript had a B-value (log odds score) calculated, where B = 0 defined a 50% chance that the transcripts is differentially expressed by chance alone. The more positive a B-value, the more likely a transcript is truly differentially expressed. This is illustrated in the volcano plot, or the log odds plot (Figure 5.7). The transcripts which show the most evidence of differential expression are high up and towards the left and right of the plot. The data clearly shows that the significant up-regulated transcripts (to the right - positive M values) are greater in magnitude (e.g. PC_7010, PC_5726).

Figure 5.7: The log odds (volcano) plot displayed the log odds of differential gene expression and revealed the outliers among thousands of transcripts. These outliers represented the most likely differentially expressed transcripts (in blue). Log odds correspond to a given value of the statistics B vs the estimated fold-change on a log scale M.
After data normalisation and statistical analyses were performed, 72 differentially expressed transcripts with a fold-change of \( \geq 2 \) were identified (Table 5.2).

**Table 5.2:** Summary of the statistical analyses of 72 differentially expressed transcripts and 2 constitutively expressed genes listed in descending order. Ranking is done by B-statistics to account for variations in M-values.

<table>
<thead>
<tr>
<th>Name</th>
<th>M</th>
<th>Fold Change</th>
<th>Expression</th>
<th>A</th>
<th>t</th>
<th>P-Value</th>
<th>B</th>
</tr>
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<tbody>
<tr>
<td>MK_PC_7010</td>
<td>3.06</td>
<td>8.32</td>
<td>Up</td>
<td>10.92</td>
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<td>8.16</td>
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<tr>
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<td>2.12</td>
<td>4.35</td>
<td>Up</td>
<td>9.66</td>
<td>14.23</td>
<td>0.00</td>
<td>7.96</td>
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<td>MK_PC_7722</td>
<td>1.64</td>
<td>3.13</td>
<td>Up</td>
<td>11.13</td>
<td>13.24</td>
<td>0.00</td>
<td>7.42</td>
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<tr>
<td>MK_PC_2954</td>
<td>3.12</td>
<td>8.7</td>
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<td>0.00</td>
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<td>12.73</td>
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<tr>
<td>MK_PC_8935</td>
<td>2.95</td>
<td>7.72</td>
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<td>12.45</td>
<td>0.00</td>
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<tr>
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<td>3.56</td>
<td>Up</td>
<td>11.54</td>
<td>12.37</td>
<td>0.00</td>
<td>6.91</td>
</tr>
<tr>
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<td>Up</td>
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<td>11.59</td>
<td>0.01</td>
<td>6.41</td>
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<td>Up</td>
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Table 5.2: Cont. Summary of the statistical analyses of 72 differentially expressed transcripts and 2
costitutively expressed genes listed in descending order. Ranking is done by B-statistics to account
for variations in M-values.

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<th>Z-score</th>
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Constitutively expressed genes

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Table 5.2: Cont. Summary of the statistical analyses of 72 differentially expressed transcripts and 2 constitutively expressed genes listed in descending order. Ranking is done by B-statistics to account for variations in M-values.

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<th>B-statistic</th>
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<td>11.91</td>
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</table>

M- the log differential expression ratio  
A- the log intensity  
t- is the moderated t-statistics  
P-value is the associated P-value after adjustment for multiple testing  
B-statistic is the log-odds that the transcript is differentially expressed  
* constitutive expression  

Provide an overall ranking of transcripts in order of evidence for differential expression.
5.4 Discussion

Custom cDNA microarrays consisting of over nine thousand cDNA transcripts, prepared from the cDNA library of phosphite-treated *Phytophthora cinnamomi*, were successfully constructed. Data extracted from the microarrays hybridised with probes from phosphite-treated and untreated mycelium were normalised to account for the systematic variations that could affect the measurement of gene expression. Seventy-two differentially expressed transcripts with a fold change of \( \geq 2 \) were identified. The majority of the transcripts were down-regulated with changes in transcription ranging from 3.5- to 2-fold. Since the arrays were made with cDNAs arising from phosphite-treated mycelium, it must be taken into consideration that the strongly down-regulated genes were absent from the arrays. The transcripts that were up-regulated exhibited the highest changes in gene expression ranging from 2- to 16-fold and were also the most likely differentially expressed transcripts (high B-value).

RNA from independent preparations, where each RNA extraction was performed from different cultures of *P. cinnamomi* isolate MP 80, was used for the preparation of probes. The experiment was repeated six times to reduce the variability by averaging, as cDNA microarrays are rather noisy at the individual gene level (Yang and Speed 2003) and to allow the use of statistical methods (Dudoit *et al.* 2002). Each microarray was analysed with probes labelled with Cy3 and Cy5, respectively. As the intensities of Cy5 tend to be higher than the Cy3 intensities, due to different biochemical properties of the two dyes (Amaratunga and Cabrera 2004), the dye assignments were reversed between the probes for half of the slide.
Weak spot intensities and irregular spot morphology were observed on the printed slides and included “doughnut” shaped spots, all very common in microarray experiments (Yang et al. 2001; Yang and Speed 2003). Poor retention of spotted DNA as well as under-estimation of transcript abundance were the likely cause of weak signal intensities, while the formation of irregular spots was likely due to the printing process (e.g. features of the print tips) and effects of the post-processing of the slides (sample evaporating too quickly after spotting). This problem was addressed during the image analysis using the software Spot (CSIRO, Australia) to cope with shapes other than circles.

Spatial position of the spots and the physical differences between the print tips, such as the size of the opening or the amount of wear of individual pin tips contribute to the variation in dye balance in the array (Ball et al. 2003). It was therefore essential to make adjustments for biases within pin groups. Print-tip Loess normalisation (WEHI, Australia) corrected for both sub-array spatial variation and for intensity-based trends, by subtracting M-values from the corresponding value of the tip group loess curve. This method is recommended as a routine normalisation method for cDNA arrays.

Before the gene expression profiles of the RNA samples were analysed and interpreted, the red and green fluorescent intensities were normalised to identify transcripts that were truly differentially expressed. The purpose of normalisation was to adjust for effects resulting from variation in the microarray technology rather than from biological differences between the RNA samples. Differences between arrays may arise from varying amounts of arrayed DNA on slides, RNA preparation, reverse transcription bias, labelling efficiency, hybridisation efficiency, saturation effects and background fluorescence (Ball et al. 2003).
Both within and between slides normalisations were performed as there were substantial differences between microarrays (Figure 5.4). This prevented the slides whose values had the greatest spread contributing most to averages. Rather than using a single gene or a set of genes such as constitutively expressed genes, data from the whole array was used for normalisation and the ratio intensity was calculated for every spot using the basic assumption that the majority of spots were not differentially expressed. This allowed the normalisation to center the array distribution at zero. Nominated controls such as constitutively expressed genes tend to be highly expressed and may not be representative of target genes with lower expression levels. Despite this, two constitutively expressed genes, EF-1 alpha and cinnamomin genes, were scattered across each array to serve as positive controls to verify that the probes were labelled to an acceptable specific activity by Cy3 and Cy5 and to ensure the specificity of hybridisations. The constitutively expressed genes performed well in all six experiments and showed no significant changes in expression in the two experimental treatments with ratio of the test and reference samples close to unity (1-fold).

Finally, summary statistics were performed with LIMMA (Linear Models for Microarray Analysis), one of the most commonly used packages for normalisation and statistical analysis for cDNA microarrays. The central idea is to fit a linear model to the expression data for each gene transcript and the results are expressed as (log) fold changes, standard errors, t-statistics and p-values. The basic statistic used for significance analysis is the moderated t-statistic, which is computed for each probe. The statistics allowed a ranking process of most likely expressed transcripts.
In conclusion, differences in gene transcription have been identified in the phosphite-treated mycelium by this study, the first large scale screening of phosphite induced gene expression and function analysis in *P. cinnamomi*. Genes encoding the phosphite regulated transcriptome (72) were sequenced and analysed to gain an insight into putative biological function (Chapter 6).
Chapter 6

Characterisation of differentially expressed genes from phosphite-treated mycelium of *Phytophthora cinnamomi*.

6.1 Introduction

The production of microarrays and the generation of gene expression profiles of the transcripts on hybridised arrays are only the first steps in understanding the data obtained by microarray experiments. The challenge of microarrays is the analysis and interpretation of the data produced. The ultimate purpose of gene expression profiling is to obtain biological relevance from differential expression. This is first achieved through a process of data mining (Draghici 2003). Bioinformatics enables the translation of the vast amount of gene expression data generated by microarray analysis into biologically significant data using software algorithms.

The process of data mining assigns a function and putative role to each gene to help identify broad biological themes and provide information about the experiment as a whole. In this way, the data from microarray experiments can provide an insight into the cellular mechanisms acting in the given condition (Baldi and Hatfield 2002; Draghici 2003; Amaratunga and Cabrera 2004). An efficient interpretation of genome-scale data from microarray experiments, particularly from diverse sources, requires the use of controlled vocabulary, referred to as Gene Ontology (GO) (Ashburner *et al.* 2000). Gene Ontology enables the unification of terms used to describe genes across all organisms, distinguishing molecular function, biological process and cellular component. This allows the extrapolation of meaningful inferences about possible
interactions between the data obtained and information available in bioinformatics databases (Yang and Speed 2003).

Microarray data can then be organised into clusters of genes of similar covariance (behaviour). As microarrays usually display patterns of expression across multiple genes and experiments, gene clusters are often identified, which are enriched in genes involved in similar biological processes (Yang and Speed 2003).

The aim of the present study was to identify transcripts that displayed \( \geq 2 \)-fold change in transcription in the mycelium of phosphite-treated *Phytophthora cinnamomi*, as determined in the microarray experiments (Chapter 5), and verify the expression by qRT-PCR. Subsequently, the function and biological significance of these genes was established, leading to the elucidation of the possible mode(s) of action of phosphite and the identification of cellular sites of significance within the pathogen.

### 6.2 Materials and Methods

#### 6.2.1 Sequencing of phosphite-regulated genes from *P. cinnamomi*

Twenty randomly selected transcripts identified with a significant fold-change (\( \geq 2 \)) in expression were located within the 384-well arraying plates using the unique ID name given to each cDNA clone (Chapter 3). The cDNA, lyophilised for safe transportation from the Adelaide Microarray Centre (South Australia) to Murdoch University (Western Australia), was used as a template for the sequencing reactions. The relevant PCR products were resuspended in sterile water to a final concentration of 25 ng/\( \mu l \) DNA and 2 \( \mu l \) aliquots were submitted for sequencing in the West Australian Genome Resource
Centre (Royal Perth Hospital). The DNA sequences were generated using the M13 Reverse primer 5’-ggAAACAgCTATgACCATg-3’ (Geneworks) and were compared by the basic local alignment search tool (BLAST) to the sequences available in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and the Joint Genome Institute database (JGI, http://www.jgi.doe.gov/), and the closest ortholog was assigned to each transcript to elucidate its putative function. The pattern of expression across the transcripts was graphically illustrated using the Comprehensive R-based Microarray Analysis web service (CARMAweb) (Rainer et al. 2006).

6.2.2 Validation of differential gene expression by qRT-PCR

The expression of 10 transcripts that showed high, medium and low level of response to phosphite was confirmed by qRT-PCR. Two independent RNA extractions used for the microarray probe preparation from phosphite-treated and untreated mycelium were used to synthesise cDNA as described in Chapter 4 (section 4.2.3). Changes in gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), where the variation in transcription between the two treatments was normalised relative to the reference gene, EF-1 alpha, for each target gene (Chapter 4, section 4.2.5).

6.3 Results

Several genes displayed changes in expression in response to phosphite in the range of 2- to 16-fold. Sequence analysis and database alignment of 20 insert sequences resulted in the identification of 17 putative transcripts of which nine encoded a unique class of protein product. Three of the nine were represented by at least two Expressed Sequence Tags (ESTs) (Table 6.1). All of the genes had significant matches to P. sojae or P.
sequences. Three out of the 20 insert ESTs encoded proteins of unknown function.

Two ESTs (clone 5726 and 7942) exhibited homology to a gene encoding adenosine diphosphate-ribosylation factor (ARF), indicating a potential role in the regulation of vesicle biogenesis involved in intracellular traffic (Protein ID 108226) (Table 6.1).

Sequence alignment revealed 100% similarity between the two sequences.

Table 6.1: Most closely related orthologs corresponding to the differentially expressed transcripts in the mycelium of phosphite-treated Phytophthora cinnamomi, as identified in the microarray experiments. Ranking was performed according to the fold-change in gene expression.

<table>
<thead>
<tr>
<th>Fold-change</th>
<th>Clone ID</th>
<th>Closest putative ortholog</th>
<th>Protein ID (JGI)/ Accession No* (NCBI)</th>
<th>E-value</th>
<th>Homology (aa/aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.27</td>
<td>5726</td>
<td><em>Ps</em> ADP-ribosylation factor (pm.C_30015)*</td>
<td>108226</td>
<td>e-110</td>
<td>166/190</td>
</tr>
<tr>
<td>8.96</td>
<td>7942</td>
<td><em>Ps</em> ADP-ribosylation factor (pm.C_30015)*</td>
<td>108226</td>
<td>9e-37</td>
<td>59/59</td>
</tr>
<tr>
<td>8.70</td>
<td>2954</td>
<td><em>Ps</em> ADP-ribosylation factor (C_scaffold_77000006)</td>
<td>158029</td>
<td>7e-25</td>
<td>43/53</td>
</tr>
<tr>
<td>8.32</td>
<td>3010</td>
<td><em>Ps</em> glycosyl transferase (pm.C_100009)*</td>
<td>108450</td>
<td>2e-19</td>
<td>38/62</td>
</tr>
<tr>
<td>6.66</td>
<td>3477</td>
<td><em>Ps</em> ABC transporter (pg.C_140062)*</td>
<td>131064</td>
<td>8e-13</td>
<td>26/43</td>
</tr>
<tr>
<td>5.83</td>
<td>3016</td>
<td><em>Ps</em> ADP-ribosylation factor (C_scaffold_77000006)</td>
<td>158029</td>
<td>7e-25</td>
<td>43/53</td>
</tr>
<tr>
<td>5.56</td>
<td>37</td>
<td><em>Ps</em> ADP-ribosylation factor (C_scaffold_77000006)</td>
<td>158029</td>
<td>5e-19</td>
<td>36/46</td>
</tr>
<tr>
<td>3.24</td>
<td>6729</td>
<td><em>Ps</em> estExt_fgenesh1_pg.C_390010</td>
<td>135523</td>
<td>9e-43</td>
<td>71/90</td>
</tr>
<tr>
<td>3.19</td>
<td>6809</td>
<td><em>Ps</em> estExt_fgenesh1_pg.C_160188</td>
<td>131644</td>
<td>2e-47</td>
<td>53/68</td>
</tr>
<tr>
<td>2.99</td>
<td>5685</td>
<td><em>Ps</em> unknown protein</td>
<td>AAO24647.1*</td>
<td>0.002</td>
<td>43/58</td>
</tr>
<tr>
<td>2.92</td>
<td>9173</td>
<td><em>Ps</em> vit. B6 bio. protein (C_scaffold_10000105)</td>
<td>156086</td>
<td>3e-18</td>
<td>37/43</td>
</tr>
<tr>
<td>2.54</td>
<td>9188</td>
<td><em>Pi</em> glutamine synthetase</td>
<td>AAN31463.1*</td>
<td>0.012</td>
<td>21/23</td>
</tr>
<tr>
<td>2.24</td>
<td>9162</td>
<td><em>Ps</em> cellulose synthase 1</td>
<td>ABP96906.1*</td>
<td>6e-21</td>
<td>59/59</td>
</tr>
<tr>
<td>2.18</td>
<td>3225</td>
<td><em>Pi</em> glutamine synthetase</td>
<td>AAN31463.1*</td>
<td>6e-12</td>
<td>38/40</td>
</tr>
<tr>
<td>2.16</td>
<td>1028</td>
<td><em>Ps</em> cellulose synthase 1</td>
<td>ABP96906.1*</td>
<td>6e-74</td>
<td>171/175</td>
</tr>
<tr>
<td>2.12</td>
<td>7099</td>
<td><em>Ps</em> cellulose synthase 1</td>
<td>ABP96906.1*</td>
<td>4e-71</td>
<td>145/147</td>
</tr>
<tr>
<td>2.09</td>
<td>5694</td>
<td><em>Ps</em> metallothionein (pg.C_1280011)*</td>
<td>143057</td>
<td>9e-59</td>
<td>89/103</td>
</tr>
<tr>
<td>2.02</td>
<td>391</td>
<td><em>Ps</em> metallothionein (pg.C_1280011)*</td>
<td>143057</td>
<td>9e-59</td>
<td>89/103</td>
</tr>
<tr>
<td>2.01</td>
<td>2043</td>
<td><em>Ps</em> annexin (C_scaffold_7000098)*</td>
<td>155769</td>
<td>8e-47</td>
<td>76/86</td>
</tr>
<tr>
<td>2.00</td>
<td>6930</td>
<td><em>Ps</em> alternative oxidase (pm.C_15460001)*</td>
<td>109753</td>
<td>5e-50</td>
<td>74/97</td>
</tr>
</tbody>
</table>

*Ps* - Phytophthora sojae, Clone ID MK_PC_number, * Protein Name (JGI) preceeded by estExt_fgenesh1_ …

Pi – Phytophthora infestans

Expression (+)
The clones 2954, 7010 and 37 (with a fold-change ranging from 8.7 to 3.6) also aligned with the protein sequence of ADP-ribosylation factor, albeit a different DNA sequence, suggesting that multiple ribosylation factors are required in phosphite response in *P. cinnamomi*.

As the cDNA clones 2954 and 7010 shared exactly the same extent of homology (44/56) to *P. sojae* ADP-ribosylation factor (Protein ID 158029) and almost identical change in fold-expression (8.7 and 8.3-fold, respectively) it is likely that they are duplicate copies of the same transcript on the microarray. This was also confirmed by sequence alignment, which revealed 100% identity of the cDNA sequence of clone 2954 to clone 7010. The cDNA clone 37 exhibited 99% identity to the two clones (clone 2954 and 7010) described above (Table 6.1).

Among the highly regulated phosphite-responsive genes was an ATP-binding cassette transporter gene (ABC transporter; clone 3477), which displayed a 6.7-fold change in expression, and a gene encoding glycosyl transferase (clone 3010, 5.8-fold up-regulation), which may be involved in protein metabolism (Protein ID 108450). Both were represented by a single EST. The least up-regulated (2.9-fold) gene of those identified as differentially expressed (Table 6.1) in response to phosphite corresponded to the gene encoding Vitamin B6 biosynthesis protein (clone 9173).

Six transcripts exhibited down-regulation in gene-expression in the range of 2- to 3.2-fold in the presence of phosphite (Table 6.1). Of these, one aligned most closely with a gene coding for annexin (clone ID 2043), whose putative function constitutes calcium ion binding and inhibition of some phospholipases (Protein ID 155769). Four ESTs (clone 9126, 1028, 7099 and 391) exhibited homology with the *CES1* gene encoding
cellulose synthase 1, with three clones aligning to different sections of the CESI gene (Figure 6.1).

![DNA alignment of four ESTs to P. sojae CESI gene. When aligned to the CESI consensus sequence, clone 7099 (nucleotides 1923-2368) was up-stream of the clone 1028 (nucleotides 2399-2926). Clones 9162 (nucleotides 2506-2738) and 1028 aligned with the same section of the consensus sequence CESI, while clone 1028 also overlapped with clone 391 (nucleotides 2831-3071).](image)

All four transcripts were down-regulated (2.04-2.24-fold) in response to phosphite treatment.

Other ESTs corresponded to P. infestans clone MY-01-A-07 encoding glutamine synthetase (clone 9188 and 3225), a gene encoding for metallothionein (clone 5694) and a gene encoding for an alternative oxidase (clone 6930). The latter is potentially involved in respiratory gaseous exchange (protein ID 108593).

6.3.1 Validation of differential gene expression by qRT-PCR

The expression of eight differentially regulated genes and two transcripts that aligned with proteins of unknown function identified from microarray analysis was confirmed using qRT-PCR (Table 6.2). The expression profiles generated by the qRT-PCR method mimicked the trend observed from the microarray hybridisation approach.
Table 6.2: Validation of expression of the putative genes/transcripts that showed high, medium and low level of response to phosphite by qRT-PCR.

<table>
<thead>
<tr>
<th>ID</th>
<th>Ortholog</th>
<th>Forward primer (5' - 3')</th>
<th>Reverse Primer (5' – 3')</th>
<th>Amplification Efficiency (%)</th>
<th>Coefficient factor (R)</th>
<th>Fold-change* ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK_PC_5726</td>
<td>ADP-ribosylation factor</td>
<td>TACCTgTgCAACAACgACgC</td>
<td>AAgATCAggTgCAgCgCC</td>
<td>97.0</td>
<td>0.995</td>
<td>47.37 ± 9.54</td>
</tr>
<tr>
<td>MK_PC_7010</td>
<td>ADP-ribosylation factor</td>
<td>TCACATATCACgAAGACAgTggC</td>
<td>TgACACACACgCTACTTgCC</td>
<td>104.0</td>
<td>0.999</td>
<td>399.49 ± 43.48</td>
</tr>
<tr>
<td>MK_PC_3477</td>
<td>ABC transporter</td>
<td>gCAAggTggCATACTACTgCgC</td>
<td>TCCAgATCCgAgAAGAgCgC</td>
<td>99.3</td>
<td>0.999</td>
<td>64.56 ± 8.08</td>
</tr>
<tr>
<td>MK_PC_3010</td>
<td>Glycosyl transferase</td>
<td>AgCAAAAgCgCACAACgTg</td>
<td>CgAAgAAgAgCgTCAATCCg</td>
<td>97.3</td>
<td>0.993</td>
<td>12.89 ± 2.37</td>
</tr>
<tr>
<td>MK_PC_9188</td>
<td>Glutamine synthetase</td>
<td>CAACgAggCCTAAgTggTCg</td>
<td>AAgCATATATAggAACCACg</td>
<td>102.7</td>
<td>1.000</td>
<td>- 3.68 ± 0.78</td>
</tr>
<tr>
<td>MK_PC_1028</td>
<td>Cellulose synthase</td>
<td>CAAgTggCTCAACAAgCgTg</td>
<td>gACgATgCCAAACACgC</td>
<td>105.3</td>
<td>0.999</td>
<td>- 3.50 ± 0.30</td>
</tr>
<tr>
<td>MK_PC_2043</td>
<td>Annexin</td>
<td>CATCAAAgATgAggTCAgCgg</td>
<td>TgACTCTTACgCAAgACgCC</td>
<td>101.5</td>
<td>1.000</td>
<td>- 3.25 ± 0.37</td>
</tr>
<tr>
<td>MK_PC_6930</td>
<td>Alternative oxidase</td>
<td>ACAATCgCCATTCgCgC</td>
<td>TgTCgAggTCgAgACgC</td>
<td>103.8</td>
<td>0.999</td>
<td>- 2.44 ± 0.21</td>
</tr>
<tr>
<td>MK_PC_6729</td>
<td>Unknown protein</td>
<td>gCTAgTACgAggTCATgCgTCg</td>
<td>TgATAggCgCATTTgCTgC</td>
<td>101.0</td>
<td>0.997</td>
<td>12.70 ± 1.34</td>
</tr>
<tr>
<td>MK_PC_5685</td>
<td>Unknown protein</td>
<td>AgAACAAgAACTgCAACAgCTgC</td>
<td>TgTgCgACCAACAgTgTg</td>
<td>96.1</td>
<td>0.999</td>
<td>5.35 ± 0.47</td>
</tr>
<tr>
<td>Reference gene</td>
<td><em>EF1-alpha</em></td>
<td>CgAgAAGAgTCgggCAACATgC</td>
<td>TgTgCAACACgggCgC</td>
<td>104.6</td>
<td>0.995</td>
<td>constitutive</td>
</tr>
</tbody>
</table>

*Fold-change represents a mean of 6 replicates averaged over 2 independent repeats of the experiment.
However, the magnitude of the differences in gene expression between phosphite-treated and untreated mycelium were much higher for some of the genes when determined by qRT-PCR compared to microarray analysis, which may be attributed to the saturation effects on the microarrays.

6.4 Discussion

This study has identified nine putative genes that were transcriptionally affected by phosphite in the mycelium of *P. cinnamomi* on the basis of EST annotation and functional analogies of differentially expressed genes from the microarray experiments. Interestingly, ESTs that matched the sequences of *P. sojae* also exhibited homology to sequences in the *P. ramorum* JGI database. Phosphite has recently been shown to be effective in preventing infection of oak trees by *P. ramorum* (Rizzo *et al.* 2005). The results of the present study may therefore be useful in helping to understand the mode of action of phosphite in other *Phytophthora* species, such as *P. ramorum*.

The length of the homologous region between the ESTs and the sequences in the databases was in some cases short (3477, 9188), which lessened the significance of the homology although the E-values remained relatively high. The alignment of all of the phosphite-regulated genes of *P. cinnamomi* to genes of the fully sequenced *Phytophthora* species, *P. sojae* and *P. ramorum* (JGI), was interesting. Most of the *P. cinnamomi* transcripts, with the exception of two ESTs (9188, 3225) having homology to *P. infestans*, showed a higher degree of similarity with *P. sojae* sequences. This is consistent with the molecular phylogenetic studies based on the ITS sequence of genomic rDNA (Crawford *et al.* 1996; Cooke *et al.* 2000), where *P. cinnamomi* and *P. sojae* have been ranked in the same clade.
The nine genes identified were grouped into functional categories (see below) according to the products they encode, however, most are likely to play a role in more than one cellular process. Each of these genes will be discussed according to their putative function.

### 6.4.1 Cell wall biosynthesis and pathogenicity

The vegetative mycelium of *Phytophthora* consists of coenocytic hyphae (Erwin and Ribeiro 1996). Apart from providing rigidity to hyphae for structural support, the cell wall also provides protection from mechanical stress. The cell walls of Oomycetes are constructed primarily from beta glucans and cellulose (Tokunga and Bartnicki-Garcia 1971; Rossman and Palm 2006). Therefore, the synthesis of these components is important in the morphogenesis and growth of these microorganisms (Bouzenzana *et al.* 2006).

Phosphite treatment of *P. cinnamomi* mycelium resulted in transcriptional suppression of a gene encoding cellulose synthase I. In its simplest form, cellulose synthase is capable of initiating, elongating and terminating glucan polymerisation in a reaction which involves glycosyl transferase in *Agrobacterium tumefaciens* (Saxena and Brown 2005). Most organisms produce crystalline cellulose defined as cellulose I. The cellulose is composed of linear polymer chains of β-1,4-linked residues, which are parallel to each other and are packed side by side to form microfibrils. This process consists of polymerisation catalysed by cellulose synthase which utilizes UDP-α-glucose as the substrate, and crystallisation (Saxena and Brown, 2005). The down regulation of *CES1* in the present study suggests that phosphite may interfere with the biosynthesis of major cell wall components of *P. cinnamomi*, which may affect the organisation of cell wall network and consequently transport of molecules across
the cell wall. This connects with the observation of a reduction in mycelial growth (70 %) by the phosphite level (40 µg/ml) used in the present study.

In contrast, the alterations in the cell wall organisation of *P. cinnamomi* by phosphite may result in the release of cell wall fragments and carbohydrates. These molecules could act as elicitors of defence responses in plants. This would suggest a direct effect of phosphite on the pathogen. Ellis *et al.* (2002) demonstrated that the *Arabidopsis* mutant cev1 plants (caused by mutation in cellulose synthase gene *CeSA3*) have reduced cellulose content in the roots and exhibited overproduction of jasmonic acid (JA) and ethylene. It was speculated that reduction of cellulose content in plant cell walls could result in the mediation of defence responses in plants (Ellis *et al.* 2002). It would therefore be useful to examine phosphite regulated gene expression of cellulose synthase genes in plants.

Interference of phosphite with the biosynthesis of the cell wall components of *P. cinnamomi* was further supported by the down-regulation of a gene encoding annexin. Bouzenza et al. (2006) have recently demonstrated that an annexin-like protein was an activator of the (1→3)-β-D-glucan synthase in *Saprolegnia monoica* (an oomycete), and suggested a novel role for annexins involving the biosynthesis of cell wall polysaccharides. The role of the annexin gene identified in the present study, however, could function in other cellular processes of *P. cinnamomi* and requires further investigation. Annexins belong to a family of structurally related calcium-dependent phospholipid binding proteins that play a role in lipid vesicle transport and mediation of intracellular calcium signals and some (lipocortins) inhibit the activation of phospholipase A₂ (Mollenhauer 1997). Annexins also play a role in scavenging reactive oxygen species *in planta* (Gidrol *et al.* 1996; Xiao *et al.* 2001).
It was revealed that phosphite down-regulated the transcription of a gene encoding glutamine synthetase. Glutamine synthetase plays a central role in amino-acid metabolism by catalysing the condensation of glutamate and ammonia to yield glutamine (Stephenson et al. 1997). This reaction is also essential for ammonia re-assimilation and de-toxification (Stephenson et al. 1997). Accordingly, suppression of the gene encoding glutamine synthetase may impair amino acid synthesis and metabolism and ammonia de-toxification in the mycelium treated with phosphite. This correlates with the elevated expression of a gene encoding Vitamin B6 biosynthesis protein. Vitamin B6 functions as an essential cofactor for enzymes involved in amino acid biosynthesis (Grogan 1988; Rottmann et al. 1991) and has been shown to be involved in the protection of phytopathogenic Cercospora fungi from the toxin, cercosporin (Denslow et al. 2007; Herrero and Daub 2007).

Transcriptional regulation of the glutamine synthetase gene by phosphite may also play a role in the pathogenicity of P. cinnamomi. Genes that are highly expressed or induced in the pathogen during the infection process may be required for disease development and therefore influence the compatibility in pathogen-host interactions (Pieterse et al. 1993; Hawthorne et al. 1994). Stephenson et al. (1997) described the cloning and expression of a gene encoding glutamine synthetase in the fungus Colletotrichum gloeosporioides. An elevated expression of glutamine synthetase occurred during disease development, particularly during the early stages of infection. The transcripts of the fungal glutamine synthetase were also induced by nitrogen starvation in axenic cultures. It may be worthwhile investigating whether the extent of transcriptional suppression of the glutamine synthetase gene in the mycelium of P. cinnamomi would be greater in nutrient limiting conditions (i.e. low phosphate medium) compared to high
phosphate medium in the absence of phosphite, and whether the glutamine synthase gene plays a role in the pathogenicity of *P. cinnamomi in planta*.

### 6.4.2 Glycosylation

In the present study, a gene coding for a glycosyl transferase, with a putative role in glycosylation, was up-regulated by almost 6-fold in phosphite-treated mycelium. In eukaryotes, it has been established that glycosylation occurs in endoplasmic reticulum (ER) and the Golgi apparatus and plays a role in protein folding and processing by sugar-sugar interaction or sugar-protein interaction (Cooper and Hausman 2004). In this process, the enzyme glycosyl transferase catalyses the transfer of a glycosyl group to an acceptor (e.g. lipids, proteins and nucleic acids). The pattern of glycosylation determines the final destination of the proteins in a cell. Proteins modified by mannose phosphorylation will leave the Golgi apparatus in transport vesicles that fuse with lysosomes. Proteins that do not receive this marker will be incorporated into the plasma membrane (e.g. integral membrane proteins and receptors) (Cooper and Hausman 2004).

Transcriptional induction of the gene encoding glycosyl transferase could result in increased and perhaps altered metabolism of proteins in the mycelium of *P. cinnamomi*. Rouhier *et al.* (1993) have reported that the growth of *P. capsici* on a medium amended with phosphite increased the percentage of water soluble cell wall fractions, corresponding to a mixture of glucans and glycoconjugates which contained mannose, galactose, and proteins. Metabolic studies have shown that phosphite altered the synthesis of lipids and their physical association within the mycelium (Grant *et al.* 1990). These observations are consistent with the proposed alterations in the cell wall (section 6.3.1) and protein metabolism. The elevated expression of the gene encoding
glycosyl transferase in *P. cinnamomi* in response to phosphite in the present study suggests that the cellular processes associated with ER and the Golgi apparatus are important in the mycelium of *P. cinnamomi* especially in times of abiotic stress resulting from phosphite application. This connects with the genes required for intracellular trafficking described below.

### 6.4.3 Cellular transport and signal transduction

The most highly responsive genes regulated by phosphite in this study encoded GTP binding proteins, which belong to the Ras GTPase superfamily (Donaldson and Honda 2005). ADP-ribosylation factors (ARFs) regulate the budding of vesicles that mediate transport from the endoplasmic reticulum (site of protein synthesis) to the Golgi apparatus (site of protein modification) in part by recruiting coat proteins to membranes (Randazzo *et al.* 2000). Common to all regulatory GTPases, the function of ARFs is regulated by the binding and hydrolysis of GTP (Randazzo *et al.* 2000), which enables them to switch a signal transduction chain on and off. The switch regulation occurs through the change of the active GTP-bound form to the inactive GDP-bound form by hydrolysis of the GTP. This reaction involves the GTPase-activating proteins (GAPs). The GTPase can be switched on again by guanine nucleotide exchange factors (GEFs) by dissociation of the GDP from the GTPase. The ratio of active to inactive GTPases determines the efficiency of the GTPase regulated signal transduction (Randazzo *et al.* 2000) (Figure 6.2).
Figure 6.2: Recruitment of coat proteins by ARF1. Dissociation of the GDP-bound ARF1 (inactive) by GEFs leads to the association of ARF1 with GTP (active). Activated ARF1 recruits coat proteins to Golgi membrane. The coat proteins organise the cargo and bring about the budding of membrane that eventually becomes a transport intermediate. The type of the coat protein (COPI, COPII and Clathrin) determines the direction of vesicle trafficking: ER to Golgi and vice versa, lysosomes, etc. (Nie and Randazzo 2006).

The up-regulation of the genes encoding ADP-ribosylation factors suggests an increase in the formation and trafficking of membrane vesicles within the phosphite-treated *P. cinnamomi* not only between ER and Golgi apparatus but also to the extracellular environment. Increased transport of molecules to and from membranes is consistent with the up-regulation of a gene encoding ABC transporter in phosphite-treated mycelium. These transporter proteins span the entire membrane and function in the transport of molecules such as metabolic products, lipids, sterols and drugs to the outside of the plasma membrane or the endoplasmic reticulum and mitochondria (Cooper and Hausman 2004). The identification of the materials transported may help to elucidate the role of phosphite in the interaction between the pathogen and the host. It is tempting to speculate that the contents of the vesicles and the molecules transported by
ABC transporters to the outside of the cell may play an important role in pathogen-host interactions (i.e. elicitors, pathogen associated molecular patterns – PAMPs, phosphatases).

Many ABC transporters may be classified as full transporters consisting of the typical two transmembrane domains (TMs) and nucleotide-binding folds (NBFs) or half transporters, which consist of only one TM and one NBF, and must combine with another half transporter to gain the ability to function. ABC transporters may also play a role in the development of multidrug resistance. The role of ABC transporters was investigated in multifungicide insensitivity in *P. infestans* using 41 full transporters and 13 half transporters. No correlation was observed between the expression of any ABC transporters and fungicide insensitivity; however, five ABC transporters were induced by several fungicides in strains with higher and lower sensitivities to fungicides, suggesting the existence of a network for protecting against toxins (Judelson and Senthil 2006). *P. cinnamomi* may also possess a mechanism for removal of natural toxic compounds from the system (e.g. ammonia); however, the role of ABC-transporters in the mycelium of phosphite-treated *P. cinnamomi* remains to be determined.

Further studies are required to establish whether the genes encoding ADP-ribosylation factors play a role in the transport of materials required for hyphal growth or transport of cell wall degrading enzymes. The observations in the present study suggest that this is unlikely, as phosphite reduced the mycelial growth by 70 %. The movement of membrane vesicles is regulated by other members of the GTPase family (e.g. Rab, Rho), which further increases the complexity of this process and extends the potential targets of phosphite.
It follows that in *P. cinnamomi*, vesicle formation and fusion is crucial in zoosporogenesis, where membrane material from the Golgi apparatus is required for subdivision of sporangial cytoplasm. An important part of the process involves microtubule dynamics, which regulates the movement of vesicles to the ventral and dorsal surface of the zoospore (Hardham 2005). During infection, the contents of the ventral vesicle enable the zoospore to stick to the root surface and the material released from the dorsal vesicle protects the cyst from drying out (Hardham 2005). Wilkinson *et al.* (2001a) have shown a reduction in zoospore production of *P. cinnamomi* in phosphite-treated *Eucalyptus marginata* and *Banksia grandis* seedlings. The up-regulation of the genes encoding ADP-ribosylation factors in the present study once again suggests that different members of the GTPase family may be involved in this process. However, further investigation is required to assess whether the change in gene expression of the ARF encoding genes in phosphite-treated mycelium is mirrored in the zoospores of *P. cinnamomi* exposed to phosphite. Previous studies on gene expression in the mycelium and zoospores of *P. nicotianae* revealed differences between the two life stages of the pathogen (Skalamera *et al.* 2004).

Finally, ARFs function not only within the secretory pathway but also as targets of signal transduction in the cell periphery. ARFs stimulate phospholipase D to produce phosphatidic acid, which facilitates regulation of signals and a mechanism for coordination of GTPases - some GEFs and GAPs are stimulated by phosphatidic acid (Randazzo *et al.* 2000; Nie and Randazzo 2006). Therefore, the ARF encoding genes in the present study may be required for phosphite signal transduction within the mycelium of *P. cinnamomi*. Further study is needed to elucidate whether phosphite has an effect on this molecular switch.
6.4.4 Stress response

It is possible that phosphite-treatment of *P. cinnamomi* may result in the imbalance of reactive oxygen species (ROS) production, as implicated by a down-regulation of a gene whose closest putative homologue was metallothionein, a protein that may be involved in ROS scavenging and metal homeostasis (zinc) in *P. cinnamomi*. Reactive oxygen species are natural by-products of oxygen metabolism (e.g. superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide) that mediate a variety of cellular responses such as programmed cell death and induction of defence responses during pathogen attack in plants (Mittler *et al.* 2004). It was demonstrated that the rice metallothionein OsMT2b protein may function as an ROS scavenger (Wong *et al.* 2004).

The presence of oxidative stress in the phosphite-treated mycelium of *P. cinnamomi* was supported by transcriptional down-regulation of a gene with homology to an alternative oxidase, an integral membrane protein that is bound to the inner mitochondrial membrane and is involved in respiratory gaseous exchange. The oxidase provides an alternative single step route (not coupled to ATP synthesis) for electrons passing through the electron transport chain to reduce oxygen and therefore ROS. The alternative oxidase pathway, which is only about 40 % as efficient as the normal energy pathway, also contributes to resistance development in *Septoria tritici* to strobilurins (Wood and Hollomon 2003). Accordingly, phosphite may have the ability to reduce the capacity of the mycelium to generate energy, and prevent scavenging of ROS. This is in accordance with the observations by Daniel and Guest (2006) who suggested that inhibition of mycelial growth and apparent hyphal distortions of phosphite-treated *P. palmivora* were due to accumulation of toxic compounds.
In conclusion, this study constitutes the first step towards understanding the molecular interaction between the fungicide phosphite and the soil-borne plant pathogen \textit{P. cinnamomi}. Several genes were identified whose transcriptional profiles have been significantly changed by phosphite treatment. They include genes required for signal transduction and vesicular transport, cell wall biosynthesis, protein metabolism, pathogenicity and stress response. It must be considered that not all the genes with changes in transcription in the presence of phosphite are involved in the phosphite response. Some genes may be induced as a result of generalised stress response imposed by phosphate starvation or phosphite toxicity on the pathogen. The role of the genes identified in the present study remains to be confirmed. Although the putative function of the genes whose expression is regulated in response to phosphite treatment had been predicted through bioinformatic analysis, the precise roles of the proteins encoded need to be verified. This can be performed through targeted gene silencing, which has previously been used in other \textit{Phytophthora} studies to produce strains deficient in particular gene products (Kamoun \textit{et al.} 1998; van West \textit{et al.} 1999b; Gaulin \textit{et al.} 2002). Although the limitations of the present study must be considered, the fact that the expression data were acquired from six independent replicate microarrays provides strong support for the validity of the data. Additionally, the expression patterns of the selected genes from the microarray analysis coincided with those generated by qRT-PCR. The project is part of an ongoing study, which includes sequencing of the remaining differentially expressed genes, confirmation of differential expression by qRT-PCR and submission of \textit{P. cinnamomi} sequences in the public database. The results offer an insight into the mode of action of phosphite in \textit{P. cinnamomi in vitro}, which may lead to more efficient use of the fungicide phosphite, and potentially develop novel means of controlling Oomycete plant pathogens. The knowledge generated in this study can be applied to prioritise between genes of interest and their products in future
studies, thus greatly accelerating the progress of our understanding of the mechanism(s) of phosphite action.
Chapter 7

General Discussion

This study is the first comprehensive investigation into the transcriptional profiling of phosphite regulated-gene expression in the mycelium of *Phytophthora cinnamomi*. Analysis of the transcriptome provided an insight into the types of biological processes that may be affected by phosphite in *P. cinnamomi*. Seventy-two transcripts with altered patterns in gene expression (fold change ≥ 2) were identified. Phosphite up-regulated expression (from 2- to 16-fold) was detected for 32 transcripts. Phosphite was also found to down-regulate expression from 2- to 3.5-fold under the conditions tested for 40 transcripts. Characterisation of relevant ESTs revealed that the most highly induced transcripts were produced from genes coding for ADP-ribosylation factors, an ABC cassette transporter, a glycosyl transferase and a vitamin B6 biosynthesis protein. In contrast, the genes that were down-regulated in response to phosphite putatively encoded cellulose synthase I, annexin, glutamine synthetase, metallothionein and an alternative oxidase. Additionally, qRT-PCR revealed that phosphite down-regulated (2.1-fold) a gene encoding ubiquitin-conjugating enzyme.

7.1 Construction and characterisation of a cDNA library

After a brief investigation into the responsiveness of six *Phytophthora cinnamomi* isolates to phosphite application (Chapter 2), the isolate MP 80, whose growth was inhibited by 70 % after four days growth in medium with 40 µg/ml phosphite, was selected for the construction of a cDNA library. The generation of a cDNA library (Chapter 3) from axenically grown mycelium treated with 40 µg/ml phosphite provided
a foundation for the identification and characterisation of the genes expressed in the phosphite-treated mycelium through Expressed Sequence Tags (ESTs).

7.2 Identification of constitutively expressed genes

Two constitutively expressed genes coding for elongation factor 1α and cinnamomin were identified in the present study by qRT-PCR. One was used as a reference gene against which the expression level of genes of interest was normalised with respect to the amount of starting material – RNA extracted from phosphite-treated (40 µg/ml) and untreated mycelium (Chapter 4 and 6). The constitutive genes and the corresponding primers designed can be applied in future gene expression studies as reference controls using the same experimental conditions.

7.3 Construction of microarrays

Significant progress towards large-scale gene expression profiling of phosphite-regulated genes in *P. cinnamomi* was made by constructing custom made cDNA microarrays consisting of 9216 transcripts (Chapter 5). The major goal of the microarray experiments was to identify genes that were either up- or down-regulated in phosphite-treated mycelium. The final number of the cDNA transcripts screened on microarrays was performed manually since available automated equipment for high-throughput processing of clones was not sensitive enough for plaque picking (a software limitation of the model, GeneTac G3). As many as 30 000 cDNA clones could have been screened on a single microarray; a feat only possible with a fully functional automated system in the time available. Additionally, the process of selecting cDNA containing phage was solely based only on a chromogenic screen to detect non-coloured
recombinants. Contributing to the redundancy of the screening was the presence of small inserts within the cDNA library. Nevertheless, the work presented here is the most comprehensive study to date of *P. cinnamomi* that has aimed at identifying genes whose transcription is regulated by the addition of phosphite.

### 7.4 Major findings

The microarray data were analysed using bioinformatics (Chapter 6) to unravel the molecular mechanisms by which phosphite regulates the growth and pathogenicity of *P. cinnamomi*. With the availability of fully sequenced genomes of *P. sojae* and *P. ramorum*, and the large collection of *P. infestans* ESTs, it was possible to assign putative functions to most of the transcripts sequenced and thus obtain appropriate information to interpret the data obtained with the microarray experiments.

#### 7.4.1 The ubiquitin/proteasome pathway (UPP) theory

The real-time PCR experiments revealed transcriptional suppression of the gene encoding ubiquitin-conjugating enzyme (Ubc) by 2.1-fold in the phosphite-treated *P. cinnamomi* mycelium (Chapter 4). This suppression may have important consequences in several cellular function of *P. cinnamomi*, including the regulation of proteins involved in cell signalling, transcriptional activation, the cell cycle, the removal of rate limiting enzymes and proteins, and cell death. Subsequently, a theory involving the regulation of elicitors/elicits by ubiquitin dependent proteolysis and the interaction with host plants was formulated. A novel mechanism for phosphite-regulated homeostasis of phosphate in plants and the pathogen was proposed (Figure 4.10).
It follows that the down-regulation of the gene encoding Ubc in phosphite-treated mycelium may result in an increase of protein elicitors, providing a possible mechanism of host-pathogen recognition, possibly through other components of the protein degradation pathway such as proteasomes in plants. Elicitors are natural compounds that are able to induce defence responses in plants similar to those observed in plant resistant-pathogen interactions (Scheel and Parker 1990). To test the proposed hypothesis it may be more appropriate to use lupins as model plants rather than Arabidopsis. Takemoto et al. (2005) assessed 53 Arabidopsis accessions for sensitivity to Phytophthora elicins and did not find any elicitin-sensitive plants and suggested that Arabidopsis may not possess an elicitin receptor. Additionally, research in our laboratory showed that Arabidopsis is not susceptible to P. cinnamomi - that is, there are no ecotypes which are killed by the pathogen (Jardine pers. comm.). Takemoto et al. (2005) reported that elicins with reduced sterol loading efficiency showed a decreased association with the elicitin binding site in N. tabacum and lower HR induction, suggesting that the binding of sterols to the elicins is important for the induction of HR. It may therefore be worthwhile investigating whether the sterol binding ability of elicins is also important for ubiquitination.

It is possible that the phosphate/phosphite uptake in both the pathogen and plants may be regulated through the ubiquitin signalling pathway and that this may be one of the mechanism(s) of how phosphite disrupts the phosphate starvation response in plants. Phosphite was previously shown to obstruct the phosphate signalling mechanism in several microorganisms and plants (Lee et al. 2005; McDonald et al. 2001; Varadarajan et al. 2002). Recent evidence suggests that the adaptive responses of plants to phosphate (Pi) deficiency are regulated by post translational regulation of E3 ligases involved in sumoylation (AtSIZ1), PHR1 transcription factor, specific microRNA (miR399) and
Ubc24 (At2g33770) encoding PHO2 (Bari et al. 2006; Chiou et al. 2006; Miura et al. 2005). The discovery of transcriptional down-regulation of Ubc by phosphite and the implications of this involvement in the molecular interaction between the pathogen and the host have opened doors for many future studies. Already there is a concurrent study on the effect of phosphite on the ubiquitin-conjugating enzyme in relation to phosphate starvation in planta as part of another PhD study (Eshraghi pers comm.). Apart from this, the UPP proteolytic pathway is involved in other mechanisms of pathogen defence such as the jasmonate and ethylene signalling (Glickman and Ciechanover 2002; Smalle and Vierstra 2004), which also warrants further investigation in correlation with phosphite.

7.4.2 Characterisation of phosphite responsive genes from the microarray

Characterisation of 20 ESTs from the microarrays resulted in the identification of nine putative genes that were transcriptionally affected in the presence of phosphite (Chapter 6). These are discussed in terms of possible roles within the mycelium of P. cinnamomi and pathogen-host interaction.

The difference in growth rate and changes in hyphal morphology observed between the mycelium cultured in medium with or without phosphite could be attributed to transcriptional changes of several putative genes identified in the present study. These include genes encoding proteins involved in biosynthesis of cell wall components, synthesis of amino acids, protein metabolism, vesicular formation and trafficking, energy metabolism, toxin detoxification and oxidative stress. These modifications could also play a role in the induction of host defence systems in susceptible plants.
In particular, the down-regulation of the gene whose closest ortholog was \textit{CESI} and a gene coding for annexin, suggest that phosphite disrupts the biosynthesis of major components of hyphal cell walls of \textit{P. cinnamomi}, cellulose and beta-glucans. This could explain the inhibition of radial growth and hyphal distortions observed in the mycelium grown in phosphite amended medium. Cell wall integrity is also important in pathogenicity and virulence. For example, the biological control of \textit{P. parasitica} (syn. \textit{P. nicotianae}) by \textit{Pythium oligandrum} is based on the ability of the control agent to produce large amounts of cellulolytic enzymes capable of degrading cellulose in the cell walls of \textit{P. parasitica} (syn. \textit{P. nicotianae}), making it easy for \textit{Pythium} to penetrate its cell walls (Picard \textit{et al.} 2000). Therefore, phosphite induced changes in cell wall construction may weaken the hyphae of \textit{P. cinnamomi} and reduce its capacity to colonise plants.

This hypothesis and a recent report of a significantly reduced zoospore production from \textit{P. cinnamomi} on phosphite-treated \textit{Eucalyptus marginata} and \textit{Banksia grandis} seedlings (Wilkinson \textit{et al.} 2001a) suggest that phosphite may reduce the probability that host plants will be infected through a reduced inoculum potential. Some studies, however, suggest that in infected plants the pathogen does not come into contact with phosphite at concentrations that could account for growth inhibition \textit{in vitro} (Darakis \textit{et al.} 1997). It was proposed that apart from protecting the host plants from further infection, the innate immune response of plants may also function in the suppression of the growth of the pathogen \textit{in planta} (Grant \textit{et al.} 1990). Therefore, the alterations in the cell wall organisation of \textit{P. cinnamomi} by phosphite may result in the release of elicitor molecules, which could stimulate defence responses in plants.
It has been shown that there is a correlation between deficiency in amino acid biosynthesis and lack of pathogenicity in several plant pathogens including *P. infestans* (Boon *et al.* 1958; Kahmann and Basse 1999; Shan *et al.* 2004). Griffith *et al.* (1990) reported a reduction in the total pool of adenylate in phosphite-treated *P. palmivora* (100 µM of phosphite), which could lead to a reduction in nucleic acid synthesis. This concurs with the finding in this study that transcriptional levels appeared to be down-regulated (2.2-2.5-fold) for the gene encoding glutamine synthetase, which is, in eukaryotes, responsible for the synthesis of glutamine. Glutamine is an amino acid utilised as a source of energy and for nucleotide synthesis and serves in the non-toxic transport of ammonia. Based on these observations and the results in the present study, phosphite may act to limit the biosynthesis of amino acids in the mycelium, which could also impact on the cell wall structure of *P. cinnamomi* (e.g. reduction of proteoglycan synthesis).

Reduction in amino acid biosynthesis may have implications in a multitude of cellular pathways such as cell signalling, energy metabolism, citric acid cycle and glycolysis/gluconeogenesis. Apparent changes in the metabolism of phosphite-treated *P. cinnamomi* were revealed by an increase in gene expression levels of the putative genes coding for glycosyl transferase and ADP-ribosylation factors. Induction of the glycosyl transferase gene, which may be involved in the process of attachment of carbohydrate moieties, in the present study coincided with the up-regulation of a proteoglycan gene in phosphite-treated *P. cinnamomi* by Wong (2006). Lipid metabolism and proteins required for secretion, plasma membrane and lysosomes are processed in endoplasmic reticulum and then transported to Golgi apparatus to undergo further modification and sorting for distribution around the cell. Vesicular transport is therefore a major cellular activity and the specificity of this process is fundamental to
maintaining functional organisation of the cell. This process is largely controlled by ADP-ribosylation factors. The massive induction (up to 16-fold) of the genes encoding ADP-ribosylation factors (ARFs) detected in the phosphite-treated mycelium of *P. cinnamomi*, suggests proliferation of vesicular trafficking and formation. The relevance of this proliferation may also be important in the response to oxidative stress, which will be discussed later.

An increase in molecular transport was further established by the up-regulation of an ABC transporter gene in phosphite-treated mycelium. ABC transporters are membrane bound proteins that facilitate transport of molecules across cell membranes such as metabolic products, lipids and sterols. The contents of the secretory vesicles and the material transported through the ATP cassette transporters remains to be determined and could be of great aid in elucidation of phosphite-induced changes in *P. cinnamomi* and possible interaction with host plants. The up-regulation of the genes encoding glycosyl transferase, ADP-ribosylation factors and transporters suggests that phosphite interferes with the synthesis, metabolism and trafficking of cellular components in *P. cinnamomi*. Alterations in metabolism and polymerisation of cell wall components could result in changes in the permeability of the cell wall that could lead to ‘leaking’ of metabolic compounds, which could account for recognition of the pathogen by the host. ARFs are also involved in regulating the microtubule dynamics and work with the Rho family of proteins to affect the actin skeleton (Randazzo *et al.* 2000), which is important in hyphal growth (Bartnicki-Garcia 2003). It is possible that other ARF proteins (there are six in mammals) or members of the GTPase family may be involved in this process and that phosphite may affect the GTPase switch that regulates the signal transduction of GTPase proteins. This warrants further study.
It is likely that the changes in transcription of the putative genes identified could be due to oxidative stress induced by phosphite in the mycelium of *P. cinnamomi*. The generation and controlled utilisation of metabolic energy in the form of ATP (adenosine 5'-triphosphate), generated from glycolysis (anaerobic) and oxidative metabolism (aerobic) (photosynthesis - in plants), is central to all cell activities (Cooper and Hausman 2004; Deacon 2006). The unavoidable consequence of aerobic respiration is the production of ROS (reactive oxygen species). The metabolism of O₂ involves the terminal oxidases: cytochrome c oxidase and the alternative oxidase. The production of superoxide anion O₂⁻ leads to the formation of hydrogen peroxide (H₂O₂) and other ROS, which can cause cellular damage such as modification of protein side chains, breakage of unsaturated fatty acid backbone and DNA mutation (Moller 2006). Induction of alternative oxidase signifies a reduction in ROS production (Camacho et al. 2004). Therefore, the suppression of the alternative oxidase gene in the present study may result in an imbalance between the ROS production and the ability of the biological system of *P. cinnamomi* to detoxify the reactive intermediates, leading to the accumulation of ROS. Interestingly, Niere et al. (1990) reported that phosphite decreased the amount of triacylglycerol and the chain length of polyphosphate present in *P. palmivora*; however, no effect on the phospholipid concentration was observed. Perhaps some of these changes could be attributed to the reaction with ROS, such as peroxidation of polyunsaturated fatty acids.

Akhter et al. (2003) reported that disruption of the alternative oxidase gene (*AOXI*) resulted in impairment of respiration and growth, and reduction of virulence in *Cryptococcus neoformans*. The ability of the alternative oxidase to bypass the strobilurin-based fungicide (Q₀,I)-induced block in the electron transport chain contributes to resistance in pathogenic fungi (Kim et al. 2003; Wood and Hollomon...
In contrast, ROS production *in planta* due to stress may lead to activation of stress-related proteins (Moller 2006).

The presence of oxidative stress in phosphite-treated mycelium was further established by the apparent suppression of a gene encoding metallothionein, a protein involved in protection against oxidative stress and metal homeostasis, and a gene encoding Vitamin B6 biosynthesis protein. Vitamin B6 has recently been implicated in defence against oxidative stress in plants (Denslow *et al.* 2005).

Yamaguchi *et al.* (2004) demonstrated that H$_2$O$_2$ induced phospholipase D in rice cells and Hui *et al.* (2004) revealed that phospholipase D suppressed DNA damage-induced apoptosis in rat fibroblast cells. Some ARFs stimulate phospholipase D (Randazzo *et al.* 2000). It is possible that the up-regulation of ARFs in the present study could be due to H$_2$O$_2$ accumulation in the mycelium and may be involved in the suppression of H$_2$O$_2$ induced cell death by stimulating phospholipase D production in *P. cinnamomi*. Suppression of programmed cell death and the removal of oxidised proteins in *P. cinnamomi* mycelium is consistent with down-regulation of a gene encoding ubiquitin-conjugating enzyme (Chapter 4). A molecular profiling of H$_2$O$_2$ induced gene expression in tobacco revealed transcriptional up-regulation of genes involved in vesicular transport, proteolysis, mitochondrial metabolism, cell death, post-translational processes and several plant defence mechanisms such as hypersensitive response and synthesis of ethylene and jasmonic acid (Vandenabeele *et al.* 2003). Lee *et al.* (2003) reported that the transcripts of *RARF1* (rice ADP-ribosylation factor 1) isolated from fungal elicitor-treated rice cells accumulated in response to H$_2$O$_2$ and suggested that *RARF1* may be involved in plant defence signalling and induction of pathogenesis-related genes. Additionally, Langlois-Meurinne *et al.* (2005) reported that the expression
of glycosyl transferase genes was induced during oxidative stress (H$_2$O$_2$) and that they may participate in maintaining cellular redox homeostasis in *Arabidopsis*. This is consistent with the elevated levels of the gene encoding glycosyl transferase in the present study. These observations and the results from the present study suggest that phosphite may cause oxidative stress such as H$_2$O$_2$ accumulation in the mycelium of *P. cinnamomi*, which could be responsible for setting off a chain of events corresponding to the transcriptional changes of the genes identified in the present study.

Finally, the possibility that the effects on gene expression may be due to a global stress or phosphate starvation must be considered. Although the level of phosphate in the present study was considered high at 10 mM, the mycelial growth was still significantly reduced (70 %), which could be manifested by phosphate uptake inhibition. Since the operation of many cellular processes are driven by phosphorylation and dephosphorylation reactions this would have widespread effects throughout the cell. A previous study has shown that phosphate starvation reduced the mycelial growth of *P. capsici* (Darakis *et al.* 1997). The advantage of using high levels of phosphate is that the responses observed *in vitro* in the present study may be similar to those *in planta* at least in the more phosphite sensitive isolates, assuming that the pathogen is exposed to sufficient concentrations of phosphite. However, to fully understand the mechanism(s) of phosphite action, it is necessary to examine the combined effect of all three, the interaction between the pathogen, the host plant and phosphite. The cellular sites that are likely to be affected by phosphite treatment in *P. cinnamomi* according to the putative genes identified in the present study are summarised in Figure 7.1 and 7.2.
Figure 7.1: Model for the interaction of differentially expressed putative genes (blue – encoding product) and cellular processes (green) in the mycelium of *Phytophthora cinnamomi* in response to phosphite, as determined by the microarray and qRT-PCR studies. Phosphite application *in vitro* regulates the expression of several putative genes, of which many may play a role in more than one cellular process. In particular, oxidative stress (ROS production), metabolism and vesicular transport appear to be of importance in phosphite response. Some changes within the mycelium of *P. cinnamomi* may account for interaction with host plants such as alterations in the cell wall biosynthesis, the release of elicitors and the ubiquitin dependent proteolysis.
Figure 7.2: Hyphal tip of *Phytophthora cinnamomi* – showing the proposed cellular processes (red) affected by phosphite application include energy metabolism, oxidative stress (ROS production), protein degradation through the UPP pathway, metabolism (glycosylation), vesicular trafficking, signal transduction, cell wall biosynthesis, hyphal growth and the release of elicitors, which may provide for the interaction with a host plant. The cytoplasm with associated organelles (green), N (nucleus), ER (endoplasmic reticulum), GA (Golgi apparatus), V (vesicle), T (transporter), R (ribosome), M (mitochondria), VC (vacuole), flows freely in coenocytic hypha.
7.5 Summary and future directions

In summary, this study is the first large scale screening of phosphite-regulated gene expression in *P. cinnamomi* and represents a significant stepping stone towards our understanding of the mode of action of phosphite. The transcriptional profiling of 9216 transcripts revealed changes in expression of 72 ESTs. Characterisation of 20 ESTs revealed genes potentially involved in a vast array of cellular functions within the mycelium of *P. cinnamomi*, all of which could play important roles in the interaction between phosphite and the pathogen as well as in pathogen-host interaction. Subsequently, a model incorporating all the genes identified was proposed (Figure 7.1).

A model for the mechanism of how phosphite may disrupt phosphate starvation responses in plants and affect phosphate uptake in both the pathogen and the host involving the components of the ubiquitin proteasome pathway was also proposed (Chapter 4). This model takes into account the interactions with host plants through the ubiquitination of elicitors/elicitins.

There are still 52 differentially expressed transcripts from the current study (Chapter 5; Table 5.2) that have not yet been characterised. The prediction of the function of these transcripts can be performed through functional category enrichment, which may help in further elucidation of the complex mode of phosphite action. The role of each gene also remains to be evaluated using RNAi to knockout expression. Future research is necessary to establish whether the changes in gene transcription are reflected in the products they encode and proteomic analysis can be performed to identify protein interactions. The genomic resources constructed (cDNA library and microarrays) can be used to study the molecular interaction between phosphite and *P. cinnamomi* in greater
detail in future studies. In addition, the ESTs from the cDNA library can be used to enrich the public database with novel ESTs unique to *P. cinnamomi*.

In conclusion, this study offers a new insight into the mode of action of phosphite in *P. cinnamomi* mycelium *in vitro*, which may lead to a more efficient use of the fungicide phosphite or the development of new control strategies through identification of potential cellular targets.
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Appendix 1

Growth medium recipes

Table 1: Phytophthora - selective agar medium (NARPH)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Ampicillin</td>
<td>0.1 g</td>
<td>Fisons</td>
</tr>
<tr>
<td>Nystatin (Nilstat)</td>
<td>1 ml</td>
<td>Wyeth-Ayerst</td>
</tr>
<tr>
<td>Rifampicin (Rifadin)</td>
<td>0.5 ml</td>
<td>Hoechst Marion Roussel</td>
</tr>
<tr>
<td>Hymexazol (Tachigaren)</td>
<td>0.05 g</td>
<td>Sunkyo Company</td>
</tr>
<tr>
<td>Pentachloronitrobenzene (Terrachlor)</td>
<td>0.1 g</td>
<td>Uniroyal</td>
</tr>
<tr>
<td>Deionised water</td>
<td>10 ml</td>
<td></td>
</tr>
<tr>
<td>Corn meal agar (Oxoid)</td>
<td>17 g</td>
<td>Unipath</td>
</tr>
<tr>
<td>Deionised water</td>
<td>1 L</td>
<td></td>
</tr>
</tbody>
</table>

Corn meal agar was autoclaved in deionised water at 15 psi for 20 min, while the rest of the ingredients were dissolved in 10 ml of sterile water and then added to the cooled (~ 50 °C) agar.
### Table 2: Modified Ribeiro’s medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
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</tr>
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<tbody>
<tr>
<td><strong>Microelement stock solution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na$_2$MoO$_4$·H$_2$O</td>
<td>41.1 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>87.8 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>7.85 mg</td>
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</tr>
<tr>
<td>MnSO$_4$·H$_2$O</td>
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<td>APS</td>
</tr>
<tr>
<td>Na$_2$B$_4$O$_7$</td>
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<td>BDH</td>
</tr>
<tr>
<td>Deionised water</td>
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<td></td>
</tr>
<tr>
<td><strong>Ferric stock solution</strong></td>
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</tr>
<tr>
<td>FeCl$_3$·6H$_2$O</td>
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</tr>
<tr>
<td>EDTA</td>
<td>2.6 mg</td>
<td>AJAX</td>
</tr>
<tr>
<td>KOH</td>
<td>1.5 mg</td>
<td>BDH</td>
</tr>
<tr>
<td>Deionised water</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td><strong>Thiamine stock solution</strong></td>
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<td></td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.1 mg</td>
<td>Sigma</td>
</tr>
<tr>
<td>Deionised water</td>
<td>100 ml</td>
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</tr>
<tr>
<td><strong>Basal medium</strong></td>
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<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>4.5 g</td>
<td>BDH</td>
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<tr>
<td>L-asparagine</td>
<td>0.10 g</td>
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<td>MgSO$_4$·7H$_2$O</td>
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<td>CaCl$_2$</td>
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<tr>
<td>Microelement stock solution</td>
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<td></td>
</tr>
<tr>
<td>Ferric stock solution</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>Deionised Water</td>
<td>1 L</td>
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</table>

The pH of the basal medium was adjusted to 6.2 with 6 M KOH and autoclaved at 15 psi for 20 min. After cooling to ~ 50 °C, 1 ml of filter-sterilised thiamine stock solution (0.22 µm filter, Millipore) was added to the autoclaved medium.

To make solid medium, 17 g/L of Agar (Grade A, BBL) was added to the basal medium prior to autoclaving.
### Table 3: Regression equations used to calculate the EC$_{50}$ values for inhibition of mycelial growth (dry weight) by phosphite of six *P. cinnamomi* isolates (Minitab 13.31).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Regression equation</th>
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<tr>
<td>MP80</td>
<td>( \text{growth} = 96.4647 - 1.41364 \text{ dose} + 0.0059834 \text{ dose}^2 )</td>
</tr>
<tr>
<td>MP 125</td>
<td>( \text{growth} = 91.2785 - 1.27647 \text{ dose} + 0.0048248 \text{ dose}^2 )</td>
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<tr>
<td>MP 97</td>
<td>( \text{growth} = 94.2721 - 1.26135 \text{ dose} + 0.0052191 \text{ dose}^2 )</td>
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<tr>
<td>MP 94-03</td>
<td>( \text{growth} = 101.214 - 0.569486 \text{ dose} + 0.0018100 \text{ dose}^2 )</td>
</tr>
<tr>
<td>MP62</td>
<td>( \text{growth} = 93.1240 - 0.802638 \text{ dose} + 0.0033750 \text{ dose}^2 )</td>
</tr>
<tr>
<td>MP32</td>
<td>( \text{growth} = 99.7235 - 1.16028 \text{ dose} + 0.0048000 \text{ dose}^2 )</td>
</tr>
</tbody>
</table>
Appendix 3

Uni Zap XR vector system

Figure 3: *In vivo* excision of pBluescript SK (−) phagemid vector. The initiator and terminator regions of the Lambda Zap vector arms are recognized by the trans-acting proteins from the helper phage, and a new DNA strand is synthesized, displacing the existing strand, which is circularized and packaged as a filamentous phage and secreted from the cell. pBluescript plasmids are recovered by infecting an F’ strain and growing in the presence of ampicillin (Stratagene, Lambda Zap II Vector product information).
### Example database of cDNA clones

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<th>Gene ID</th>
<th>Comments</th>
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1 (1/2/6-2; 7-12)  H9  MK_PC_136  Unknown
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1 (1/2/6-2; 7-12)  J9  MK_PC_138  Unknown
1 (1/2/6-2; 7-12)  K9  MK_PC_139  Unknown
1 (1/2/6-2; 7-12)  L9  MK_PC_140  Unknown
1 (1/2/6-2; 7-12)  M9  MK_PC_141  Unknown
1 (1/2/6-2; 7-12)  N9  MK_PC_142  Unknown
1 (1/2/6-2; 7-12)  O9  MK_PC_143  Unknown
1 (1/2/6-2; 7-12)  P9  MK_PC_144  Unknown
1 (1/2/6-2; 7-12)  A10  MK_PC_145  Unknown
1 (1/2/6-2; 7-12)  B10  MK_PC_146  Unknown
1 (1/2/6-2; 7-12)  C10  MK_PC_147  Unknown
1 (1/2/6-2; 7-12)  D10  MK_PC_148  Unknown
1 (1/2/6-2; 7-12)  E10  MK_PC_149  Unknown
1 (1/2/6-2; 7-12)  F10  MK_PC_150  Unknown
1 (1/2/6-2; 7-12)  G10  MK_PC_151  Unknown
1 (1/2/6-2; 7-12)  H10  MK_PC_152  Unknown
1 (1/2/6-2; 7-12)  I10  MK_PC_153  Unknown
1 (1/2/6-2; 7-12)  J10  MK_PC_154  Unknown
Appendix 5

Quantitative RT-PCR raw data

Table 4: Raw data (C_T values) from two separate qRT-PCR experiments of four candidate reference genes from the mycelium of *Phytophthora cinnamomi* in the presence and absence of phosphite.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gene</th>
<th>Untreated 1:10</th>
<th>Phosphite-treated 1:10</th>
<th>Untreated 1:100</th>
<th>Phosphite-treated 1:100</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>EF-1 alpha</td>
<td>17.14</td>
<td>17.23</td>
<td>20.56</td>
<td>20.43</td>
</tr>
<tr>
<td>2</td>
<td>EF-1 alpha</td>
<td>17.04</td>
<td>17.15</td>
<td>20.62</td>
<td>20.85</td>
</tr>
<tr>
<td>1</td>
<td>GAPDH</td>
<td>24.55</td>
<td>25.49</td>
<td>28.06</td>
<td>29.06</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH</td>
<td>24.22</td>
<td>25.01</td>
<td>27.34</td>
<td>28.47</td>
</tr>
<tr>
<td>1</td>
<td>Ubc gene</td>
<td>21.73</td>
<td>22.9</td>
<td>24.78</td>
<td>26.25</td>
</tr>
<tr>
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<td>Ubc gene</td>
<td>22.02</td>
<td>23.3</td>
<td>25.44</td>
<td>26.8</td>
</tr>
<tr>
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<td>beta-tubulin gene</td>
<td>21.18</td>
<td>21.34</td>
<td>24.6</td>
<td>25.08</td>
</tr>
<tr>
<td>2</td>
<td>beta-tubulin gene</td>
<td>21.68</td>
<td>21.89</td>
<td>25.08</td>
<td>25.72</td>
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