Genetic analysis of host and phosphite mediated resistance to *Phytophthora cinnamomi* in *Arabidopsis thaliana*

by

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

2012
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.

....................................
Leila Eshraghi
March 2012
Abstract

Phosphite (Phi), an analogue of phosphate (Pi) is highly effective for the control of *Phytophthora cinnamomi*, a devastating necrotrophic pathogen worldwide. This study describes the effect of phosphite (Phi) on the induction of defence responses in *Phytophthora cinnamomi*-infected *Arabidopsis thaliana* accessions Ler and Col-0, and mutants defective in salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), phosphate starvation response (PSR) and auxin response signalling pathways. The inoculation of the resistant Col-0 with *P. cinnamomi* induced a rapid increase in callose deposition (by 6 h after inoculation) and hydrogen peroxide (H$_2$O$_2$) production (by 24 h after inoculation) whereas inoculation of susceptible Ler showed a delayed and reduced response. Treatment of Ler with Phi produced a response to *P. cinnamomi* inoculation similar to that observed in Col-0 in terms of timing and magnitude suggesting Phi primes the plant for a rapid and intense response to infection involving heightened activation of a range of defence responses.

A reliable method for measuring disease progression is important when evaluating susceptibility in host–pathogen interactions. A sensitive quantitative polymerase chain reaction (QPCR) assay was developed for the quantitative measurement of *P. cinnamomi* DNA (biomass) *in planta* that avoids problems caused by variation in DNA extraction efficiency and degradation of host DNA during host tissue necrosis. Purified plasmid DNA, containing the pScFvB1 mouse gene, was added during DNA extraction and the pathogen’s biomass was normalized based on plasmid DNA rather than host DNA or sample fresh weight. It was demonstrated that normalization of pathogen DNA to sample fresh weight or host DNA in samples with varying degrees of necrosis led to an overestimation of the pathogen’s biomass.

Inoculation of mutants in the SA, JA, and ET defence signalling pathways did not affect the resistance of Col-0 suggesting alternative pathways are involved. A high level
susceptibility was observed in the *aba2-4* mutant suggesting a role for ABA signalling in the induction of resistance to *P. cinnamomi*. Phi treatment of *aba2-4* increased resistance but not to the wild type levels indicating a possible role for ABA-dependent and ABA independent signalling in Phi mediated resistance. Application of Phi to non-inoculated *A. thaliana* seedlings elevated transcription of defence genes in the SA (*PRI* and *PR5*) and JA/ET (*THI2.1* and *PDF1.2*) pathways. Furthermore, analysis of gene expression in Col-0 revealed that either Phi or *P. cinnamomi* caused the down-regulation of the transcriptional level of *AtMYC2* (a positive regulator of ABA signalling which also negatively regulates JA-related genes) and increased the transcriptional abundance of *PDF1.2*. Together these results suggest that the resistance response of Col-0 and Phi treatment both act partially through an ABA dependent mechanism which is independent of the antagonism between ABA and elements of the JA/ET pathway such as *PDF1.2*.

Phosphite has been suggested to interfere with various plant processes including Pi homeostasis therefore the potential involvement of the Pi and auxin signalling pathways in resistance to *P. cinnamomi* was investigated using several PSR and auxin response pathway mutants. The mutants *tir1-1*, an auxin response mutant deficient in the auxin-stimulated SCF (Skp1−Cullin−F-Box) ubiquitination pathway and *phr1-1*, a mutant defective in response to Pi starvation were highly susceptible to *P. cinnamomi* compared to their parental background Col-0. Complementation restored resistance to the level observed in Col-0. Moreover, inhibition of auxin transporters by TIBA (2,3,5-triiodobenzoic acid) led to a significant increase in susceptibility of *Lupinus angustifolius* seedlings to *P. cinnamomi* supporting the importance of the auxin signalling pathway in *P. cinnamomi* resistance. The 26S proteasome subunits mutants; *rpn10-1* (Defective in ubiquitin/26S proteasome-mediated proteolysis) and *pbe1-1* (proteasome subunit beta type-5-A) were also susceptible to *P. cinnamomi*. The *rpn10-1*
mutant has also been associated with the auxin signalling pathway and the susceptibility of \textit{rpn10-1} and \textit{pbe1-1} indicates that the 26S proteasome and auxin signalling could play a role in resistance to \textit{P. cinnamomi}. Given the apparent involvement of auxin and PSR signalling in the resistance to \textit{P. cinnamomi}, the possible involvement of these pathways in Phi mediated resistance was also investigated. Application of Phi at both low and high concentrations attenuated some of the Pi starvation inducible genes such as \textit{At4}, \textit{AtACP5} and \textit{AtPT2}. However, in phosphate sufficient plants, Phi treatment mimicked Pi starvation responses in terms of enhanced expression of \textit{PHR1}, \textit{AUX1}, \textit{AXR1}, \textit{AXR2} and \textit{SGT1B}; suppression of primary root elongation, and increased root hair formation. Together, these results suggest that the auxin response pathway, particularly auxin sensitivity and transport, plays a role in the plant’s resistance to \textit{P. cinnamomi} and suggest that phosphite-mediated resistance may in some part be through its effect on stimulation of the auxin response pathway.
Statement of the contributions of jointly authored papers

The following manuscripts have either been published or have been prepared/Submitted to scientific journals.


The contribution of work for this paper is 90% by the candidate of this thesis, Leila Eshraghi including the design, performance and analysis of experiments and writing of the manuscript, and 10% for all other authors in terms of advice on the experimental design, approach and revising the manuscript.


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Candidate, Leila Eshraghi

Signature: ……………………………………………………………………………………

Coordinating Supervisor, Professor Giles Hardy

Signature: ……………………………………………………………………………………
Conference publications pertaining to this thesis


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MY PARENTS

for their ongoing support and
keen interest in my study and
teaching me the values of hard work
and discipline.
## List of abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>Δct</td>
<td>Change in threshold cycle</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AGI</td>
<td><em>Arabidopsis</em> Genome Initiative</td>
</tr>
<tr>
<td>ARG</td>
<td>Auxin responsive genes</td>
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<tr>
<td>ARF</td>
<td>Auxin response factor</td>
</tr>
<tr>
<td>ASK</td>
<td><em>Arabidopsis</em> SKP1-like</td>
</tr>
<tr>
<td>AUX/IAA</td>
<td>Auxin/indole-3-acetic-acid</td>
</tr>
<tr>
<td>AXR</td>
<td>Auxin resistant</td>
</tr>
<tr>
<td>COI</td>
<td>Coronitine insensitive</td>
</tr>
<tr>
<td>COP</td>
<td>Constitutive photomorphogenic</td>
</tr>
<tr>
<td>CTR</td>
<td>Constitutive triple response</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CUL</td>
<td>Cullin</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>E1</td>
<td>Enzyme 1 (same as UBA, ubiquitin activating enzyme)</td>
</tr>
<tr>
<td>E2</td>
<td>Enzyme 2 (same as UBC, ubiquitin conjugating enzyme)</td>
</tr>
<tr>
<td>E3</td>
<td>Enzyme 3 (same as ubiquitin protein ligase)</td>
</tr>
<tr>
<td>EIN</td>
<td>Ethylene insensitive</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector-triggered immunity</td>
</tr>
<tr>
<td>ETR</td>
<td>Ethylene receptor</td>
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<tr>
<td>ERF</td>
<td>Ethylene response factor</td>
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<tr>
<td>FBX2</td>
<td>F-box protein 2</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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GUS  Beta-glucuronidase
GMO  Genetically Modified Organisms
HR  Hypersensitive response
IAA  Indole-3-acetic acid
ISI  Induces systemic resistance
JA  Jasmonic acid
LRR  Leucine-rich repeat
MAPK  Mitogen-activated protein kinase
MES  2-morpholinoethanesulfonic acid
Myc  Epitope tag from c-Myc protein
NIM  Non-inducible immunity
NO  Nitric oxide
NPR  Non-expressor of pathogenesis-related genes
PAMP  Pathogen associated molecular pattern
Pc  *Phytophthora cinnamomi*
PCR  Polymerase chain reaction
PCD  Programmed cell death
PDF  Plant defensin
Pi  Phosphate
Phi  Phosphite
PHR1  Phosphate starvation response 1
PIN  Pin-formed
PPCK1  Phosphoenolpyruvate carboxylase kinase 1
PR  Pathogenesis-related
PSR  Phosphate starvation response
PTI  PAMP-triggered immunity
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>QPCR</td>
<td>Quantitative polymerase change reaction</td>
</tr>
<tr>
<td>R gene/protein</td>
<td>Resistance gene/protein</td>
</tr>
<tr>
<td>RAR</td>
<td>Required for MIA12 resistance</td>
</tr>
<tr>
<td>RBX</td>
<td>RING-box protein, same as ROC1 and Hrt1p</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene protein domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reaction oxygen species</td>
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<tr>
<td>ROC</td>
<td>Regulator of cullins</td>
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<td>RT</td>
<td>Reverse transcription</td>
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<tr>
<td>RNAase</td>
<td>Ribonuclease</td>
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<tr>
<td>SA</td>
<td>Salicylic acid</td>
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<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
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<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1-Cullin1-F-box</td>
</tr>
<tr>
<td>SGT</td>
<td>Suppressor of G2 allele of skp1</td>
</tr>
<tr>
<td>SKP</td>
<td>S phase kinase-associated protein</td>
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<tr>
<td>SON</td>
<td>Suppressor of nim1-1</td>
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<tr>
<td>T-DNA</td>
<td>Transfer DNA</td>
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<tr>
<td>THI</td>
<td>thionin</td>
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<tr>
<td>TIBA</td>
<td>2,3,5-triiodobenzoic acid</td>
</tr>
<tr>
<td>TIR1</td>
<td>Transport inhibitor response1</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin activating enzyme</td>
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<tr>
<td>UBC</td>
<td>Ubiquitin conjugating enzyme</td>
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<td>U-box</td>
<td>UFD2-homology domain</td>
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<td>UPP</td>
<td>Ubiquitin proteasome pathway</td>
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Chapter 1

Literature Review
1.1 General Introduction

*Phytophthora* is a genus of oomycetes that is one of the most economically important group of plant pathogens worldwide (Erwin & Ribeiro, 1996). Species of *Phytophthora* are water mould algal relatives, and are not true fungi (Mycota) even though they reproduce by spores and grow filamentous hyphae. *Phytophthora* species disperse and infect by zoospores and survive unfavourable conditions as thick-walled chlamydospores or oospores (McCarren *et al*., 2005). Most *Phytophthora* species cause root diseases, but some can infect stems and leaves, causing cankers, fruit rots and foliar blights, respectively (Hansen, 2008). Although many *Phytophthora* species are relatively host-specific, *Phytophthora cinnamomi* has a host range of more than 3000 plant species and is one of the most pathogenic *Phytophthora* species (Hardham, 2005) causing considerable damage to native ecosystems, agriculture and horticulture worldwide (Shearer & Fairman, 2007, Brasier, 2008, Hansen, 2008). Shearer *et al.* (2004) estimated that of the 5710 described plant species in south-western Australia, 2284 species are susceptible and 800 species are highly susceptible to *P. cinnamomi*. *Phytophthora cinnamomi* is particularly known for its impact on jarrah (*Eucalyptus marginata*) forest in Western Australia, giving rise to the name “jarrah dieback”. The impact of *P. cinnamomi* on biodiversity especially on sand-plain floras in Western Australia is recognized as a “biological disaster of global significance” (Podger *et al*., 1996) and the Commonwealth Government recognized it as a “Key threatening process to Australia’s biodiversity” under the 1999 EPBC Act.

Recent efforts to control *P. cinnamomi* have focused on the use of the systemic chemical phosphite (Phi; a salt of phosphorous acid) an analogue of phosphate (Pi) (Chiou & Lin, 2011). A greater understanding of how Phi affects the plant-pathogen interactions is necessary to develop more effective Phi treatments. The following literature review discusses the current knowledge of i) the mechanisms of plant
resistance and other factors influencing plant-pathogen interactions, including plant-
*Phytophthora* interactions, ii) the mode of action of Phi and its effect on the induction of plant defence responses and subsequent increase in plant resistance, and iii) the interactions of Phi and phosphate in terms of phosphate sensing and the phosphate starvation response.

### 1.2 What is resistance?

Plants defend themselves against pest and pathogen attacks (biotic stress) and environmental changes (abiotic stress) by the induction of a series of tailored defence responses. Such responses include callose deposition, production of reactive oxygen species (ROS), thickening of cell walls and activation of defence signalling pathways through phytohormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) (Chisholm *et al.*, 2006, Jones & Dangl, 2006). Plants are often described as either being resistant or susceptible to a particular pathogen, although these terms are the extremes on a continuum (Guest & Brown, 1997). Cahill *et al.* (1989) clearly demonstrated the plant resistant continuum from histological changes induced in response to *P. cinnamomi* infection in a range of species. Susceptible hosts were unable to impede infection by *P. cinnamomi*, which results in secondary symptoms of wilting, yellowing and death. Resistant hosts slowed or prevented the growth and activity of the pathogen and no secondary symptoms of infection formed.

Plant defence mechanisms may stop a pathogen’s initial penetration or may restrict the pathogen’s colonization and reproduction after its establishment within the host tissue (Parlevliet, 1979). Generally, there are three main types of defence systems in plants; the pre-existing/constitutive defence system, a non-specific basal defence or PAMP (pathogen-associated molecular patterns)-triggered immunity (PTI) and gene-for-gene resistance or effector triggered immunity (ETI) (Spoel & Dong, 2012). In addition, systemic acquired resistance (SAR) may be induced by either PTI or ETI and
induced acquired resistance (ISR) which is typically induced by non-pathogenic rhizobacteria (Dreher & Callis, 2007).

In order to initiate penetration into a host cell, the pathogen needs to overcome the plant’s pre-existing defence barriers such as suberized periderm, cuticle and phenolic compounds (Kolattukudy, 1985). Plants also possess the ability to perceive and initiate a defence response to a wide range of potential pathogenic microorganisms based on the microbial or pathogen-associated molecular patterns (MAMPs or PAMPs) they present to the plant. In some cases, PTI stops the growth of potential pathogens at an early stage of infection and is often associated with the induction of defence-responsive genes, deposition of callose papillae and production of reactive oxygen species (ROS) to strengthen the cell walls at the sites of infection (Schwessinger & Zipfel, 2008, Barna et al., 2012) and activation of defence signalling pathways through phytohormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) (Chisholm et al., 2006, Jones & Dangl, 2006). A successful pathogen is able to avoid triggering PTI, suppresses PTI or overcomes the resistance responses, often through the delivery of effectors to the plant (Jones & Dangl, 2006).

In host specific resistance, when PTI is overcome by pathogen effectors, a second system which relies on the specific recognition of pathogen effectors by plant R genes is activated (Ingle et al., 2006). The R gene-mediated defences are termed effector-triggered immunity (ETI) or gene-for-gene resistance (Jones & Dangl, 2006) and R gene product recognition of pathogen effectors leads to the rapid activation of specific defence related pathways and results in incompatible interactions. Indirect or direct recognition of pathogen effectors by R genes initiates ETI, which may resemble an accelerated and amplified PTI response, but often includes a hypersensitive cell death response (HR), leading to disease resistance (Jones & Dangl, 2006). The hypersensitive cell death response at the site of infection is characterized by a continuous burst of ROIs
(reactive oxygen intermediates), elevation of SA accumulation, and systemic induction of pathogenesis-related (PR) genes in tissues (Durrant & Dong, 2004, Jones & Dangl, 2006).

In addition to PTI and ETI, pathogen attack also activates systemic acquired resistance (SAR), which decreases the plant’s susceptibility to a range of potential pathogens such as fungi, bacteria and viruses (Durrant & Dong, 2004) by accumulation of SA and through the activity of the NPR1 (non-expressor of PR genes) regulatory protein which stimulates expression of many PR genes. Induced systemic resistance (ISR) is also a NPR1-dependent but SA-independent response which is not only stimulated by non-pathogenic root-colonizing bacteria, but also heightens the plant’s ability to defend itself from the future attack of bacteria and fungi (Figure 1) (Feys & Parker, 2000, Durrant & Dong, 2004).

1.3 Plant defence signalling

The plant defence hormones (phytohormones) such as SA, JA, ET, auxin (IAA) and ABA are involved in activation of defence pathways and modulating plant-pathogen interactions. Microarray analysis in Arabidopsis thaliana has revealed significant cross-talk between defence pathways induced by these phytohormones indicating a considerable network of regulatory interactions (Schenk et al., 2000, Adie et al., 2007a) which has been broadly reviewed (Beckers & Spoel, 2006, Robert-Seilaniantz et al., 2011a). The following summarizes the role of these molecules/phytohormones in the induction of defence pathways.

SA is associated with the induction of the HR which restricts the spread of biotrophic pathogens locally and produces programmed cell death (PCD) around the infected area. SA functions through feedback loops both upstream and downstream of the HR thus creating a SA-dependent gradient leading to the restriction of cell death development in the primary infection site (Hofius et al., 2007). Local SA levels regulate
HR development and downstream signalling events which are involved in defence gene expression leading to SAR and enhance resistance to secondary pathogen infection on parts distant from the initial primary infection site (Durrant & Dong, 2004, Thatcher et al., 2005, Beckers & Spoel, 2006). Induction of SAR is dependent on the endogenous SA accumulation and transmission of the SA signal through the activity of the regulatory protein NPR1 (Figure 1). In response to SA, the NPR1 protein moves into the nucleus and interacts with TGA transcription factors to up-regulate expression of various PR genes (Dong, 2004) some of which have direct antimicrobial activity (Eckardt, 2003).

Jasmonic acid (JA) and its derivatives, collectively known as jasmonates, are lipid-derived signalling molecules which originate from the plastid membrane α-linolenic acid (Schaller & Stintzi, 2009, Wasternack & Kombrink, 2010). Jasmonates are involved in developmental processes such as flower and fruit development, root growth and senescence (Beckers & Spoel, 2006), as well as being involved in responses to several different biotic/abiotic stresses (Schaller & Stintzi, 2009). Although JA is an important modulator of defence against necrotrophic pathogens, it seems to be involved in other aspects of plant-pathogen interactions such as SAR (Truman et al., 2007). JA-related signalling responses are mediated through an SCF F-box subunit; CORONATINE INSENSITIVE1 (COI1) (Afek & Sztejnberg, 1989, Fonseca et al., 2009, Sheard et al., 2010) and largely through JASMONATE RESISTANT 1 (JAR1) which conjugates isoleucine to JA to form the active signalling compound JA-Ile (Turner et al., 2002). Arabidopsis thaliana coi1 mutants are susceptible to the necrotrophic bacterial leaf pathogen Erwinia carotovora (Norman-Setterblad et al., 2000) and the necrotrophic fungi Alternaria brassicicola and Botrytis cinerea (Thomma et al., 2001), and exhibit enhanced SA levels (Kloek et al., 2001). The importance of
jasmonates in plant defence against wounding (Pozo et al., 2005, Beckers & Spoel, 2006) and in response to pathogens (Pozo et al., 2005) has been well documented.

Ethylene (ET) is a gaseous hormone involved in the regulation of many developmental and physiological processes in plants such as leaf and flower senescence and fruit ripening (van Loon et al., 2006a). ET is also a signalling hormone associated with plant defence responses to pathogen attack (Chen et al., 2005, Broekaert et al., 2006, Adie et al., 2007b), insect attack (Kahl et al., 2000) or abiotic stresses (Francia et al., 2007). In addition, ET stimulates specific types of PR proteins and phytoalexin enzymes which lead to resistance in various plants (Chen et al., 2002, van Loon et al., 2006a). In many circumstances, the amount of ethylene synthesized is negatively correlated with the progression of the disease (Broekaert et al., 2006, van Loon et al., 2006b). However, ET mediates different types of induced resistance and differentially affects resistance against pathogens with different lifestyles (van Loon et al., 2006a).

The Arabidopsis genome encodes five ET receptors including ETR1, ERS1, ETR2, ERS2, and EIN4 (ETHYLENE INSENSITIVE 4) that are localized in the endoplasmic reticulum (ER) and act as negative regulators of ET signalling (Hua & Meyerowitz, 1998, Stepanova & Alonso, 2009). ET receptors interact with CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) that negatively regulates downstream components of the ET pathway and is inactivated by ET, resulting in suppression of downstream ET signalling components such as EIN2 and EIN3 (Stepanova & Alonso, 2009). EIN3 is a short-lived transcription factor and EIN3-related ET response transcription factors (EIL; EIN3-Like) play a key role in defence pathways (Zhong et al., 2009) by regulating the expression of ERF (ETHYLENE RESPONSE FACTOR) genes such as ERF1. The ERF1 genes encode ET-response element binding protein (AP2/EREBP) (Riechmann et al., 2000) transcription factors involved in plant defence against necrotrophic pathogens and in both JA and ET signalling and regulating expression of the PR proteins including
**PDF1.2** (PLANT DEFENSIN 1.2) and **THI2.1** (THIONIN 2.1) (Figure 1) (Lorenzo *et al.*, 2003).

The phytohormone abscisic acid (ABA) plays a key role in the induction of resistance to abiotic stresses (Nambara & Marion-Poll, 2005) and is involved in root geotropism, seed and bud dormancy and the closure of stomata through stomatal guard cells (Yoshida *et al.*, 2006, Jones, 2008, Mori & Murata, 2011, Takezawa *et al.*, 2011). ABA also plays a multifaceted and important role in plant immunity (Ton *et al.*, 2009, Cao *et al.*, 2011). ABA is involved in a complicated network of antagonistic and synergistic interactions and its positive or negative impact on the outcome of plant-pathogen interactions seems to depend on the pathogen’s lifestyle and the timing of the defence response (Ton *et al.*, 2009). ABA has been shown to enhance the susceptibility of plants to biotrophic/hemibiotrophic pathogens such as *Cladosporium cucumerinum* and *Phytophthora infestans* (Henfling *et al.*, 1980), *Fusarium oxysporum* (Anderson *et al.*, 2004), and *Phytophthora sojae* (McDonald & Cahill, 1999), whilst it increases resistance to the necrotrophic pathogens *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Ton *et al.*, 2009). These outcomes highlight the different function of ABA in response to necrotrophic and biotrophic/hemibiotrophic infections.

The plant phytohormone auxin is a central player in the regulation of plant growth and is involved in almost all aspects of plant development (Hay *et al.*, 2006, Benjamins & Scheres, 2008, Petricka & Benfey, 2008, Vanneste & Friml, 2009). Indole-acetic acid (IAA) is the most common natural auxin found in plants and at the cellular level controls elongation, division, and differentiation of the plant cell as well as formation of lateral roots (Casimiro *et al.*, 2003). In addition to being involved in the growth of plants, auxin plays an important role in plant–pathogen interactions (Yamada, 1993, Robert-Seilanianz *et al.*, 2011a). Elevation of auxin signalling correlates with enhancement of susceptibility to biotrophic pathogens (Robert-Seilanianz *et al.*, 2011a).
However, the Arabidopsis auxin signalling mutants; axr1, axr2 and axr6 that are defective in the auxin-stimulated SCF (SKp1-Cullin-F-box) ubiquitination pathway exhibited an increased susceptibility to the necrotrophic pathogens Plectosphaerella cucumerina and B. cinerea indicating that inhibition of auxin signalling in some instances leads to susceptibility of plants to necrotrophic pathogens (Llorente et al., 2008).

1.4 Interactions between plant hormones and defence responses

The interactions between phytohormones and their effect on the outcome of plant-pathogen interactions are complex (Figure 1). Some of the observations and insights on the interactions between these hormones and their effect on the induction of resistance against pathogens are summarised below.

Although, JA can antagonize ET signalling to promote lesion containment (Hofius et al., 2007), the altered susceptibility of A. thaliana mutants defective in JA and ET biosynthesis or signalling indicates that JA and ET can work synergistically to induce defence responses. The classic example of their positive interaction is the pathogen-induced expression of the PDF1.2 gene in A. thaliana, which requires a parallel induction of the JA and ET signalling pathways for full expression (Penninckx et al., 1998). Lorenzo et al. (2003) demonstrated that the JA and ET signalling pathways join in the activation of ERF1 (encoding ethylene-response factor 1) transcription and ERF1 regulated a large group of genes responsive to both ET and JA indicating that in the activation of some pathogen-defence responses the JA and ET signalling pathways work synergistically (Pozo et al., 2005).

SA signalling is commonly associated with resistance to biotrophic pathogens, whilst a combination of JA and ET trigger resistance to necrotrophic pathogens (McDowell et al., 2000, Thomma et al., 2001, Oliver & Ipcho, 2004, Glazebrook, 2005). However, there are some exceptions among hemibiotrophs (Thaler et al., 2004,
van Loon et al., 2006b, Francia et al., 2007). The SA and JA/ET signalling pathways are usually antagonistic and therefore activation of resistance to a biotrophic pathogen is often associated with an increase in susceptibility to necrotrophic pathogens (Robert-Seilanianz et al., 2011a).

The antagonistic interaction between the SA and ABA signalling pathways has been suggested in different studies (Ward et al., 1989, de Torres Zabala et al., 2009, Fan et al., 2009). ABA interferes with SA-related defences in the soybean–P. sojae pathosystem (Ward et al., 1989) and ABA was shown to have a suppressive effect on SAR in a JA/ET independent pathway (Yasuda et al., 2008). Moreover, activation of SAR represses both ABA biosynthetic and ABA-responsive genes.

SA and auxin signalling pathways also mostly interact antagonistically. Induction of auxin signalling increases susceptibility to biotrophic pathogens and auxin treatments resulted in the suppression of PRI gene expression (SA-responsive gene) (Park et al., 2007, Robert-Seilanianz et al., 2011b). Furthermore, the suppression of the auxin signalling pathway increased the susceptibility to some necrotrophic pathogens (Llorente et al., 2008). Auxin signalling affects SA synthesis and SA treatment stabilises the auxin AUX-IAA negative regulators including AXR3 and AXR2 (Wang et al., 2007) indirectly through suppression of the F-box auxin receptor TIR1 and therefore decreases the auxin sensitivity and limits ubiquitination of AXR2 and other AUX-IAA proteins. Moreover, activation of the auxin signalling pathway suppresses SA biosynthesis and the SA signalling pathway (Robert-Seilanianz et al., 2007).

Auxin also interacts with the JA signalling pathway although this interaction appears complex. Nagpal et al. (2005) demonstrated a decrease in JA concentration in an auxin loss-of function mutant (tir1), while the ARF6 and ARF8 (auxin response regulators), promoted JA biosynthesis (Tabata et al., 2010). Auxin treatment also
suppressed the JA signalling pathway (Rojo et al., 1998) and similarly microarray analysis showed that auxin treatment represses JA biosynthesis (Liu & Nester, 2006).

AtMYC2 is a positive regulator of the ABA signalling pathway and allelic to JASMONATE INSENSITIVE1 (JIN1) (Abe et al., 2003). AtMYC2 has a crucial role in biotic/abiotic stress responses and differently modulates the expression of JA-induced genes. AtMYC2 negatively regulates the genes involved in JA/ET defence responses against pathogens but activates the genes involved in JA-mediated responses to herbivory (Lorenzo et al., 2004). Furthermore, the exogenous application of ABA represses the transcription of the JA/ET-related genes such as PDF1.2, CHI, and PR4 in a MYC2 dependent manner while over expression of AtMYC2 suppressed the expression of the PDF1.2 gene (Anderson et al., 2004, Lorenzo et al., 2004). PDF1.2 is a JA/ET responsive defence gene which is negatively regulated by AtMYC2 and ABA while other JA responsive defence genes such as VSP and PI are positively regulated by both JA and AtMYC2/ABA (Lorenzo et al., 2004). Anderson et al. (2004) demonstrated an antagonistic interaction between ABA and ET signalling mutants. Consistent with this, the exogenous application of ABA on tomato ABA-deficient and maize ABA biosynthesis mutants resulted in suppression of ET production (Beaudoin et al., 2000, Spollen et al., 2000, LeNoble et al., 2004).
Figure 1
**Figure 1** An overview of the interactions between the salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and auxin signalling pathways in *Arabidopsis* and their role in the induction of resistance against pathogens. ROS, reaction oxygen species; SAR, Systemic acquired resistance; ISR, induced systemic resistance; HR, hypersensitive response. The figure was assembled based on references cited in the text.
1.5 Ubiquitin proteasome pathway (UPP) and defence signalling

There is growing evidence on the importance of the ubiquitin proteasome pathway (UPP)-mediated events both in plant defence mechanisms (PTI, ETI, SAR, and ISR) and in pathogen virulence. UPP is required for various steps in SA, JA, ET, ABA and auxin signal transduction cascades which impact on defence signalling in plants (Table 1-1) (Zeng et al., 2006). The ubiquitin/26S proteasome system represents the major protein degradation pathway in the cell and plays an essential role in the degradation of proteins that are abnormal or no longer required (Zeng et al., 2006). UPP is also involved in the fine tuning of cellular functions and signalling pathways by regulating the intracellular levels of a wide range of proteins and hormones, including those involved in the control of the cell cycle, transcriptional activation, apoptosis, cell signalling (Glickman & Ciechanover, 2002, Smalle & Vierstra, 2004), organ initiation and patterning (Samach et al., 1999, Shen et al., 2002, Imaizumi et al., 2005) and plant defence (Kim & Delaney, 2002, Xu et al., 2002).

In A. thaliana, ET up-regulates the transcription of many defence-related genes encoding cell-wall-modifying enzymes or protein components, oxidative burst regulators, and PR proteins (Zhong & Burns, 2003). ET acts through the EIN3 (Ethylene-insensitive 3) family of transcription factors which are substrates of the UPP (van Loon et al., 2006b). Jasmonic acid also regulates many of above defence-related genes by acting through the ERF/AP2 transcription factors which are involved downstream of the EIN3 family (reviewed in Guo & Ecker (2004)). Treating plants with elicitors of pathogens induces transcription of not only defence genes, but also of a number of UPP genes including ubiquitin conjugating enzyme OsUBC5b and a RING E3 ligase ELS in rice (Takai et al., 2002). On the other hand, it has been suggested that ubiquitination may be a mechanism of recognition of the pathogen on the plant cell surface (Craig et al., 2009). If this is the case, products from proteolysis of pathogen
elicitins by the host plant may be a signal that leads to stimulation of defence responses. Recognition of elicitins by plants may lead to the induction of the hypersensitive response (HR) at the site of pathogen infection (Ricci et al., 1989, Kamoun et al., 1993). Elicitin like proteins from Pythium induced defence gene expression in A. thaliana and this induction required the RAR1 and SGT1 genes (Kawamura et al., 2009). Elicitins produced by pathogens such as Phytophthora are sterol binding proteins, and the importance of sterols for auxin signalling (Pan et al., 2009) indicates a potential link between elicitins and auxin signalling regulated by the UPP.

Three distinct enzymatic activities are required in the UPP. Ubiquitin is first catalysed by the enzyme E1 (ubiquitin-activating enzyme, UBA) and then transferred to a cysteinyl sulphydryl on E2 (ubiquitin conjugating protein, UBC). Finally, E3 (ubiquitin ligase) helps to recruit the substrate and transfer ubiquitin from E2 to the target protein (substrate) (Pickart & Eddins, 2004). The Arabidopsis genome encodes only two E1s, at least 45 E2-like proteins and nearly 1200 E3 components (Vierstra, 2003).

Most of the complexity in the UPP in Arabidopsis is within the E3s which confer substrate specificity. The E3s were named SCF based on the name of their three subunits, SKP1, Cdc53 (or Cullin; CUL1), and F-box protein (Deshaiies, 1999). The RBX1 (or ROC1) is the fourth subunit of SCF which contains a Ring H2-type Ring finger that binds E2-Ub. The SCF are complexes of four polypeptides which together have ubiquitin ligase activity. SCF E3s function as scaffolds that bring the activated Ub-E2 intermediate together with the targets to promote transfer without forming E3-Ub intermediate (Vierstra, 2003). The specificity of the SCF complex is determined by the F-box subunit which contains a protein interaction motif at its C-terminus and a signature F-box motif at its N-terminus (Jackson et al., 2000a, Gagne et al., 2002). The RING-finger protein RBX1 is a core element of the SCF complex which interacts
directly with CUL1 and SKP1 links CUL1 to a F-box protein which is involved in recruiting special substrates to the SCF complex for ubiquitination (Craig & Tyers, 1999). Although, Arabidopsis contains 694 F-box proteins, only a few have a known function. Some of the E3s and SCF-F-box complexes in A. thaliana are involved in the auxin response, Pi signalling and pathogen resistance (Table 1-1).

### 1.5.1 SCF\(^{\text{TIR1}}\) complex

**TIR1** encodes an auxin receptor which mediates auxin-regulated transcription. TIR1 contains a leucine-rich repeat and an F-box and interacts with ASK1, ASK2 and AtCUL1 to shape SCF-TIR1, a SCF ubiquitin ligase complex. As part of the SCF complex and in the presence of auxin, TIR1 interacts with the AUX/IAA transcriptional repressor proteins and mediates their degradation. Previous investigations showed that the SGT1B protein is required for SCF\(^{\text{TIR1}}\) mediated auxin response (Gray et al., 2003). SGT1B functions in both plant disease resistance signalling and SCF-TIR1 mediated degradation of AUX/IAA proteins (Austin et al., 2002, Tor et al., 2002, Gray et al., 2003).

### 1.5.2 SCF\(^{\text{COII}}\) complex

COI1 is a positive regulator of JA signalling and it is thought to act as a selective de-repressor of JA-responsive genes by targeting repressor proteins for degradation (Sheard et al., 2010, Robert-Seilanianz et al., 2011a). Importantly, COI1 interacts with SKP1 and CUL1 proteins *in planta*, indicating that COI1 is indeed part of a SCF-COI1 ubiquitin ligase complex (Devoto et al., 2002). Thus, JA activates the regulatory protein COI1 that is part of the E3 ubiquitin ligase-containing complex SCF\(^{\text{COII}}\), which is thought to de-repress JA-responsive genes involved in plant defence (Xu et al., 2002).
Table 1: Selected ubiquitin/26S proteasome pathway component genes from *Arabidopsis* and their functions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>AGI</th>
<th>Protein</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIR1</td>
<td>AT3G6</td>
<td>E3 (F-box)</td>
<td>SCF^{TIR1}\textsuperscript{;} Auxin responses</td>
<td>Gray <em>et al.</em> (2001)</td>
</tr>
<tr>
<td>SGT1B</td>
<td>AT4G1</td>
<td>E3 (SGT1-)</td>
<td>pathogen resistance</td>
<td>Austin <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>COI1</td>
<td>AT2G3</td>
<td>E3 (F-box)</td>
<td>SCF^{COI1}\textsuperscript{;} Jasmonate and ethylene</td>
<td>Xu <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>SON1</td>
<td>AT2G1</td>
<td>E3 (F-box)</td>
<td>SCF^{SON1}\textsuperscript{;} pathogen resistance</td>
<td>Kim &amp; Delaney (2002)</td>
</tr>
<tr>
<td>FBX2</td>
<td>AT5G2</td>
<td>E3 (F-box)</td>
<td>SCF^{FBX2}\textsuperscript{;} Pi signalling</td>
<td>Chen <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>ASK1</td>
<td>AT1G7</td>
<td>E3 (SKP)</td>
<td>Auxin responses</td>
<td>Yang <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>RBX1</td>
<td>AT5G2</td>
<td>E3 (RBX)</td>
<td>Auxin responses</td>
<td>Gray <em>et al.</em> (2002)</td>
</tr>
</tbody>
</table>
1.5.3 SCF$^{\text{SON1}}$ complex

SON1 is a constituent of the SCF ubiquitin ligase complex and encodes an F-box protein which regulates a novel induced defence response independent of SA and SAR (Kim & Delaney, 2002). The transcriptional level of $\text{SON1}$ does not change in response to pathogen or the analogue of SA (2,6-Dichloroisonicotinic acid; INA). However, post-translational modifications to SON1 following a biotic challenge decrease SON1’s activity and therefore enable the plant to mount a successful defence response (Kim & Delaney, 2002).

1.5.4 SCF$^{\text{FBX2}}$ complex

F-BOX PROTEIN 2 (FBX2) is a sub-unit of the SCF ubiquitin ligase complex and encodes an F-box containing protein that interacts with BHLH32 protein. FBX2 is involved in mediating the PSR and cellular response to phosphate starvation (Chen et al., 2008). FBX2 like BHLH32 negatively regulates several Pi starvation responses such as expression of $\text{PPCK1}$, formation of root hairs and the accumulation of anthocyanins (Chen et al., 2008).

1.6 Phosphate and the phosphate starvation response (PSR)

Phosphorus (P) plays a vital role in various basic biological functions as a structural element in nucleic acids and phospholipids, as a facilitator in energy metabolism and the activation of metabolic intermediates, as a component in signal transduction cascades and the regulation of enzymes (Ticconi et al., 2001, Hammond et al., 2004). Despite its importance, phosphorus is one of the most limiting nutrients for plants because inorganic orthophosphate (Pi), the preferential assimilated form, is more than 80% immobile in soil and not readily available to roots (Marschner, 1995, Holford, 1997, Ticconi et al., 2001).
Plants show many adaptations to allow growth under conditions of low Pi availability including biochemical responses and morphological changes in root architecture (Figure 2). Under Pi deficiency stress, roots increase secretion of protons or organic acids that enhance the solubilisation of insoluble inorganic Pi complexes (Poirier & Bucher, 2002). The Pi starvation response 1 (PHR1) is activated under Pi deficiency conditions (Bari et al., 2006) and up-regulates a series of Pi-responsive genes (Franco-Zorrilla et al., 2004, Rubio et al., 2007). Pi starvation also increases the expression of a series of Pi acquisition enzymes and some regulatory proteins such as nucleases and phosphatases to acquire Pi from organic sources (Poirier & Bucher, 2002). In addition, Pi-binding proteins, high-affinity Pi transporters, and Pi sensor protein kinases that check extracellular Pi availability increase due to Pi starvation (Torriani-Gorini, 1994). An increase in the synthesis of anthocyanins which regulate photosynthetic light reactions to the Pi-dependent Calvin cycle is also one of many biochemical adaptations to Pi starvation (Trull et al., 1997, Raghothama, 1999).

Numerous Pi-responsive genes have been identified including three common marker genes such as AtPT2, a Pi transporter (Muchhal & Raghothama, 1999); AtACP5, an acid phosphatase (del Pozo et al., 1999), and At4 (Burleigh & Harrison, 1999). At4 is “a cDNA representing a Pi-starvation-inducible gene” which is up-regulated in A. thaliana roots in response to Pi deficiency and was down regulated in response to Pi fertiliser (Burleigh & Harrison, 1999). Alternately, AT4 could not be down-regulated in phol mutants which are blocked in loading Pi from roots to shoots even after Pi fertilisation (Burleigh & Harrison, 1999, Hamburger et al., 2002), indicating that down-regulation of the At4 gene depended on translocation of Pi from roots to shoots (Figure 2).

Phosphate starvation in A. thaliana inhibits the growth of the primary root, causes elongation of lateral roots (Williamson et al., 2001, Linkohr et al., 2002, Lo´pez-Bucio et al., 2002, Al-Ghazi et al., 2003) and the formation of root hairs (Bates & Lynch,
Pi starvation also promoted the formation of proteoid roots in *Lupinus albus* (Johnson *et al.*, 1994, Johnson, 2004). Auxin signalling is one of the many pathways involved in the Pi starvation response (Rietz *et al.*, 2010) and is strongly involved in stimulating root development to Pi limiting conditions (Lo´pez-Bucio *et al.*, 2002, Al-Ghazi *et al.*, 2003). Auxin influences root morphology by inhibiting primary root elongation and increasing lateral root formation (Perez-Torres *et al.*, 2008, Yoon *et al.*, 2009, Rietz *et al.*, 2010). Primary root elongation and lateral root formation can either be induced or inhibited by the application of exogenous auxin (Evans *et al.*, 1994, Himanen *et al.*, 2002), while auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) can radically suppress lateral root formation (Fujita & Syono, 1996, Casimiro *et al.*, 2001). Furthermore, *Arabidopsis* mutants such as sur1/alf1 (SUPERROOT 1 PROTEIN /ABERRANT LATERAL ROOT FORMATION 1) with high concentrations of endogenous auxin showed an increase in the number of lateral roots (Boerjan *et al.*, 1995). Auxin signalling is mediated largely by the SCF<sup>TIR1</sup> E3 ubiquitin ligase complex that accelerates AUX/IAA repressor protein degradation in response to auxin, thus altering gene expression (Wooward & Bartel, 2005, Perez-Torres *et al.*, 2008). TIR1 (Transport Inhibitor Response 1), an auxin receptor F-box protein is a part of the SCF complex (SCF<sup>TIR1</sup>) which is required for normal response to auxin is involved in cellular response to Pi starvation (Gray *et al.*, 2001, Dharmasiri *et al.*, 2005, Kepinski & Leyser, 2005, Perez-Torres *et al.*, 2008). Pi deprivation increases the expression of the TIR1 gene in *Arabidopsis* seedlings and causes AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) auxin response repressors to be degraded (Figure 3) (Perez-Torres *et al.*, 2008). The finding that the tir1-1 mutant is impaired in its ability to increase lateral root formation in response to low Pi availability and is compromised in auxin responses (Gray *et al.*, 1999, Ruegger *et al.*, 2010) indicates that TIR1 modulated auxin sensitivity is important in Pi starved roots (Perez-Torres *et al.*, 2008).
Figure 2 The morphological, biochemical and hormonal changes in response to phosphate starvation in Arabidopsis thaliana. The figure was compiled from papers cited in the text. AtPT2, a Pi transporter; AtACP5, an acid phosphatase; At4, a Pi-starvation-inducible gene.
SGT1B protein is required for SCF<sub>TIR1</sub> mediated auxin response (Gray <em>et al.</em>, 2003) and functions in both plant disease resistance signalling and SCF<sub>TIR1</sub> mediated degradation of AUX/IAA proteins (Austin <em>et al.</em>, 2002, Tor <em>et al.</em>, 2002, Gray <em>et al.</em>, 2003). SGT1B also interacts with RAR1; a component of R-gene-mediated resistance (Azevedo <em>et al.</em>, 2002, Dodds & Schwechheimer, 2002, Tor <em>et al.</em>, 2002). SGT1B is required for responses that are mediated by a diverse range of R-gene structural types, which induce resistance against a variety of pathogens (Austin <em>et al.</em>, 2002, Tor <em>et al.</em>, 2002, Azevedo <em>et al.</em>, 2006). Both RAR1 and SGT1 interact with the COP9 signalosome in plants which is a multi-protein complex mediating a number of physiological responses controlled by SCF-type E3 ligases and is involved in protein degradation by the 26S proteasome (Dodds & Schwechheimer, 2002). This interaction between RAR1, SGT1B and COP9 indicates that these multi-protein complexes may have a vital role in establishing disease resistance (Vierstra, 2003) (Figure 3) and supports a possible link between Pi signalling and the outcome of plant pathogen interactions.

1.7 Phosphite

Phosphite (Phi) is a salt of phosphorous acid (H<sub>3</sub>PO<sub>3</sub>) which is systemically mobile within the plant. Neutralizing phosphorous acid with a base (KOH) results in production of a salt which is Phi (KH<sub>2</sub>PO<sub>3</sub> or K<sub>2</sub>HPO<sub>3</sub>):

\[
\text{KH}_2\text{PO}_3 + \text{K}_2\text{HPO}_3
\]

The anionic form of phosphorous acid (phosphate or phosphonate) is used in the formulation of fungicides containing either the potassium salt of Phi (Foli-R-Fos) or the aluminum salt of ethyl-phosphonate (Fosetyl-Al) (Dunhill, 1990, Martin <em>et al.</em>, 1998). Grant <em>et al.</em> (1990) stated that phosphonate must refer to the salts of phosphonic acid but according to IUPAC nomenclature both terms Phi and phosphonate are acceptable (Roos <em>et al.</em>, 1999). Therefore, the term Phi will be used in this thesis for both Phi and phosphonate.
**Figure 3.** The links/interactions between auxin signalling and ubiquitin proteasome pathways in relationship to phosphate starvation and systemic acquired resistance (SAR) in *Arabidopsis*. This figure was assembled based on papers cited in the text.
Phi is effective in the control of a range of diseases caused by oomycetes and in particular by *Phytophthora* species (Martin *et al*., 1998, McDonald *et al*., 2001). Phi is translocated in both the phloem and xylem after being taken up through roots, stems, leaves or flowers and inhibits but does not kill the pathogen (Ouimette & Coffey, 1990, Hardy *et al*., 2001). The protective function of Phi in plants is more effective if used as a preventive tool (Hardy *et al*., 2001). Detection of Phi in the roots after foliar spray or trunk injections proved the phloem mobility of Phi (Ouimette & Coffey, 1989b, Schutte *et al*., 1991, Whiley *et al*., 1995, Jackson *et al*., 2000b). In some Australian native species, *Banksia telmetia* and *Lambertia multiflora*, a greater concentration of Phi is found in roots than shoots (Komorek & Shearer, 1997). Trunk injections are effective for longer than foliar applications, persisting several years, depending on the species (Shearer & Fairman, 2007). In Western Australia, Phi is applied either as foliar spray (5 g/L per tree) in 6-12 month intervals, 100-400 g/L as an autumn aerial spray in plant communities with high conservation value or as a trunk injection in spring (50-200 g/L per tree) (Hardy *et al*., 2001).

### 1.8 Mode of action of Phosphite

Despite the common use of Phi as a fungicide, knowledge of its complex mode of action and the mechanisms underlying Phi ability to protect plants against infection by *Phytophthora* species is incomplete. Although Phi concentrations in treated plants were high enough to inhibit the pathogen directly, plant defence responses were also likely to contribute to the pathogen growth inhibition (Smillie *et al*., 1989). Guest (1986) also described a mixed direct and indirect mode of action for Phi. Several studies have also suggested that Phi demonstrates a complex mode of action including (i) acting directly by suppression of pathogen growth as a result of accumulated Phi in plant tissue, (ii) acting indirectly by stimulating the release of stress metabolites (elicitors) from the pathogen to elicit the plant defence responses, and (iii) directly by stimulating host
defence responses (Coffey & Bower, 1984, Jackson et al., 2000b, Daniel & Guest, 2006).

1.8.1 Direct effect on pathogen

Phi and fosetyl-Al inhibit the growth and reproduction of many Phytophthora species (Coffey & Bower, 1984, Coffey & Joseph, 1985, Fenn & Coffey, 1985, Dolan & Coffey, 1988, Ouimette & Coffey, 1989a, Komorek & Shearer, 1997, Wilkinson et al., 2001). A direct effect of Phi on P. cinnamomi in vitro is evident from suppression of mycelia growth, oospores and sporangia formation, zoospore release and chlamydospore production (Coffey & Joseph, 1985). Grant et al. (1990) also showed that Phi can directly inhibit the growth of P. palmivora. Phi tolerant mutants of P. palmivora and P. capsici showed an enhanced level of resistance to Phi both in vitro and in vivo (Dolan and Coffey 1988) supporting direct action of Phi on the pathogen. The sensitivity of Phytophthora to Phi varies within species (Coffey & Bower, 1984, Fenn & Coffey, 1984, Coffey & Joseph, 1985, Ouimette & Coffey, 1989a) and the efficiency of Phi uptake could be the cause of these variations (Barchietto et al., 1989). The concentration of Pi, a competitive inhibitor of Phi uptake, in the growth medium can also greatly influence the inhibitory effect of Phi on mycelial growth so that a higher concentration of Phi is required for inhibition of mycelium as the concentration of Pi increases (Fenn & Coffey, 1984, Komorek & Shearer, 1997).

1.8.2 Indirect effect by stimulating plant defence mechanisms

The enhanced stimulation of defence responses in Phi-treated plants following pathogen challenge has been demonstrated in several plant-pathogen interactions (Grant et al., 1990, Jackson et al., 2000b, Daniel & Guest, 2006). Phosphite treatment increased the synthesis and accumulation of phenolics in Eucalyptus marginata (Jackson et al., 2000b), phytoalexins in P. cryptogea-infected cowpea (Saindrenan et
al., 1988) and phytoalexins in citrus species inoculated with *P. citrophthora* (Afek & Sztejnberg, 1989). Daniel *et al.*, (2005) also showed that Phi-treated *Xanthorrhoea australis* seedlings accumulated high levels of lignin-like and phenolic compounds particularly in vascular cell walls of infected tissue in response to *P. cinnamomi* infection.

### 1.9 Phosphite (Phi) and phosphate signalling pathway

Phi exclusively interferes with the manifestation of a wide range of biochemical and developmental phosphate (Pi) starvation responses (PSR) in *A. thaliana* and other plant species (Ticconi *et al.*, 2004, Lee & Tsai, 2005, Fang *et al.*, 2009, Li *et al.*, 2010). Phi at concentrations lower than 2.5 mM repressed induction of the Pi starvation-inducible enzymes, phosphoenolpyruvate phosphatase, pyrophosphate-dependent phosphofructokinase and high-affinity Pi uptake, and altered the root to shoot ratio of seedlings under Pi deficient conditions (Ticconi *et al.*, 2001). Similar results were reported for *Brassica nigra* (Carswell *et al.*, 1997). Application of Phi also repressed induction of the Pi-responsive mRNAs, *AtPT2*, *AtACP5* and *At4*, in *A. thaliana* under Pi deficient conditions (Ticconi *et al.*, 2001). Phi at concentrations above 2.5 mM inhibited *A. thaliana* growth in both phosphorus-sufficient (+Pi or Pi/+RNA) and deficient (-Pi) media. However, Phi at concentrations lower than 2.5 mM had only a minor effect on growth of seedlings in different Pi conditions (Ticconi *et al.*, 2001). Similar results were found for *B. nigra* seedlings and *B. napus* cell suspension cultures (Carswell *et al.*, 1996). Phi-inhibited growth was strongly correlated with reduced internal Pi concentrations suggesting competitive action of Phi on Pi assimilation as a cause for growth inhibition (Carswell *et al.*, 1996). Together these Phi effects indicate that Phi may be a useful inhibitor to manipulate the Pi starvation response pathways in plants.
1.10 Plant-Phytophthora interaction

Plants normally respond to Phytophthora attack by the formation of cell wall appositions and restructuring of the cytoskeleton around the invading hyphae. Reactive oxygen species (ROS) are then produced to initiate hypersensitive cell death and strengthen the cell wall, as well as the induction of pathogenesis related (PR) proteins and phytoalexins to fight against the progression of the pathogen (Cahill et al., 2002, Walker et al., 2006, Smertenko & Franklin-Tong, 2011). Although a range of defence genes are up-regulated in response to Phytophthora attack, the pathogen lifestyle generally dictates the type and efficiency of the defence responses activated in plants and different plant defence pathways are involved with different Phytophthora species (Roetschi et al., 2001, Khatib et al., 2004).

Typically JA-dependent defence responses are considered important in defence against necrotrophic pathogens, while SA-responsive defence responses are typically associated with resistance to biotrophic pathogens (McDowell et al., 2000, Thomma et al., 2001, Oliver & Ipcho, 2004, Glazebrook, 2005). Significant interaction between the core defence response pathways ET, JA, SA and isoflavonoid phytoalexins induced by these pathways are important plant responses to various Phytophthora species (Cosio et al., 1996, Subramanian et al., 2005, Graham et al., 2007). Molecular studies on A. thaliana-Phytophthora interactions showed the existence of substantial cross-talk and connection between the SA, JA and ET pathways or alternative pathways in resistance to Phytophthora species. Khatib (2004) suggested different involvement of all three (SA, JA and ET) signalling pathways in the A. thaliana response to the biotroph P. parasitica. In the JA perception mutant (coi1) and the ET mutant (ein2), CBEL (an elicitor from P. parasitica) failed to induce necrosis and the expression of defence genes regulated by SA was affected in nahG; SA deficient transgenic plants (Khatib et al., 2004). However, resistance to the facultative biotroph P. porri was independent of SA,
JA and ET signalling pathways because *A. thaliana* mutants defective in JA (*jar1*), ET (*ein2*), SA (*npr1* and *sid2*) and nahG transgenic plants remained resistant to *P. porri* (Roetschi *et al*., 2001).

**1.11 Screening methods for Phytophthora**

Studying interactions between *Phytophthora* pathogens and their hosts is an essential step in disease management strategies and a reliable method for measuring the disease progression is an important component of these studies. The model plant *A. thaliana* has been used to study various fungal, bacterial and oomycete pathogen–plant interactions (Glazebrook, 2001, Thomma *et al*., 2001). However, *Arabidopsis* exhibits a strong non-host resistance to most *Phytophthora* species, including *P. infestans* and *P. sojae* (Kamoun *et al*., 1999, Kamoun, 2001, Takemoto *et al*., 2003). Currently, the assessment of *Phytophthora* disease progression depends on symptomology, lesion measurements or sporangia counting (Rookes *et al*., 2008). However, these techniques are of restricted use in studies where the extent of lesion development is limited or in the early stages of infection when resistance is first expressed.

To overcome the problems associated with visual assessments, quantitative polymerase chain reaction (QPCR) assays have been developed for several oomycete pathogens, such as *P. infestans* and *P. citricola* (Bohm *et al*., 1999), *P. capsici* (Silvar *et al*., 2005), *P. cinnamomi* (Kong *et al*., 2003), and other pathogens (Bohm *et al*., 1999, Schaad & Frederick, 2002, Brouwer *et al*., 2003, McCartney *et al*., 2003, Schaad *et al*., 2003, Lievens *et al*., 2006). QPCR determination of pathogen biomass is rapid, sensitive, specific and very efficient for detecting even early stages of infection when there is little pathogen biomass. Despite the benefits of the technique, some existing problems, such as the requirement for a reliable normalization technique, may confound results. Frequently, in QPCR assays, the pathogen biomass is measured and normalized either to host plant DNA, the sample surface area or the weight of the collected sample.
However, measurement of the pathogen biomass relative to the amount of plant DNA may lead to an overestimation of pathogen biomass especially at late stages of infection by necrotrophs, where host DNA degradation is often associated with necrosis (Brouwer et al., 2003, van Wees et al., 2003). Furthermore, the validity of pathogen biomass measurements based on sample surface area or weight may also be significantly affected by variable DNA extraction yield between the samples (Gachon & Saindrenan, 2004). Therefore, developing a sensitive QPCR assay to detect pathogen biomass at any stage of pathogen-host interaction, avoiding problems caused by tissue necrosis including host DNA degradation and variable DNA extraction yield within biological samples is required.

1.12 Summary

Understanding interactions between Phytophthora pathogens and their host plants is an essential step in disease control strategies and the model plant A. thaliana has been used extensively to study such interactions (Khatib et al., 2004, Senchou et al., 2004, Wang et al., 2011). Although some ecotypes of A. thaliana are resistant to P. cinnamommi, this pathogen can infect both roots and leaves and complete its life cycle on some A. thaliana ecotypes (Robinson & Cahill, 2003) making Arabidopsis an attractive model plant to investigate mechanisms of resistance to this pathogen.

The plant phytohormones such as SA, JA, ET, auxin and ABA are involved in activation of defence pathways and modulating plant-pathogen interactions. UPP is required for various steps in SA, JA/ET, ABA and auxin signal transduction cascades which are involved in defence signalling in plants (Zeng et al., 2006). Microarray analysis in A. thaliana has revealed significant cross-talk between defence pathways induced by these phytohormones (Schenk et al., 2000, Beckers & Spoel, 2006, Adie et al., 2007a, Robert-Seilaniantz et al., 2011a). The interactions between these
phytohormones/signalling pathways and their effect on the outcome of plant-
\textit{Phytophthora} interactions are complicated and the synergistic and antagonistic
interactions between different signalling pathways and their effect on the induction of
resistance to biotrophic or necrotrophic pathogens have been well documented (Ward \textit{et al.}, 1989, Pozo \textit{et al.}, 2005, Park \textit{et al.}, 2007, de Torres Zabala \textit{et al.}, 2009, Fan \textit{et al.},
indicates the involvement of different plant defence signalling pathways with different
\textit{Phytophthora} species (Cosio \textit{et al.}, 1996, Roetschi \textit{et al.}, 2001, Khatib \textit{et al.}, 2004,

Phi which is highly effective in controlling \textit{P. cinnamomi} (Hardy \textit{et al.}, 2001,
Shearer & Fairman, 2007), interferes with a wide range of biochemical and
developmental PSR in \textit{A. thaliana} and other plant species (Ticconi \textit{et al.}, 2004, Lee &
Tsai, 2005, Fang \textit{et al.}, 2009, Li \textit{et al.}, 2010) indicating a possible role in Pi
homeostasis. Pi status that is very important in determining root architecture mediated
through the auxin signalling pathway and auxin signalling is required for the full Pi
starvation response (Lo´pez-Bucio \textit{et al.}, 2002, Al-Ghazi \textit{et al.}, 2003, Nacry \textit{et al.},

Auxin plays a key role in plant–pathogen interactions and induction of resistance
to necrotrophic pathogens \textit{Plectosphaerella cucumerina} and \textit{Botrytis cinerea} (Yamada,
mediated by the SCF$^{TIR1}$ E3 ubiquitin ligase complex that accelerates AUX/IAA
repressor degradation in response to auxin (Wooward & Bartel, 2005, Perez-Torres \textit{et al.},
2008). The \textit{SGT1B} protein which functions in SCF-TIR1 mediated degradation of
AUX/IAA proteins (Austin \textit{et al.}, 2002, Tor \textit{et al.}, 2002, Gray \textit{et al.}, 2003) interacts
with RAR1; a component of R-gene-mediated resistance (Azevedo \textit{et al.}, 2002, Dodds
& Schuchheimer, 2002, Tor \textit{et al.}, 2002) and both RAR1 and SGT1B interact with the
COP9 which is involved in protein degradation by the 26S proteasome (Schwechheimer et al., 2002). This interaction between RAR1, SGT1B and COP9 suggests a possible link between Pi signalling and plant defence through the UPP.

In conclusion, auxin responsive mutants are susceptible to necrotrophic pathogens and auxin and ABA signalling pathways have positive interactions while they both antagonistically interact with the SA and JA/ET signalling pathways. Therefore, it is relevant to investigate the possible involvement of auxin and ABA as well as SA and JA/ET signalling pathways in the outcome of *A. thaliana*—*P. cinnamomi* interactions. Also the involvement of the auxin signalling pathway in Pi signalling and the interference of Phi in the PSR suggest that Phi mediated resistance could be through its effect on Pi signalling and in particularly on the auxin signalling pathway which is involved in both Pi and defence signalling. Therefore, analysis of gene regulation and screening the *A. thaliana* mutants/transgenic lines defective in the SA, JA, ET, ABA and auxin signalling pathways grown under Pi sufficient and deficient conditions for their response to *P. cinnamomi* infection and Phi treatments will lead to a better understanding of the molecular mechanisms involved in plant—*P. cinnamomi* interactions and the effect of Phi on these interactions. Figure 4 summarizes and links the different sections of this literature review and gives an overview on how these signalling pathways may interact.
Figure 4. The cross talks between different defence signalling pathways (SA, JA, ABA, and auxin), the ubiquitin proteasome pathway (UPP) and Pi signalling and their role in induction of resistance (ISI and SAR) to biotrophic and necrotrophic pathogens.
1.13 Thesis Aims

The overall aim of this research was to study the indirect effect of Phi in conferring resistance to *P. cinnamomi* and to find which plant gene(s) or pathway(s) are involved. The information on gene regulation and alteration in plant metabolites in response to Phi and understanding the link between these changes and Phi induced resistance may ultimately contribute to better management strategies for more successful control of diseases caused by *Phytophthora* species.

To achieve this aim, the following objectives were set to:

1. Examine the effect of Phi on the induction of defence responses in *A. thaliana* with and without *P. cinnamomi* infection and to determine whether this pathosystem can be used for in depth studies on the mechanisms of action of Phi (Chapter 2).

2. Develop a sensitive quantitative PCR (QPCR) assay which enables accurate quantification of *in planta* *P. cinnamomi* biomass at all stages of the plant-pathogen interaction (Chapter 3).

3. Apply this QPCR technique to identify genes and pathways involved in *P. cinnamomi* resistance through screening of *A. thaliana* mutants and germplasm (Chapter 4).

4. Understand the mechanism(s) through which Phi modulates the plant defence response through Phi treatment of *Arabidopsis* defence pathway mutants (Chapter 4).

5. Investigate the concomitant effect of Phi and Pi on the induction of resistance to *P. cinnamomi* by examining the effect of Phi on the Pi starvation response, root morphology, ABA and auxin signalling pathways (Chapter 5).
Chapter 2

Phosphite primed defence responses and enhanced expression of defence genes in *Arabidopsis thaliana* infected with *Phytophthora cinnamomi*

**This chapter has been published:**

Phosphite primed defence responses and enhanced expression of defence genes in *Arabidopsis thaliana* infected with *Phytophthora cinnamomomi*

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This paper describes the effect of phosphite (Phi), a systemic chemical, on the induction of defence responses in Phytophthora cinnamomi-infected Arabidopsis thaliana accessions Ler and Col-0. Application of Phi to non-inoculated A. thaliana seedlings of accession Ler elevated transcription of defence genes in the salicylic acid (PRI and PR5) and jasmonic acid/ethylene (THI2.1 and PDF1.2) pathways. Furthermore, a systemic increase in the expression of the PRI gene was demonstrated in Phi-treated seedlings using the transgenic line PRI::GUS in the presence/absence of the pathogen by 72 h after inoculation. The cells of Phi-treated A. thaliana Ler leaves responded to P. cinnamomi zoospore inoculation with a rapid increase in callose deposition and hydrogen peroxide (H$_2$O$_2$) production. Phi treatment resulted in the production of callose papillae 6 h earlier than in non-Phi-treated inoculated seedlings and enhanced the production of H$_2$O$_2$ in the leaves of A. thaliana at the site of hyphal penetration and in cells away from the inoculation point. By 24 h after infection, clear differences in the amount of H$_2$O$_2$ production were observed between the Phi-treated and non-Phi-treated plants. These rapid host responses did not occur in non-Phi-treated inoculated seedlings. There was also a significant ($P < 0.001$) decrease in lesion size in Phi-treated plants. These results indicate that Phi primes the plant for a rapid and intense response to infection involving heightened activation of a range of defence responses.

**Keywords:** callose deposition, defence response, hydrogen peroxide, potassium phosphonate (phosphite), reactive oxygen species (ROS)
Introduction

The oomycetes are a diverse branch of eukaryotic microorganisms with many destructive plant pathogens, including biotrophs such as the downy mildews (e.g. Bremia lactucae, Hyaloperonospora arabidopsidis), white rusts (Albugo spp.), hemibiotrophs (e.g. Phytophthora infestans, Phytophthora sojae) and necrotrophs (e.g., Pythium spp., Phytophthora cinnamomi) (Hardham, 2007). Phytophthora cinnamomi, a soil-borne pathogen with a host range of more than 3000 plant species, is a major threat to the sustainability of plant communities in South West Australia and is also causing considerable damage to agriculture, horticulture and forestry worldwide (Shearer & Fairman, 2007, Brasier, 2008, Hansen, 2008, Rookes et al., 2008).

Plants have the ability to defend themselves against different pathogens by induction of tailored defence responses including callose deposition, thickening of the cell walls, production of reactive oxygen species (ROS) and signalling through the hormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) (Jones & Dangl, 2006). Despite the plants’ innate immune system, chemical controls are sometimes required to protect plants against pathogens.

Phosphite (a salt of phosphorous acid; Phi) is a systemically mobile chemical that has been used since the late 1970s in the management of Phytophthora diseases in many native plant ecosystems (Guest & Grant, 1991, Hardy et al., 2001). It has been demonstrated that Phi is highly effective in controlling P. cinnamomi in native plant communities in Australia (Hardy et al., 2001, Shearer & Fairman, 2007). Application of Phi to plants either as a spray or by injection protects the plant from infection by oomycete pathogens. Several studies have suggested that Phi exhibits a complex mode of action including (i) acting directly by inhibition of pathogen growth as a result of accumulated Phi in plant tissue, (ii) acting indirectly by inducing the release of stress
metabolites from the pathogen to elicit the defence response and (iii) directly stimulating the hosts defence responses (Coffey & Joseph, 1985, Jackson et al., 2000, Daniel & Guest, 2006). Phi was previously observed to enhance the production of phytoalexins and ROS in plants and a reduced effect was observed in NahG-expressing plants (Molina et al., 1998, Daniel & Guest, 2006). Despite the common use of Phi, knowledge of its complex mode of action and the biochemical mechanisms underlying its ability to protect plants against infection by Phytophthora species is incomplete.

The diversity of genetic and genomic tools available for the model plant Arabidopsis thaliana makes it a good system in which to study the in planta action of Phi. There have been a number of reports on the interaction of A. thaliana and Phytophthora species including P. infestans (Huitema et al., 2003), P. sojae (Kamoun et al., 1999), P. palmivora (Daniel & Guest, 2006) and P. cinnamomi (Robinson & Cahill, 2003). Robinson and Cahill (2003) reported that P. cinnamomi can infect roots and leaves of A. thaliana, express virulence and complete its life cycle on A. thaliana. They also showed that A. thaliana can mount defence responses as a result of P. cinnamomi infection. Arabidopsis thaliana accession Columbia (Col-0) has previously been identified as susceptible to P. palmivora (Daniel & Guest, 2006) but resistant to P. cinnamomi (Robinson and Cahill 2003), while accession Ler, which is highly resistant to P. palmivora, is highly susceptible to P. cinnamomi (Robinson & Cahill, 2003). This highlights the role of the host genotype in determining the outcome of the interaction between A. thaliana and the Phytophthora species. Robinson and Cahill (2003) showed that although P. cinnamomi is not usually considered a leaf pathogen, it has the ability to penetrate and colonize leaves of A. thaliana, and they developed a detached-leaf assay that was used in the present study.
The objective in this study was to elucidate the role of Phi in priming the systemic defence responses in the *P. cinnamomi*--*A. thaliana* pathosystem, with an emphasis on the regulation of gene expression related to the various defence signalling pathways.

**Materials and methods**

**Plant and pathogen materials**

*Arabidopsis thaliana* accessions Landsberg erecta (Ler) and Columbia (Col-0) were used as Robinson and Cahill (2003) showed them to be the most and the least susceptible, respectively to *P. cinnamomi* amongst the twenty accessions tested. *Arabidopsis thaliana* accessions Ler and Col-0 were purchased from LEHLE SEEDS and *PRI:*::*GUS* (stock no. N6357), a transgenic line, was purchased from the European *Arabidopsis* Stock Centre (NASC) (Shapiro & Zhan, 2001).

*Phytophthora cinnamomi* isolate MP 94.48 was obtained from the Centre for *Phytophthora* Science and Management (CPSM) culture collection at Murdoch University. The isolate was maintained on 20% V8-juice agar at 25°C in the dark and subcultured every 7 days. *Phytophthora cinnamomi* zoospores were produced aseptically using the method described by Byrt and Grant (1979). The zoospore density was determined using a bright line haemocytometer and adjusted to a concentration of $1 \times 10^5$ zoospores mL$^{-1}$ using sterile distilled water.

**Plant growth and inoculation procedure for histochemical assessment of defence responses**

Seeds of Ler and Col-0 were planted on the surface of a seed raising mix (Debco) in 5-cm free-draining polyurethane pots premoistened with distilled water. Seeds were stratified for 3 days at 4°C in the dark before being transferred to a growth cabinet with a 10-h photoperiod with 100 $\mu$mol m$^{-2}$ s$^{-1}$ at 21 ± 1°C. A stock solution of filter-
sterilised 100 mM potassium Phi (pH 5.5, adjusted with KOH) was freshly prepared from phosphorous acid (Aldrich Chemicals) and mixed with sterilized distilled water to give the specified Phi concentration. Four-week-old seedlings were sprayed to runoff with a solution of 20 mM Phi and maintained in the dark for 1 day prior to challenge with *P. cinnamomi*. Control plants were sprayed with sterile distilled water.

Leaves of 4-week-old *A. thaliana* seedlings were washed with sterile distilled water 48 h after application of Phi and then excised and placed into Petri dishes, abaxial surface upward, onto 2.5- cm sterile filter papers (Whatman No. 1) (Robinson & Cahill, 2003) moistened with 1 mL of sterilized liquid MS medium without sucrose (Murashige & Skoog, 1962). Each leaf was inoculated with 3-µL drops of 1 x 10⁵ zoospores mL⁻¹ on the abaxial surface in a completely randomized block design. Plates were sealed with Parafilm and incubated in the dark at 25°C for 12 h then transferred to a growth cabinet with a 10-h photoperiod at 21 ± 1°C to allow lesions to develop. Control leaves were inoculated with 3 µL sterile distilled water. The samples were collected for histochemical assessments (lesion size, callose deposition and hydrogen peroxide production) at different time points, as mentioned specifically for each assessment.

*Lesion size assessment*

The percentage of leaf area affected by the *P. cinnamomi* lesion was calculated using the program Image-Pro Express (Media Cybernetics, Inc.) with photographs taken using a binocular microscope (Olympus SZ40) with a digital camera (Nikon Cool PIX 995). A minimum of 20 leaves (first two fully developed leaves from 4-week old plants) in each treatment was assessed for lesion development 5 days after inoculation, and the experiment was conducted twice.
**Callose visualization**

For callose visualization within the cells, leaves were collected at 3, 6, 12, 24, 48 and 72 h after inoculation and stained using the method described by Eschrich and Currier (1964). Leaves were examined for callose production using Olympus BX51 microscope fitted with a UV filter set (excitation filter 330-385 nm, emission filter 420 nm, dichromic mirror 400 nm). The number of callose papillae per inoculation point was measured for all treatments. A minimum of 15 leaves was examined for each collection time for each treatment and the experiment was repeated twice.

**DAB staining for observation of H$_2$O$_2$ production**

The oxidative burst was detected using 3, 3-diaminobenzidine (DAB), a stain for H$_2$O$_2$. The presence of H$_2$O$_2$ causes polymerization of DAB, yielding a brown colour. Following inoculation, H$_2$O$_2$ production was visualized in leaves according to Thordal-Christensen et al. (1997) and samples were counterstained with trypan blue to visualize the extend of hyphal growth (Keogh et al., 1980). Samples were examined microscopically for H$_2$O$_2$ production using an Olympus BX51 photomicroscope. Photographs were captured using an Olympus DP 70 camera and associated software. H$_2$O$_2$ was measured quantitatively 24, 48 and 72 h after inoculation by measuring the number of DAB-positive cells (Robinson & Cahill, 2003) at the site of infection and at remote sites using the program ASSESS (APS Press). A minimum of 10 leaves was examined for each collection time for each treatment and the experiment was repeated twice.

**Quantitative measurements of defence genes using qRT-PCR**

For quantitative measurement of defence gene transcription, seeds of accession Ler were germinated on 0.8% (w/v) Phytagar-MS medium (Murashige & Skoog, 1962) with modifications, which included the addition of 2.5 mM KH$_2$PO$_4$ and 1 mL prepared
vitamin stock solution (10 g myo-inositol, 100 mg thiamine, 100 mg pyridoxine and 100 mg nicotinic acid to 100 mL dH2O). In addition, 2.5 mM MES [2-(N-morpholino)-ethanesulfonic acid]-KOH (pH 5.5) and 0.5% (w/v) sucrose were included. After sowing the seeds on MS, the plates were transferred to a growth cabinet at 21 ± 1°C with a 10-h photoperiod at a photon fluorescence rate of 100 µmol m⁻² s⁻¹. The plants were grown on MS medium for 3 weeks and then transferred to fresh MS medium with the different Phi treatments (0, 2.5 and 20 mM) in a completely randomized design for 7 days. The leaf samples were collected 7 days after treatment, snap-frozen in liquid nitrogen and stored in a -80°C freezer for RNA extraction.

**RNA extraction and cDNA synthesis**

Three biological samples per treatment were randomly chosen for RNA extraction. Each sample was a pool of two plants. Tissue samples were homogenized using Tissuelyser® (QIAGEN) and total RNA was isolated using the RNeasy plant mini kit (QIAGEN) according to the manufacturer’s instructions. Total RNA was further treated with RNase-free DNase Set (QIAGEN) to remove any genomic DNA, according to the manufacturer’s instructions. Approximately, 1 µg of DNA-free RNA was used for first-strand cDNA synthesis using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The sequences of genes were obtained from NCBI, and primer pairs (Table 1) were designed using PRIMER EXPRESS 1.5 software (Applied Biosystems Inc.).

**qRT-PCR conditions**

After first-strand cDNA synthesis, a LightCycler® 480 Real-Time PCR System (Roche Applied Science) was used to assess the transcription of selected defence genes. RT-PCR was performed in a volume of 10 µL containing 1x Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 10 µM primers, and 50-fold diluted first-strand
cDNA. PCR conditions were 2 min at 50°C and 2 min at 95°C, followed by 40 cycles each consisting of denaturation for 5 s at 95°C, annealing for 10 s at 55°C and extension at 72°C for 10 s. The quality of the dissociation curves was analysed using following programme: 95°C for 10 s, followed by a constant increase in temperature from 55°C to 95°C. PCR efficiency for each target gene was checked via the slope of a standard curve constructed from amplification of a dilution series of the cDNA. Quantification of relative gene transcription was then determined by amplifying the target genes and the reference genes in triplicate per treatment along with a no-template control. In addition, RT-negative samples were used to test the effectiveness of the DNase treatment. The relative level of mRNA for the defence genes were analyzed using qbase\textsuperscript{PLUS} software (Biogazelle), and the transcript abundance of defence genes was normalized based on elongation factor 1 alpha (EF-1\textalpha) and actin 2 (ACT2) as reference genes. The reference genes were selected from a previous experiment in which they were shown not to be affected by Phi (data not shown). The specificity of the PCR reactions was confirmed by melting curves analysis of the products, as well as by size verification of the amplicons in a conventional agarose gel.

**Observation of GUS activity**

The transgenic *A. thaliana* line (*PR1::GUS*) carrying the transcriptional fusion of *PR1* promoter to the β-glucuronidase (*GUS*) reporter gene in the Col-0 background (Shapiro & Zhan, 2001) was used to examine expression of the *PR1* gene during *P. cinnamomi* infection in the absence and presence of Phi. *PR1::GUS* seeds were sown on MS medium as previously described. After 24 days the seedlings were transferred to new MS medium plates containing different concentrations of Phi (0, 2.5 or 20 mM) for a further 4 days. Leaves of *PR1::GUS* seedlings were then inoculated with *P. cinnamomi* zoospores as previously described in a completely randomized design.
Leaf samples were collected 24, 48 and 72 h after inoculation and immersed in a GUS staining solution [2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc; Rose Scientific) in 0.2% Triton X-100, 50 mM NaHPO₄ buffer (pH 7.2), and 2 mM potassium ferricyanide]. A vacuum was applied for 15 min and the leaves were incubated at 37°C for 12 h and examined macroscopically for staining patterns using a binocular microscope (Olympus SZ40) with a digital camera (Nikon Cool PIX 995). Leaf samples from a minimum of 15 seedlings were examined for each treatment and the experiment was repeated twice.

Data analysis

ANOVA was used to compare the treatments in all measurements and the normality of residuals was tested using GENSTAT software for Windows™, release 10.2. Microsoft Excel 2007 was used to graph the charts. Figures were assembled using Photoshop version CS5 (Adobe).

Results

Interaction between A. thaliana and P. cinnamomi

Infection of detached A. thaliana leaves by P. cinnamomi zoospores resulted in the appearance of lesions on leaves in both accessions Columbia (Col-0) and Landsberg erecta (Ler) (Fig. 1). There was a significant ($P < 0.001$) reduction of $P. \textit{cinnamomi}$-affected leaf area in leaves treated with 20 mM Phi (1.07 %) compared to non-treated leaves (5.01 %) in accession Ler (Fig. S1). In accession Col-0, Phi treatment did not show a significant effect on lesion development in leaves compared to non-treated leaves (Fig. 1a,b). In contrast, in accession Ler, lesion development was much more limited in Phi-treated leaves (Fig. 1d) than in leaves without Phi treatment (Fig. 1c). No lesions were formed in non-inoculated leaves of either Col-0 or Ler.
Callose production during the interaction

Callose formation became apparent from 6 to 12 h after inoculation in Phi-treated (20 mM) and non-Phi-treated leaves, respectively, of accession Ler (Fig. 2a). The number of callose papillae significantly differed within Phi-treated and non-treated leaves of accession Ler at 24 h and 48 h after inoculation, with the greatest difference occurring at 48 h ($P < 0.001$). In accession Col-0, callose formed earlier, from 3 h in both Phi-treated (20 mM) and non-treated leaves, and there was a significant ($P < 0.001$) difference in the number of callose papillae between them at 3, 6 and 12 h after inoculation (Fig. 2b). In most cases, non-Phi-treated leaves of accession Ler displayed few callose papillae at the site of infection (Fig. S2a), while the epidermal cells of Phi-treated leaves showed whole-cell fluorescence or fluorescence along entire walls (Fig. S2b) suggesting a more intense lignifications response. Callose formation was not observed in Phi-treated non-inoculated leaves of accessions Col-0 or Ler.

Production of hydrogen peroxide

Production of $H_2O_2$ was detected in DAB-stained leaf tissue as a brownish-red coloration. By 72 h after inoculation of accession Ler, when there was a clear lesion, DAB staining occurred in many cells (Fig. 3). $H_2O_2$ accumulation in non-Phi-treated leaves of accession Ler was mostly restricted to the lesion and sites of hyphal penetration (Fig. 3a). However, in leaves of accession Ler treated with 20 mM Phi, $H_2O_2$ accumulation was found at sites of hyphal penetration and in cells outside of visible lesions (Fig. 3b). In leaves of accession Col-0 not treated with Phi, $H_2O_2$ production was evident by 6 h after inoculation with $P. cinnamomi$ (Fig. 3e). Quantitative measurement of DAB staining in accession Ler showed a significant ($P < 0.05$) increase in the number of DAB positive cells in Phi-treated leaves compared to leaves without Phi treatment at all time points (Fig. S3). $H_2O_2$ production was not observed in Phi-treated non-inoculated leaves of either Col-0 or Ler.
**Induction of PRI gene during P. cinnamomi infection**

To study the induction of defence gene expression by Phi, a transgenic line carrying transcriptional fusion of the PRI promoter to the β-glucuronidase (GUS) reporter gene (Shapiro & Zhan, 2001) was used. The PRI gene (pathogenesis-related gene 1) is one of the SA-responsive genes that functions in the SA defence signalling pathway. Light GUS staining was evident around the inoculated sites in non-Phi-treated leaves by 72 h after inoculation (Fig. 4a). Application of Phi resulted in induction of PRI::GUS expression in infected leaves and the level of induction depended on the concentration of Phi applied (Fig. 4b,c). For example, the lesioned area and PRI::GUS expression were larger and less evident, respectively, in leaves of seedlings treated with 2.5 mM Phi (Fig. 4b) than in leaves of seedlings treated with 20 mM Phi (Fig. 4c). Application of Phi also caused a systemic induction of PRI::GUS in non-inoculated leaves (Fig. 4e,f), which was consistent between 15 samples per treatment. A higher level of GUS expression was observed in both inoculated and non-inoculated leaves treated with 20 mM Phi compared to those treated with 2.5 mM Phi. There was no indication of GUS activity around the mock-inoculated sites (Fig. 4d).

**Relative gene transcription quantification using qRT-PCR**

The induction of PRI::GUS in Phi treated but non-inoculated leaves suggested that Phi not only potentiates/primes the defence responses for a rapid response following pathogen challenge, but also constitutively induces defence gene expression in the absence of a pathogen. To examine this possibility further, the level of transcription of PRI and several other defence genes was analysed in Phi-treated plants. Defence genes in the SA (PRI, PR5, and NPR1) and JA/ET (THI2.1, PDF1.2) pathways were quantitatively measured using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). In this experiment, changes in the transcription of defence genes were measured in both Phi-treated (2.5 and 20 mM) and non-Phi-
treated samples. The PRI gene was included in the experiment to verify the response of the PRI::GUS transgenic line to Phi treatments. The extent of the increase in PRI transcription depended on the concentration of Phi applied (Table 2). The relative transcription of PRI in non-inoculated seedlings treated with 2.5 and 20 mM Phi was 561- and 384-fold higher, respectively, than that in non-Phi treated, non-inoculated seedlings (control). Of the other four defence genes tested, PR5, THI2.1 and PDF1.2 showed a significant ($P < 0.05$) increase in transcription whereas NPR1 did not change (Table 2). In seedlings treated with 20 mM Phi the relative transcription of THI2.1 was the highest among defence genes tested.

**Discussion**

This study investigated the effect of phosphite (Phi) on the interaction between the model plant A. thaliana and P. cinnamomi. Phi increased the transcription of defence genes relating to the SA and JA/ET signalling pathways (PRI, PR5, THI2.1 and PDF1.2) in non-inoculated plants and enhanced the induction of defence responses such as callose deposition and hydrogen peroxide production following pathogen challenge. Phi application also restricted lesion development in the susceptible accession Ler, and caused its responses to be more similar in terms of intensity and timing to that of the resistant accession Col-0. Furthermore, application of Phi enhanced expression of PRI::GUS in both inoculated and non-inoculated leaves.

Phi treatment primed the plant for enhanced production of H$_2$O$_2$ in the leaves of A. thaliana at the site of hyphal penetration and in cells outside of the infection area. Daniel and Guest (2006), who studied host defence responses in Phi-treated P. palmivora-infected A. thaliana also observed a similar stimulation of ROS (superoxide; O$_2^-$ and H$_2$O$_2$). However, they did not observe any O$_2^-$ release in the non-Phi-treated plants after infection, while in the present study, production of H$_2$O$_2$ occurred within 24
h in response to *P. cinnamomi* infection, even in non-Phi-treated plants. These results could be caused by differences in the kinetics of the defence induction in *A. thaliana* in response to different *Phytophthora* spp. The production of ROS (NO, H$_2$O$_2$ and O$_2^-$), which is shown to be a fundamental mechanism in a series of physiological and biochemical changes, is often observed prior to the hypersensitive response (HR) and cell death (Kotchoni & Gachomo, 2006). Localized generation of H$_2$O$_2$ is shown to be one of the earliest cytologically detectable defence responses against penetration of plant cell walls by different pathogens and can be an important indicator of the failure of a pathogen to invade epidermal cells (Mellersh *et al.*, 2002). H$_2$O$_2$ is also involved in signal transduction leading to SAR, which confers systemic resistance against a broad range of pathogens following localized infection (Conrath *et al.*, 2002).

Deposition of callose within the cells has been well documented as a defence response in plant-*Phytophthora* interactions (Bonhoff *et al.*, 1987). In accession Ler, Phi treatment not only considerably increased the callose deposition within and around the cell walls following inoculation of leaves, but also resulted in the production of callose papillae 6 h earlier than in non-Phi-treated seedlings. These results, together with the observation that Phi-treated non-inoculated leaves did not produce callose, suggests that Phi primes the host for a more rapid and more intense defence response following recognition of a potential pathogen. Guest (1986) also showed that Phi induced both callose papillae and the HR in tobacco inoculated with *P. nicotianae*, while Daniel and Guest (2006) showed that Phi increased the release of O$_2^-$ but did not stimulate callose production in *P. palmivora*–challenged *A. thaliana*. This suggests that the mechanism of action of Phi is not to directly induce a defined set of defences but is largely dependent on the plant’s natural regulation of its defence response to different pathogens. In *A. thaliana*, callose deposition is associated with resistance to the fungal pathogens *Plectosphaerella cucumerina* and *Alternaria brassicicola* and the oomycete pathogens
Hyaloperonospora parasitica (Flors et al., 2005) and P. cinnamomi (Robinson & Cahill, 2003). Conversely, a recent study of the A. thaliana–P. cinnamomi pathosystem (Rookes et al., 2008) found that a callose deficient pmr-4 mutant of A. thaliana did not display increased susceptibility to infection. However, this mutant also possesses constitutive activation of SAR (Vogel & Somerville, 2000) that may have compensated to some extent for the loss of callose.

The SAR pathway provides nonspecific resistance in non-inoculated tissues to secondary infection by a broad range of pathogens (Durrant & Dong, 2004). To determine whether Phi treatment may also act through the activation of SAR, the effect of Phi on the expression of the SA-responsive PRI gene during P. cinnamomi infection was examined using the transgenic line PRI::GUS. Application of Phi enhanced the expression of PRI::GUS in infected leaves and caused a systemic induction of PRI::GUS in non-inoculated leaves. Other defence genes in the SA (PR5) and JA/ET (THI2.1, PDF1.2) pathways also showed elevated transcription in non-inoculated Phi-treated plants. The elevated transcription of genes of these pathways showed that in addition to priming the plant for callose and H₂O₂ production, Phi induces defence gene expression in A. thaliana. However, the induction of these defence genes in the absence of P. cinnamomi suggests that rather than simply priming the plant for a more rapid and intense response to infection, Phi is able to up-regulate the expression of defence genes in the absence of a pathogen. The enhanced defence gene expression in the absence of a pathogen suggests that the effect of Phi on the plant’s defence system is not only mediated through greater release of elicitors from the invading pathogen, but that Phi itself modulates the defence response. The activation of genes from both the SA and JA/ET pathways shows that while these pathways are typically thought of as antagonistic (Thatcher et al., 2005), Phi up-regulates a range of defence pathways in a coordinated way. However, the significance of this up-regulation of defence genes is
unclear because inoculation of *Arabidopsis* defence-related gene mutants with *P. cinnamomi* by Rookes *et al.* (2008) did not exhibit any increase in susceptibility to the pathogen, suggesting the involvement of other pathways in resistance to *P. cinnamomi*. Therefore, Phi may confer resistance to *P. cinnamomi* through mechanisms independent of the common signalling pathways.

Phi may also indirectly affect induction of defence responses through its effect on the pathogen. Phi is not metabolized by the plant but accumulates in the plant tissue, where it may persist for some time (McDonald *et al.*, 2001). A number of studies have shown that *in vitro* Phi inhibits growth of oomycetes and causes morphological changes in the hyphal wall, e.g. enhanced secretion of elicitors by *P. capsici* and *P. cryptogea* including the release of glycoconjugate elicitors from the hyphal cell wall (Rouhier *et al.*, 1993, Perez *et al.*, 1995, Wilkinson *et al.*, 2001). This enhanced secretion could lead to a more rapid elicitation of plant defence responses and thereby contribute to the enhanced resistance of Phi-treated plants. However, the enhanced expression of *PR1::GUS* and other defence genes in this study following treatment of plants with Phi in the absence of *P. cinnamomi* cannot be explained by enhanced release of elicitors from the pathogen.

In conclusion, the results of this study showed that Phi primes the plant for a rapid and intense response to infection involving heightened activation of a range of defence responses. In addition to its priming activity, Phi also induces some aspects of the defence response, such as the expression of defence genes relating to the SA and JA/ET pathways, in the absence of a pathogen. The heightened defence response in Phi-treated, *P. cinnamomi*-inoculated Ler, including the more rapid and intense production of callose and H$_2$O$_2$, closely resembles the responses of the resistant line Col-0 in the absence of Phi treatment. Previous studies have shown the importance of the timing of defence induction, with resistant plants showing the same defences as susceptible
plants, but earlier and to a higher degree (Onate-Sanchez & Singh, 2002). These findings further support the hypothesis that the enhanced resistance conferred by Phi treatment may at least in part be caused by the augmentation of the defences that would otherwise be induced in the plant, but at a later stage and to a lesser degree. Furthermore, the induction of defence genes of both the SA and JA/ET pathways suggests that Phi facilitates the recruitment of a broad array of defences. Further studies, including an analysis of the effects of Phi treatment in A. thaliana defence-pathway mutants, are required to shed light on the mechanism through which Phi modulates the recruitment of plant defences.

**Acknowledgments**

We thank Mr Gordon Thomson for his technical support on microscopic assessments and Dr Oliver Berkowitz for helpful comments on the manuscript.

**References**


Guest DI, 1986. Evidence from light microscopy of living tissue that Fosetyl-Al modifies the defence response in tobacco seedlings following inoculation by *Phytophthora nicotianae* var *nicotianae*. *Physiological and Molecular Plant Pathology* 29, 251-61.


Huijema E, Vleeshouwers VGAA, Francis DM, Kamoun S, 2003. Active defence responses associated with non-host resistance of *Arabidopsis thaliana* to the


Table 1 Sequences of the gene-specific primer pairs used in quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>AGI number</th>
<th>Forward and reverse primers (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>Amplicon Tm b (°C)</th>
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<td>AT2G14610</td>
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<td>82</td>
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<tr>
<td>PR5</td>
<td>AT1G75040</td>
<td>TGGCGGCAAGATTTCTACG/TTTGCAATCTCCGATCCTC</td>
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<tr>
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<td>79</td>
</tr>
<tr>
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<td>79</td>
</tr>
<tr>
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*PR1: pathogenesis-related 1; PR5: pathogenesis-related 5; THI2.1: thionin 2.1; NPR1: nonexpresser of PR genes 1; PDF1.2: plant defensin 1.2; EF-1 alpha: elongation factor 1 alpha; and ACT2: actin 2

bMelting temperature.
Table 2 Expression analysis of defence genes in *Arabidopsis thaliana* accession Landsberg erecta (Ler) subjected to different phosphite (Phi) treatments. The transcript level of defence genes was determined using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) in samples treated with 2.5 or 20 mM Phi. Results are expressed as both normalized relative transcript level (mean ± SE) and the factor of increase in transcription compared with the control (0 mM Phi) and data are means of three biological samples per treatment.

<table>
<thead>
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<th>Defence pathway</th>
<th>Normalized relative transcript level</th>
<th>Relative fold difference to control</th>
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<tbody>
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<td></td>
<td></td>
<td>0 mM Phi (control)</td>
<td>2.5 mM Phi</td>
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<tr>
<td>PR1</td>
<td>SA</td>
<td>0.0003 ± 0.0001</td>
<td>0.1683 ± 0.0183</td>
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<td>PR5</td>
<td>SA</td>
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<td>0.259 ± 0.0183</td>
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<td>SA</td>
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<tr>
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<td>JA/ET</td>
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<td>0.418 ± 0.059</td>
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</table>

aSee table 1.

bSA: salicylic acid; JA/ET: jasmonic acid/ethylene.
**Figure Captions**

**Figure 1** Lesion formation on phosphite (Phi)-treated (20 mM) and non-Phi-treated excised leaves of *Arabidopsis thaliana* accessions Columbia (Col-0) and Landsberg erecta (Ler), 5 days after inoculation with *Phytophthora cinnamomi* zoospore suspension (3-µL droplet with $1 \times 10^5$ zoospores mL$^{-1}$). (a) Localized necrotic lesion on a non-Phi-treated leaf of accession Col-0. (b) Localized necrotic lesion on a Phi-treated leaf of accession Col-0. (c) Large non-localized infected area on a non-Phi-treated leaf of accession Ler. (d) Restricted and small lesion on a Phi-treated leaf of accession Ler. Scale bar = 2 mm.

**Figure 2** Number of callose papillae per inoculation point developed over time in phosphite-treated (20 mM) and non-phosphite-treated leaves of *Arabidopsis thaliana* accessions Landsberg erecta (Ler) (a) and Columbia (Col-0) (b), following inoculation with *Phytophthora cinnamomi* (PC) zoospore suspension. Leaves were inoculated with a 3-µL drop of $1 \times 10^5$ zoospores mL$^{-1}$. Data represent the mean and standard error (bar) of results obtained for 15 leaves per treatment.

**Figure 3** Hydrogen peroxide (H$_2$O$_2$) production in phosphite (Phi)-treated (20 mM) and non-Phi-treated leaves of *Arabidopsis thaliana* accessions Landsberg erecta (Ler) and Columbia (Col-0) following inoculation with *Phytophthora cinnamomi*; stained with 3,3-diaminobenzidine (DAB) and counterstained with trypan blue. Leaves were inoculated with a 3-µL drop of $1 \times 10^5$ zoospores mL$^{-1}$. Photographs show accumulation of H$_2$O$_2$ around the lesion site in non-Phi-treated (a) and Phi-treated (b) leaves of accession Ler, 72 h after inoculation. Scale bar for (a) and (b) = 100 µm. (c) Higher magnification of (a), bar = 50 µm. (d) H$_2$O$_2$ stained at the site of pathogen ingress on a Phi-treated leaf of accession.
Ler, 12 h after inoculation; bar = 50 μm. (e) H$_2$O$_2$ stained within the cell in the non-Phi-treated leaf of accession Col-0 following the penetration of hyphae into the cell, 6 h after inoculation; bar = 20 μm. (f) H$_2$O$_2$ stained within the cell in a Phi-treated leaf of accession Col-0 following the penetration of hyphae into the cell, 12 h after inoculation. Scale bar = 20 μm. h: hyphae; z: zoospore; gt: germ tube; and dc: dead cell.

**Figure 4** Enhanced expression of *PRI1::GUS* in leaves of a transgenic *Arabidopsis thaliana* line following phosphite (Phi)-treatments (2.5 or 20 mM), in the presence and absence of *Phytophthora cinnamomi* inoculation. Leaves were inoculated with *P. cinnamomi* zoospore suspensions (3-μL droplets with 1 × 10$^5$ zoospores mL$^{-1}$). Leaves were harvested and stained with X-Gluc 72 h after inoculation and local expression of GUS was detected in both Phi-treated and non Phi-treated ones. Photographs show *PRI1::GUS* expression around the inoculated area on a non-Phi-treated (a), 2.5 mM Phi-treated (b) and 20 mM Phi-treated (c) leaves of the transgenic line. The lesioned area and *PRI1::GUS* expression were larger and less evident, respectively, in leaves of seedlings treated with 2.5 mM Phi (b) than in leaves of seedlings treated with 20 mM Phi (c). Scale bar for (a), (b) and (c) = 0.5 mm. (d) Non-Phi-treated and non-inoculated sites did not show any apparent staining. (e) Systemic induction of *PRI1::GUS* expression on a non-inoculated, 2.5 mM Phi-treated leaf. (f) Systemic induction of GUS expression on a non-inoculated, 20 mM Phi-treated leaf. Scale bar for (d), (e) and (f) = 1 mm.

**Supplementary Figure Captions:**

**Figure S1** Average area of lesions on leaves (expressed as the percentage of leaf area affected), in phosphite-treated (20 mM) and non-phosphite-treated excised
leaves of *Arabidopsis thaliana* accessions Landsberg erecta (Ler) and Columbia (Col-0), 72 h after inoculation with *Phytophthora cinnamomi* (Pc) zoospore suspension (3-µL droplet with 1 x 10⁵ zoospores mL⁻¹). Data represent the mean and standard error (bar) of twenty leaves assessed for each treatment. Bars with the same letter are not significantly different according to Tukey test.

**Figure S2** Callose deposition in phosphite (Phi)-treated (20 mM) and non-Phi-treated excised leaves of *Arabidopsis thaliana* accession Landsberg erecta (Ler) following inoculation with *Phytophthora cinnamomi* zoospores. Leaves were inoculated with a drop of 3 µL of 1 x 10⁵ zoospores mL⁻¹. Blue-light-induced fluorescence spots are callose. (a) Callose papillae (p) in non-Phi-treated leaf 48 h after inoculation. (b) Callose papillae (p) in a Phi-treated leaf 48 h after inoculation. Scale bar for (a) and (b) = 200 µm. (c), (d), Callose deposition in a Phi-treated leaf at penetration site 6 h after inoculation. z = zoospore, p = callose papillae. Scale bar for (c) = 50 µm and for (d) = 20 µm. (e) Callose deposition in a Phi-treated leaf 24 h after inoculation. Scale bar = 100 µm.

**Figure S3** Effect of phosphite (Phi) on hydrogen peroxide production (detected by 3,3-diaminobenzidine (DAB) staining) over time at the inoculation point of leaves of *Arabidopsis thaliana* accession Landsberg erecta (Ler), following inoculation with *Phytophthora cinnamomi* (Pc) zoospores. Leaves were inoculated with a 3-µL drop of 1x10⁵ zoospores mL⁻¹. Graph shows the number of DAB positive cells in non Phi-treated and Phi-treated leaves at different time points (24, 48 and 72 h). Data represent the mean and standard error (bar) of results obtained for 10 leaves per treatment. The number of DAB positive cells in Phi-treated leaves were significantly (*P* < 0.05) higher than that of non Phi-treated leaves in all time points. Asterisk (*) shows significant difference between treatments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure S1
Figure S2
Figure S3
Chapter 3

A quantitative PCR assay for accurate in planta quantification of the necrotrophic pathogen *Phytophthora cinnamomi*

This chapter has been published:

A quantitative PCR assay for accurate in planta quantification of the necrotrophic pathogen *Phytophthora cinnamomi*

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Abstract

A reliable method for measuring disease progression is important when evaluating susceptibility in host–pathogen interactions. We describe a sensitive quantitative polymerase chain reaction (QPCR) assay that enables quantitative measurement of \textit{in planta} DNA of the necrotrophic pathogen, \textit{Phytophthora cinnamomi}, that avoids problems caused by variation in DNA extraction efficiency and degradation of host DNA during host tissue necrosis. Normalization of pathogen DNA to sample fresh weight or host DNA in samples with varying degrees of necrosis led to overestimation of pathogen biomass. Purified plasmid DNA, containing the pScFvB1 mouse gene, was added during DNA extraction and pathogen biomass was normalized based on plasmid DNA rather than host DNA or sample fresh weight. This method is robust and improves the accuracy of pathogen measurement in both resistant (non-host \textit{A. thaliana–P. cinnamomi}) and susceptible (host \textit{Lupinus angustifolius–P. cinnamomi}) interactions to allow accurate measurement of pathogen biomass even in the presence of substantial host cell necrosis.

\textbf{Keywords:} \textit{Arabidopsis thaliana}, Internal control, \textit{Lupinus angustifolius}, Non-host resistance
Introduction

The oomycetes comprise many destructive plant pathogens including those employing a biotrophic lifestyle such as the downy mildews (e.g. *Bremia lactucae, Hyaloperonospora parasitica*), white rusts (*Albugo* spp.), the hemibiotrophs (e.g. *Phytophthora infestans, P. sojae*) and necrotrophs such as *Pythium* spp. and *P. cinnamomi* (Hardham 2007). Due to the global impacts of plant diseases caused by *Phytophthora* species, it is the most economically important genus of plant pathogens worldwide (Erwin and Ribeiro 1996). Among these, *P. cinnamomi* with a host range of more than 3000 plant species is considered to be one of the most pathogenic and causes considerable damage to natural ecosystems, agriculture, horticulture and forestry worldwide (Hardham 2005, Shearer and Fairman 2007, Brasier 2008, Hansen 2008, Rookes et al. 2008).

Studying interactions between *Phytophthora* pathogens and their host plants is an important step in disease control strategies, however, studying interactions with native and crop plants is challenging because of the limited knowledge of their genome structure. To address this issue model plants such as *Arabidopsis thaliana* have been used extensively for studying various fungal, bacterial and oomycete pathogen–plant interactions (Glazebrook 2001, Thomma et al. 2001). However, most *Phytophthora* species, including *P. infestans* and *P. sojae*, cannot infect *Arabidopsis* suggesting that *Arabidopsis* exhibits a strong non-host resistance to these species (Kamoun et al. 1999, Kamoun 2001, Takemoto et al. 2003). Although some ecotypes of *A. thaliana* are resistant to *P. cinnamomi*, this pathogen can infect both roots and leaves of *A. thaliana*, express virulence and complete its life cycle on some *A. thaliana* ecotypes (Robinson and Cahill 2003) making *Arabidopsis* an attractive model plant to investigate mechanisms of resistance to this necrotrophic *Phytophthora* species. Although clear
differences between resistant and moderately susceptible ecotypes exist, the limited extent of lesion development, even on susceptible ecotypes, makes traditional methods of visual assessment of the symptoms and spore or colony counting difficult, particularly in the early stages of infection when resistance is first being expressed.

To overcome the problems associated with visual assessments, quantitative polymerase chain reaction (QPCR) assays have been used in a number of other pathosystems (Böhm et al. 1999, Schaad and Frederick 2002, McCartney et al. 2003, Schaad et al. 2003). QPCR determination of in planta pathogen biomass is rapid, sensitive, specific and very efficient for detecting pathogen biomass even at early stages of infection when little pathogen biomass exists. Despite the benefits of the technique, some inherent problems, such as the requirement for a reliable normalization technique, may confound results. Many studies normalize the amount of pathogen DNA to host DNA, however, DNA degradation during plant cell collapse, such as that occurring during infection by a necrotrophic pathogen, could lead to an overestimation of pathogen biomass. To avoid this, some studies have chosen to normalize their results to sample surface area or weight (Brouwer et al. 2003, van Wees et al. 2003). However, these comparisons are still confounded by variability in DNA extraction yields between samples of the same surface area or weight (Gachon and Saindrenan 2004).

The objective of this study was to develop a sensitive QPCR assay which can be used to quantify P. cinnamomi, or other pathogen biomass at all stages of the necrotrophic pathogen–host interaction by avoiding problems caused by tissue necrosis including host DNA degradation and variable DNA extraction yield between samples. The method is based on the addition of plasmid DNA as an internal control during the DNA extraction stage and subsequently normalizing pathogen biomass using the internal control. Several animal, human and food studies have previously described the addition of an internal control for accurate quantification of target DNA (Klerks et al.
2006, Damen et al. 2008, Halliday et al. 2010). In this study, we tested the potential for overestimation of pathogen DNA when measuring pathogen DNA relative to sample fresh weight or host DNA without the use of an internal control and the efficacy of the use of an internal control using two different plant–pathogen interactions, the resistant *A. thaliana–P. cinnamomi* interaction and the susceptible *Lupinus angustifolius–P. cinnamomi* interaction.

**Materials and methods**

**Plant materials, growth conditions and inoculation**

*Arabidopsis thaliana* ecotypes *Landsberg erecta* (Ler) and *Colombia* (Col-0) were purchased from Lehle Seeds (Round Rock, TX). Seeds were planted on the surface of seed raising mix (Debco, Australia) in 5 cm free-draining polyurethane pots pre-moistened with distilled water. Seeds were stratified for 3 days at 4°C in the dark before being transferred to a growth cabinet with a 10/14 h day/night photoperiod with 100 µmol m$^{-2}$ s$^{-1}$ at 21±1°C. The pots were watered every second day. Leaves of four-week old *A. thaliana* seedlings were inoculated with 5 µL of 1x10$^5$ *P. cinnamomi* zoospores mL$^{-1}$ on the abaxial surface and pots were transferred into a box which was sealed with aluminum foil (to maintain high humidity). After 12 h incubation in the dark at 25°C, the seedlings were transferred to a growth cabinet with a 10/14 h day/night photoperiod at 21±1°C to allow lesions to develop. Tissue samples were collected 24, 48 and 72 h after inoculation.

Blue lupin (*L. angustifolius* L., cv. Mandalup) seeds were obtained from Department of Agriculture and Food, Western Australian (DAFWA). Seeds were surface-sterilized in 70% ethanol for 2 min followed by immersion in 50% bleach solution (6.25% available chlorine) for 5 min. The sterilized seeds were germinated on filter paper pre-moistened with distilled water at 25°C in dark for 3 days. The seedlings
were transferred to filter papers pre-moistened with half strength MS medium (Murashige and Skoog 1962) in plastic Petri-dishes (14 cm in diameter) and grown for a further 4 days. The seedlings were inoculated by placing a 2 mm diameter plug of \textit{P. cinnamomi} mycelium at the tips of roots and covering with filter paper pre-moistened with sterile distilled water. Mycelial tissue was removed from the surface of the roots and root tissue samples collected at 24, 48, 72 and 96 h after inoculation. It should be noted that different inoculation procedures were used for \textit{Arabidopsis} and lupin and this should be taken into account if comparing the degree of susceptibility of the two species.

\textbf{Inoculum preparation}

\textit{Phytophthora cinnamomi} (isolate MP 94.48) was obtained from the Centre for \textit{Phytophthora} Science and Management (CPSM) culture collection at Murdoch University. The isolate was maintained on 20\% V8-juice agar (Erwin and Ribeiro 1996) at 25\(^\circ\)C in the dark and sub-cultured every 7 days. Preliminary trials found isolate MP 94.48 to show similar infection characteristics to other \textit{P. cinnamomi} isolates tested but was more pathogenic on \textit{A. thaliana} ecotype Ler and more consistently produced zoospores in culture (data not shown). \textit{Phytophthora cinnamomi} zoospores were produced aseptically using the method described by Byrt and Grant (1979). The zoospore density was determined using a haemocytometer and adjusted to a concentration of \(1 \times 10^5\) zoospores mL\(^{-1}\) with sterile distilled water and used for inoculation of \textit{A. thaliana} leaves. For genomic DNA extraction, \textit{P. cinnamomi} hyphae was harvested from patches of 5-day old hyphae grown on filter papers placed on the surface of V8 medium.
**Plasmid DNA extraction**

For the preparation of plasmid DNA, *E. coli* HB2151 containing the plasmid pScFvB1 (Manatunga et al. 2005) was grown in LB broth medium (Sambrook et al. 1989) with 50 µg mL\(^{-1}\) ampicillin for 24 h at 37ºC with shaking. Plasmid DNA was extracted using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) following the manufacturer’s instructions.

**Plant DNA extraction using plasmid DNA as an internal control**

Eight *A. thaliana* leaf discs (7 mm in diameter with an average total weight of 50 mg per eight discs) that included the entire area of a lesion were collected in a 2 mL tubes 72 h after inoculation. In the case of lupin, either 50 mg or 100 mg of the infected roots were weighed for each sample and transferred to a 2 mL tube. Tissue samples were homogenized using Precellys 24 lysis and homogenizer (Bertin Technologies). DNA was extracted from the tissue samples using the Nucleon™ PhytoPure™ Genomic DNA Extraction Kits (GE Healthcare) according to the manufacturer’s instructions. Based on a preliminary experiment, 1 ng of ScFvB1 plasmid DNA was added to all samples just before the first centrifugation step.

**Primer design**

*Phytophthora cinnamomi* primers (forward 5´–GCT AGC AAG CAC GTA TGA GG and reverse 5´–CGC CCC AAC TAT ACG ACA AC) were designed for the PDN gene (FJ493007). Actin (AT3G18780) primers (forward 5´–CTT GCA CCA AGC AGC ATG AA; reverse 5´–CCG ATC CAG ACA CTG TAC TTC CTT) were used for amplification of both *A. thaliana* and lupin genome and the plasmid was detected using the primers forward 5´–GGA TCG GAC ATC GAG CTC AC; reverse 5´–CAC TTG AGC TGG CAC TGC AG. All primers were designed using primer 3 (v. 0.4.0) software (http://frodo.wi.mit.edu/) and tested for their specificity by (a) comparing with
the genome sequences of *A. thaliana*, *L. angustifolius*, and *P. cinnamomi* and (b) running PCR using all other DNAs used in this study as templates. On the basis of these analyses the primers were judged to be specific for their target DNA templates. The *P. cinnamomi* primers were designed to be specific to *P. cinnamomi* and not to bind to other *Phytophthora* sequences in Genbank however when used on samples potentially containing other *Phytophthora* species, such as field soil samples, the specificity of the primers should be experimentally determined.

**QPCR amplification**

The QPCR assays were conducted using a LightCycler 480 (Roche Applied Science, Mannheim, Germany). All DNA samples were analyzed by QPCR assays using the specific primers and SYBR Green 1 (Roche Applied Science, Mannheim, Germany). QPCR was performed in a volume of 10 μl containing 5 μM forward primer, 5 μM reverse primer, 5 μl of 2x SYBR Green 1 Master Mix and 20 ng of DNA template. Thermal cycling conditions were as follows; 2 min at 50°C, 2 min at 95°C followed by 40 cycles each consisting of 5 s at 95°C, and then 10 s at 55°C and 10 s at 72°C. Melting curve analysis was carried out using the following program: 95°C for 10 s, followed by a constant decrease in temperature from 95°C to 55°C at a rate of 3.3°C per 30 s. The amplification efficiency for each target gene was checked via the slope of a standard curve constructed from amplification of 10-fold dilution series of DNA (10,000 pg, 1,000 pg, 100 pg, 10 pg and 1 pg). The QPCR assay was tested with DNA from pure tissues of *A. thaliana*, *P. cinnamomi*, plasmid, and *L. angustifolius*. 
**Results**

**Assay specificity and sensitivity**

To assess the specificity of primers for the plasmid pScFvB1, the primers were tested for amplification in the presence of DNA from *P. cinnamomi*, *A. thaliana*, or lupin, and in a no-template control. No amplification occurred in any of these assays (data not shown). Similarly no amplification products were obtained when the *P. cinnamomi* specific primer pair (FJ493007) was used with *A. thaliana*, pScFvB1, or *L. angustifolius* DNA templates. Amplification with the plant actin primer pair occurred only in the presence of *A. thaliana* DNA or lupin DNA (data not shown).

To test the sensitivity of amplification with the *P. cinnamomi* primer pairs and the effect of plant and plasmid DNA on amplification by these primers, a series of 10-fold dilutions of *P. cinnamomi* (ranging from 10 ng to 1 pg) were prepared. To each PCR reaction, 200 pg of plasmid DNA and 20 ng of *A. thaliana* DNA were added and QPCR was performed. In additional, *P. cinnamomi* (100 pg) was assayed in the absence of non-target DNA (*A. thaliana* and plasmid) as a control. The concentrations of *A. thaliana* and plasmid DNA were chosen to give Ct values ranging from 18 to 20 based on a preliminary experiment (data not shown). The results showed that the presence of the plant and plasmid DNA in the QPCR reaction did not affect amplification of *P. cinnamomi* DNA over this range of concentrations as low as 1 pg for *P. cinnamomi*. Furthermore, amplification of 10-fold dilution series of plasmid DNA (ranging from 1 ng to 0.1 pg) was not affected by the presence of 20 ng of *A. thaliana* DNA and 1 ng of *P. cinnamomi* DNA compared to controls without non-target DNA (*A. thaliana* and *P. cinnamomi*). The efficiency of amplifications for *P. cinnamomi* and plasmid primers were 98% (slope = -3.399; $R^2 = 0.999$) and 96.5% (slope = -3.434; $R^2 = 0.999$), respectively.
Testing the inhibitory effect of DNA extracts on plasmid amplification

To determine the presence of PCR inhibitors in the DNA extracts from the pathogen culture, and plant, amplification of plasmid DNA (10 pg) was carried out in the presence of a 10-fold dilution series of *P. cinnamomi* and 20 ng of *A. thaliana* DNA. Furthermore, the amplification of plasmid DNA (10 pg) was also measured in the presence of DNA extracts from infected *A. thaliana* leaf samples (eight leaf discs; around 50 mg) or lupin roots (100 mg) in four biological samples. The Ct value for amplification of the plasmid in the absence of *P. cinnamomi* and *A. thaliana* DNA (control) was 19.28, and for amplification of the plasmid in the presence of five dilution series of *P. cinnamomi* and 20 ng of infected/non-inoculated *A. thaliana* DNA was 19.31 to 19.46, with the plasmid ΔCt ranging from 0.03 to 0.18 (Table 1). The Ct value for amplification of the plasmid in the presence of infected/non-inoculated lupin DNA was ranged from 19.18 to 19.42 (Table 1). The similarity in the Ct values shows that the plasmid amplification was not affected by the presence of *A. thaliana*, lupin or *P. cinnamomi* DNA, nor DNA extracted from necrotic tissue.

Using plasmid DNA as an internal control for measuring pathogen biomass

We demonstrated the efficiency of using plasmid DNA as the internal control in two different plant–pathogen pathosystems including the resistant *A. thaliana*–*P. cinnamomi* interaction and the susceptible *L. angustifolius*–*P. cinnamomi* interaction.

The *A. thaliana*–*P. cinnamomi* combination

To test the efficiency of this method, we took two different approaches. In the first approach, either 100 ng or 1,000 ng of *P. cinnamomi* DNA were added into tubes containing 0.1, 0.05 or 0.025 g of non-inoculated *A. thaliana* leaves and DNA extracted with the addition of 1 ng of plasmid DNA just before the first centrifugation step. The concentrations of the plasmid DNA (1 ng) added to samples were calculated in such a
way that after re-suspending DNA samples in 100 µL of distilled water, the QPCR fluorescence passed the threshold line (Ct value) close to cycle 20 when using 20 ng of sample in the PCR reaction.

In the second approach, 0.1, 0.05 or 0.025 g of non-inoculated A. thaliana leaves were weighed and collected in 2 mL tubes. Then, either 0.01 g or 0.005 g P. cinnamomi hyphae (2-fold differences) was added to the tubes containing the A. thaliana leaves. One nanogram of plasmid DNA was then added to each sample and DNA extraction was carried out as previously described.

*Phytophthora cinnamomi* biomass was calculated based on either plasmid or *Arabidopsis* DNA using Ct values according to Gao et al. (2004) with some modifications as follows:

\[
\Delta Ct_{P. cinnamomi} = Ct_{P. cinnamomi} - Ct_{Plasmid/Arabidopsis}
\]

\[
\Delta \Delta Ct = \Delta Ct_{P. cinnamomi\ (S1)} - \Delta Ct_{P. cinnamomi\ (S2)}
\]

S1 and S2 refer to different amounts of *P. cinnamomi* mixed with *Arabidopsis* leaf samples.

\[
\text{Fold Pc amount} = E^{-\Delta \Delta Ct}
\]

where E is the efficiency of primer amplification for *P. cinnamomi*

The results of these approaches are presented in Table 2. In both approaches, there was no significant difference between the obtained and the expected \(\Delta \Delta Ct\) values when the *P. cinnamomi* DNA amount was normalized to *Arabidopsis* DNA \( (X^2 = 0.771, P = 0.380) \) or to plasmid DNA \( (X^2 = 0.830, P = 0.362) \).

*The Lupin—P. cinnamomi combination*

To demonstrate the validity of this method in the susceptible lupin—*P. cinnamomi* pathosystem, the above two approaches were repeated using lupin tissue.
In the first approach, *P. cinnamomi* DNA (100 ng or 1,000 ng) was added to tubes containing 0.1 g or 0.05 g of non-inoculated lupin root tissue and 1 ng plasmid DNA added prior to the first centrifugation step of DNA extraction. In the second approach, *P. cinnamomi* hyphae (0.01 g or 0.002 g) were added into tubes containing 0.1 g or 0.05 g of non-inoculated lupin root tissue. The results for analysis of the lupin–*P. cinnamomi* combination are presented in Table 3.

In both approaches, there was no significant difference between the obtained and expected ∆∆Ct values when the *P. cinnamomi* DNA amount was normalized to lupin ($X^2 = 0.845, P = 0.358$) or plasmid DNA ($X^2 = 0.841, P = 0.359$) showing that in samples with no pathogen infection, normalization to plasmid DNA is a valid method of pathogen biomass quantification.

**Measurement of pathogen biomass in infected plants**

The results showed that the relative amount of pathogen can be measured according to either plasmid or plant DNA as internal controls in the absence of an infection when pathogen DNA or hyphae is added to the plant DNA extraction. To answer the question of whether degradation of host DNA in infected tissue would lead to an overestimation of pathogen biomass when normalized to plant DNA, we conducted the following experiment.

*Arabidopsis thaliana* and lupin were grown and inoculated as described. 50 mg of *A. thaliana* leaf (eight discs; 7 mm in diameter) were collected as a biological sample from infected areas of inoculated leaves. Five independent biological samples were assessed for pathogen biomass quantification. For lupin, either 50 mg or 100 mg of infected root tissue was collected in 2 mL tubes and eight independent biological samples collected. DNA extraction was conducted as described previously. Relative pathogen biomass was measured based on both plant and plasmid DNA. The results
showed that there was considerable overestimation when pathogen biomass was calculated based on plant DNA for both the *A. thaliana*–*P. cinnamomi* and lupin–*P. cinnamomi* pathosystems. In all infected samples tested, there were differences between ΔCt values when normalization was based on host DNA compared to that of based on plasmid DNA. The calculated overestimation \( (E^{\Delta \Delta Ct}) \) of pathogen biomass for *Arabidopsis* samples ranged from 1.56 to 3.19 fold (Table 4). Overestimation of pathogen biomass for the lupin samples was much greater than that of *Arabidopsis* ranging from 6.03 to 17.72 fold (Table 4). This high overestimation range in lupin root occurs since *P. cinnamomi* extensively infested lupin root tissues within 72 h causing considerable tissue necrosis. These results suggest that the calculation of pathogen biomass based on plant DNA can be misleading (usually overestimated) in situations where extensive plant cell death has occurred, such as during infection by necrotrophic pathogens.

**Differences between DNA yield during DNA extraction**

There were some variations between DNA yields during DNA extraction in both lupin and *Arabidopsis* samples (Table 4). DNA yields for *Arabidopsis* leaves ranged from 9.7 to 21.1 µg when eight discs per sample were used for DNA extraction. In the case of lupin, the DNA yield was from 3.3 to 8.2 µg and 20.1 to 37.0 µg from 0.05 g and 0.1 g of infected root tissue, respectively. These results showed that normalization of pathogen biomass only based on tissue weight or tissue surface area can generate biased results. Adding plasmid as an internal control during DNA extraction makes it possible to normalize not only DNA extraction yield but also relative pathogen biomass.
Relative and total measurement of pathogen biomass over time in susceptible/resistant hosts

Leaves of four-week-old *A. thaliana* ecotypes; Ler and Col-0 were inoculated with *P. cinnamomi* zoospores as described. For each ecotype, five biological samples were collected and snap frozen at 24, 48 and 72 h after inoculation. Each biological sample was a bulk of four randomly chosen infected leaf discs (7 mm in diameter). QPCR was conducted and relative and total pathogen biomass calculated as follows:

Relative pathogen biomass = [pathogen DNA amount in sample (pg)] / [plasmid DNA amount in sample (pg)].

Total pathogen biomass in initial sample = pathogen DNA in reaction (pg) x plasmid dilution factor

Plasmid dilution factor = plasmid amount used in DNA extraction (pg) / plasmid amount in QPCR reaction (pg).

There were considerable differences in relative pathogen biomass between ecotype Ler and Col-0 when pathogen DNA was normalized to host DNA (Fig. 1a). Ecotype Col-0 was highly resistant to *P. cinnamomi* and showed no significant differences among relative pathogen DNA over the different time points; whereas ecotype Ler was moderately susceptible to *P. cinnamomi*, showing a significant ($P<0.001$) increase in pathogen DNA between 24 and 72 h (Fig. 1a).

There was a significant ($P<0.001$) difference between the two ecotypes (Ler and Col-0) of *A. thaliana* with regard to total pathogen biomass. In ecotype Ler, the total pathogen biomass reached to 3.7 ng pathogen DNA in 72 h compared to 1.1 ng in ecotype Col-0 (Fig.1b).

Root tips of 4-day-old blue lupin (*L. angustifolius*) seedlings were inoculated using *P. cinnamomi* mycelial plugs. Root samples (0.1 g) were collected at 24, 48, 72
and 92 h after inoculation. QPCR results revealed that lupin was highly susceptible to *P. cinnamomi* showing a large increase in pathogen biomass in the course of 24 h. Relative pathogen biomass reached more than 7 ng within 96 h (Fig. 1c).

In lupin, pathogen growth was much faster than that in *A. thaliana* and total pathogen biomass reached to 335 ng by 72 h (Fig. 1d). The results of relative pathogen biomass (normalized based on plasmid) and total pathogen biomass were highly (*r*=0.98 *P*<0.001) correlated. The visual assessments of lesion size (Fig. 1e and f) supported the QPCR results (Fig. 1a and c).

**Discussion**

Early detection and quantification of pathogen biomass is one of the essential steps for determination of disease resistance. To date, several QPCR assays have been developed to detect different oomycete pathogens *in planta* such as *Phytophthora infestans* and *Phytophthora citricola* (Böhm et al. 1999), *Phytophthora capsici* (Silvar et al. 2005), *P. cinnamomi* (Kong et al. 2003), and other pathogens (Brouwer et al. 2003, Lievens et al. 2006). In these QPCR assays, the pathogen biomass was measured and normalized either to host plant DNA or the sample surface or weight of collected sample. However, measurement of the pathogen biomass relative to the amount of plant DNA may lead to an overestimation of pathogen biomass especially at late stages of infection, where host DNA degradation is often associated with host cell death (Brouwer et al. 2003, van Wees et al. 2003). Furthermore, the validity of pathogen biomass measurements based on sample surface or weight may also be significantly affected by variable DNA extraction yield between the samples (Gachon and Saindrenan 2004).

The use of host plant DNA for normalisation of pathogen biomass was shown to lead to overestimation of growth of *P. cinnamomi*, in both resistant (*A. thaliana*) and
susceptible (lupin) interactions. Moreover, the degree of overestimation was shown to be related to the degree of tissue necrosis as susceptible lupin, with extensive tissue necrosis, showed a higher impact on the accuracy of pathogen DNA quantification than resistant *Arabidopsis* (Fig. 1). The occurrence of overestimation during the quantification of *in planta* levels of necrotrophic pathogens relative to plant DNA has been suggested previously; however, solutions including normalizing pathogen DNA according to tissue weight or surface area were implemented in some pathosystems (Brouwer et al. 2003, van Wees et al. 2003). In our study, DNA yield was shown to vary considerably in samples for which the equivalent amount of starting tissue was used, even when the degree of disease did not differ significantly according to lesion size suggesting the potential for inaccuracy in relative pathogen quantification.

To address these issues as they relate to the *Arabidopsis–P. cinnamomi* pathosystem, a QPCR based assay that enables the measurement of both relative and total amount of *P. cinnamomi* was developed. The method described in this study where 1 ng of plasmid DNA was added during DNA extraction, accounted for the variation in DNA yields and enabled the calculation of the total amount of pathogen DNA present in the initial leaf or root sample. Furthermore, the broad dynamic range of the QPCR technique allowed useful information to be gained from both resistant/moderately susceptible interactions (e.g. *A. thaliana–P. cinnamomi*) where pathogen biomass may remain low throughout the experiment and susceptible interactions (e.g. *L. angustifolius–P. cinnamomi*) where *in planta* pathogen biomass may reach high levels.

The results demonstrating the utility of adding an internal control DNA during sample DNA extraction are in accordance with previous results from several studies investigating human and animal systems (Beld et al. 2004, Damen et al. 2008, Erdner et al. 2010). In a recent study, Diguta *et al.* (2010) also noted the potential for inaccuracy of pathogen quantification using normalization to host DNA or sample fresh weight and
they added intact yeast, *Yarrowia lipolitica*, as an internal control prior to DNA extraction for detection of the fungal pathogen *Botrytis cinerea* on grapes. However, variation in the efficiency and reproducibility of a given DNA extraction method for extracting the yeast DNA may still influence the accuracy of pathogen quantification. The use of previously purified and quantified plasmid DNA in the methods described in this study avoids this possible additional variation. Plasmid DNA was chosen as the internal control because a) the small size of the plasmid requires a little amount of plasmid (1 ng) to be added to each sample and b) a QPCR target sequence within the plasmid can be selected that has no homology to either host or pathogen to ensure the specificity of the primers. The plasmid used in this study contains the mouse ScFvB1 sequence and thus should be useful for a wide range of plant–pathogen systems, having no significant homology in known plant and fungal genomes. We showed that plant and plasmid DNA in the QPCR reaction did not affect amplification of pathogen DNA over a range of different concentration as low as 1 pg and similarly that plasmid DNA amplification was not affected by extracts from *P. cinnamomi*, plant host or necrotic tissue in the QPCR. The use of internal controls in the form of intact bacteria containing plasmid vector or plasmid DNA have been successfully demonstrated in several studies quantifying human or animal diseases/pathogens (Klerks et al. 2006, Damen et al. 2008, Halliday et al. 2010).

In conclusion, we demonstrate the potential for overestimation of pathogen biomass both in resistant interactions, with limited pathogen growth and necrosis, and susceptible interactions, with a high degree of necrosis, when pathogen DNA is normalized to either sample weight or host DNA. The addition of plasmid DNA into the biological samples during DNA extraction was an efficient way to overcome issues of variation in DNA extraction efficiency and degradation of host DNA. This method described herein should be applicable to a wide range of plant–pathogen systems.
Acknowledgements

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Table 1 Testing the efficiency of plasmid DNA amplification in the presence of DNA extracts from *Phytophthora cinnamomi* culture, inoculated/non-inoculated *Arabidopsis* or *Lupinus angustifolius* tissues with *P. cinnamomi* using QPCR.

<table>
<thead>
<tr>
<th>Infectiona</th>
<th><em>Arabidopsis</em> DNA</th>
<th><em>P. cinnamomi</em> DNA (pg)</th>
<th>Ct value for plasmid</th>
<th>ΔCt c</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>20 ng</td>
<td>10,000</td>
<td>19.32</td>
<td>0.04</td>
</tr>
<tr>
<td>–</td>
<td>20 ng</td>
<td>1,000</td>
<td>19.31</td>
<td>0.03</td>
</tr>
<tr>
<td>–</td>
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<td>0.03</td>
</tr>
<tr>
<td>–</td>
<td>20 ng</td>
<td>10</td>
<td>19.32</td>
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</tr>
<tr>
<td>–</td>
<td>20 ng</td>
<td>1</td>
<td>19.34</td>
<td>0.06</td>
</tr>
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<td>+</td>
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<td>Unknown</td>
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<tr>
<td>+</td>
<td>20 ng</td>
<td>Unknown</td>
<td>19.37</td>
<td>0.09</td>
</tr>
</tbody>
</table>

| – d | – | – | 19.28 | – |

a Infected leaves of *A. thaliana* or roots of lupin were collected 72 h after inoculation with *P. cinnamomi* (+) or distilled water (–).

b Ct (threshold cycle) is the number of PCR cycles at which a fluorescence intensity crosses a threshold set as a statistically significant increase in the SYBR fluorescence over all reactions within a plate.

c ΔCt = (Ct of plasmid in samples tested) – (Ct of plasmid DNA in reactions without *Arabidopsis*, lupin or *P. cinnamomi* DNA).

d Control is the pure plasmid DNA in the absence of both plant and pathogen DNA.
### Table 2 Accuracy of QPCR using plasmid as internal control in *Arabidopsis thaliana–Phytophthora cinnamomi* combination

<table>
<thead>
<tr>
<th>Plasmid DNA (ng)</th>
<th>Arabidopsis leaf (g)</th>
<th><em>P. cinnamomi</em></th>
<th>Mean ΔCt</th>
<th>SE ΔCt</th>
<th>ΔΔCt</th>
<th>$E^{ΔΔCt}$ (Fold Pc Amount)</th>
<th>EXP. Fold</th>
<th>$X^2$ Value</th>
<th><em>P</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1, 0.05, 0.025</td>
<td>1,000 ng DNA</td>
<td>2.85</td>
<td>0.24</td>
<td>3.39</td>
<td>9.9</td>
<td>10</td>
<td>0.830</td>
<td>0.362</td>
</tr>
<tr>
<td>1</td>
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<td>100 ng DNA</td>
<td>6.25</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.01 g hyphae</td>
<td>3.56</td>
<td>0.02</td>
<td>0.77</td>
<td>1.7</td>
<td>2</td>
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<tr>
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<td>1</td>
<td>0.1, 0.05, 0.025</td>
<td>100 ng DNA</td>
<td>8.42</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.01 g hyphae</td>
<td>2.06</td>
<td>0.18</td>
<td>1.3</td>
<td>2.41</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.025</td>
<td>0.005 g hyphae</td>
<td>0.76</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\Delta C_t = (C_t$ of *P. cinnamomi*) – (C_t of plasmid or *A. thaliana*); the values are the mean of five biological replicates

SE ΔCt is the standard errors of ΔCt.

ΔΔCt is the variation between ΔCts of *P. cinnamomi* pairs (either *P. cinnamomi* DNA or *P. cinnamomi* hyphae)

E is the efficiency of *P. cinnamomi* primer amplification (1.95)
### Table 3 Accuracy of QPCR using plasmid as internal control in *Lupinus angustifolius–Phytophthora cinnamomi* combination

<table>
<thead>
<tr>
<th>Plasmid DNA (ng)</th>
<th>Lupin root (g)</th>
<th><em>P. cinnamomi</em></th>
<th>Mean ΔCt</th>
<th>SE ΔCt</th>
<th>ΔΔCt</th>
<th>$E^{-\Delta\Delta C_t}$ (Fold Pc Amount)</th>
<th>EXP. Fold</th>
<th>$X^2$ Value</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Values based on plasmid DNA as an internal control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>1,000 ng DNA</td>
<td>1.87</td>
<td>0.11</td>
<td>3.33</td>
<td>9.42</td>
<td>10</td>
<td>0.841</td>
<td>0.359</td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>100 ng DNA</td>
<td>5.20</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>0.01 g hyphae</td>
<td>-1.89</td>
<td>0.09</td>
<td>2.34</td>
<td>4.82</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>0.002 g hyphae</td>
<td>-4.23</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Values based on lupin DNA as an internal control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>1,000 ng DNA</td>
<td>0.33</td>
<td>0.16</td>
<td>3.51</td>
<td>10.58</td>
<td>10</td>
<td>0.845</td>
<td>0.358</td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>100 ng DNA</td>
<td>3.83</td>
<td>0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>0.01 g hyphae</td>
<td>-3.41</td>
<td>0.34</td>
<td>2.35</td>
<td>4.85</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1, 0.05</td>
<td>0.002 g hyphae</td>
<td>-5.75</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\Delta C_t = (Ct\ of\ P.\ cinnamomi) - (Ct\ of\ plasmid\ or\ lupin);$\ the \ values \ are \ the \ mean \ of \ five \ biological \ replicates

SE $\Delta C_t$ is the standard errors of $\Delta C_t$

$\Delta \Delta C_t$ is the variation between $\Delta C_t$s of *P. cinnamomi* pairs (either *P. cinnamomi* DNA or *P. cinnamomi* hyphae)

$E$ is the efficiency of *P. cinnamomi* primer amplification (1.95)
Table 4 Measurement of *Phytophthora cinnamomi* DNA in infected samples collected from the resistant *Arabidopsis thaliana–P. cinnamomi* pathosystem and susceptible lupin–*P. cinnamomi* pathosystem

<table>
<thead>
<tr>
<th>Sample amount</th>
<th>Total DNA yield (µg)</th>
<th>Values based on plant DNA</th>
<th>Values based on plasmid DNA</th>
<th>ΔΔCt</th>
<th>E$^{ΔΔCt}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ΔCt=Pc – CtAt/Lupin</td>
<td>ΔCt=Pc – CtPlasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis–P. cinnamomi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg (8 leaf discs)</td>
<td>17.1</td>
<td>7.12</td>
<td>8.16</td>
<td>-1.05</td>
<td>2.03</td>
</tr>
<tr>
<td>50 mg (8 leaf discs)</td>
<td>21.1</td>
<td>7.20</td>
<td>7.87</td>
<td>-0.67</td>
<td>1.56</td>
</tr>
<tr>
<td>50 mg (8 leaf discs)</td>
<td>19.6</td>
<td>7.78</td>
<td>8.71</td>
<td>-0.93</td>
<td>1.88</td>
</tr>
<tr>
<td>50 mg (8 leaf discs)</td>
<td>10.3</td>
<td>6.96</td>
<td>8.54</td>
<td>-1.58</td>
<td>2.90</td>
</tr>
<tr>
<td>50 mg (8 leaf discs)</td>
<td>9.7</td>
<td>6.84</td>
<td>8.55</td>
<td>-1.72</td>
<td>3.19</td>
</tr>
<tr>
<td><em>lupin–P. cinnamomi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg</td>
<td>6.1</td>
<td>10.21</td>
<td>5.95</td>
<td>-4.26</td>
<td>17.72</td>
</tr>
<tr>
<td>50 mg</td>
<td>3.3</td>
<td>10.17</td>
<td>6.34</td>
<td>-3.83</td>
<td>13.32</td>
</tr>
<tr>
<td>50 mg</td>
<td>8.0</td>
<td>11.29</td>
<td>7.44</td>
<td>-3.86</td>
<td>13.55</td>
</tr>
<tr>
<td>50 mg</td>
<td>8.2</td>
<td>11.59</td>
<td>7.98</td>
<td>-3.61</td>
<td>11.42</td>
</tr>
<tr>
<td>100 mg</td>
<td>37.0</td>
<td>12.30</td>
<td>8.95</td>
<td>-3.35</td>
<td>9.59</td>
</tr>
<tr>
<td>100 mg</td>
<td>24.7</td>
<td>10.63</td>
<td>7.97</td>
<td>-2.66</td>
<td>6.03</td>
</tr>
<tr>
<td>100 mg</td>
<td>20.1</td>
<td>9.27</td>
<td>6.54</td>
<td>-2.73</td>
<td>6.32</td>
</tr>
<tr>
<td>100 mg</td>
<td>25.4</td>
<td>6.31</td>
<td>3.35</td>
<td>-2.96</td>
<td>7.40</td>
</tr>
</tbody>
</table>

Ct (threshold cycle) is the number of PCR cycles at which a statistically significant increase in the SYBR fluorescence (greater than background) can be detected; ΔCt is the variation between the mean of five Ct values obtained after amplification of *P. cinnamomi* DNA and the mean of Ct values obtained after amplification of *A. thaliana* or plasmid DNA; ΔΔCt is the difference between ΔCt of *P. cinnamomi* based on *Arabidopsis* DNA and ΔCt of *P. cinnamomi* based on plasmid DNA; E is the efficiency of *P. cinnamomi* primer amplification (1.965); E$^{ΔΔCt}$ is the fold pathogen biomass difference between ΔCt values obtained for *P. cinnamomi* using either *Arabidopsis* or plasmid DNA as internal control.
Figure Caption

**Fig. 1** Relative and total QPCR quantification of *Phytophthora cinnamomi* biomass over time in *Arabidopsis thaliana* and lupin normalized based on plasmid DNA or host plant DNA. (a) *P. cinnamomi* biomass (pg DNA) relative to the plasmid DNA (Pd) or *Arabidopsis* DNA (At) in *A. thaliana* ecotypes Ler (moderately susceptible) and Col-0 (resistant) after inoculation with *P. cinnamomi* zoospores. (b) The total amount of *P. cinnamomi* biomass (DNA) in the initial infected tissue samples from *A. thaliana* leaves. (c) *P. cinnamomi* biomass (pg DNA) in lupin (susceptible) relative to plasmid DNA (Pd) or lupin DNA (Lp). (d) The total amount of *P. cinnamomi* biomass (DNA) in the initial infected tissue samples from lupin roots. Data represent the mean of four biological replicates and bars represent the standard error of the mean. Lesion development in *P. cinnamomi* infected tissues in *A. thaliana* ecotypes Ler and Col-0 (e) and lupin (f). The scale bars for (e) and (f) are 2 mm and 1 cm, respectively.
Fig 1
Chapter 4

Defence signalling pathways involved in plant resistance and phosphite-mediated control of
Phytophthora cinnamomi

This chapter has been submitted to Planta:
Defence signalling pathways involved in plant resistance and phosphite-mediated control of *Phytophthora cinnamomi*

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Abstract

*Phytophthora cinnamomi* is one of the most devastating plant pathogens worldwide. Current control of *P. cinnamomi* in natural ecosystems primarily relies on chemical phosphite (Phi). To investigate host and Phi mediated resistance, *A. thaliana* ecotypes and mutants defective in salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) signalling pathways were screened for susceptibility to *P. cinnamomi*. In contrast to Col-0, the *aba2-4* mutant, deficient in the synthesis of ABA, was susceptible suggested a role for ABA in resistance to *P. cinnamomi*. Phosphite treatment increased resistance in *aba2-4*, but not to the level of Col-0, suggesting that Phi may act through both ABA dependent and independent pathways. Phi treatment or *P. cinnamomi* inoculation of Col-0 down-regulated *AtMYC2*, a positive regulator of ABA signalling which negatively regulates JA/ET-related pathogenesis related genes such as *PDF1.2* while positively regulating JA-mediated herbivore responses such as *VSP* and *PI*. Consistent with this, *P. cinnamomi* or Phi treatment caused up-regulation of *PDF1.2* and *THI2.1* and down regulation of *VSP2* and the ABA responsive gene *RD22*. Despite the up-regulation of JA/ET dependent defence genes, the JA defective mutant, *jar1-1* and ET defective mutants, *ein2-1* and *etr1-3* showed wild type levels of resistance to *P. cinnamomi* suggesting these JA/ET defences are not required for resistance to *P. cinnamomi*. Together these results suggest that the resistance response of Col-0 and Phi treatment both act, at least in part, through a mechanism dependent on ABA synthesis which appears independent of the interaction between ABA and elements of the JA/ET pathway.

**Keywords:** abscisic acid, oomycete pathogen, *Arabidopsis thaliana*, necrotrophic pathogen, plant defense signalling, abiotic and biotic stress.
Introduction

*Phytophthora* is a genus of oomycetes that is the most economically important genus of plant pathogens worldwide (Erwin and Ribeiro 1996). Among these, *P. cinnamomi* is considered to be one of the most pathogenic and causes considerable damage to agriculture, horticulture and forestry worldwide (Shearer and Fairman 2007; Brasier 2008; Hansen 2008; Rookes et al. 2008).

Understanding interactions between *Phytophthora* pathogens and their host plants is an essential step in developing disease control strategies. Model plants such as *A. thaliana* have been used to study interactions with various *Phytophthora* species (Khatib et al. 2004; Senchou et al. 2004; Wang et al. 2011). However, for the wide host range necrotroph pathogen, *P. cinnamomi*, comparatively little is known about the mechanisms involved in host resistance and the mode of action of the effective chemical, phosphite (Phi). A better understanding of the molecular mechanisms of plant—*Phytophthora cinnamomi* interactions and the effect of Phi on these interactions may allow the design of molecular strategies to improve disease resistance or the more effective use of Phi against *Phytophthora* in both agriculture and the management of natural ecosystems.

Plants defend themselves against different pathogens by induction of tailored defence responses including physiological changes like thickening of the cell walls and biochemical responses such as callose deposition, production of reactive oxygen species (ROS) or activation of signalling pathways including salicylic acid (SA), ethylene (ET), jasmonic acid (JA), and/or abscisic acid (ABA) (Chisholm et al. 2006; Jones and Dangl 2006). Microarray analysis in *A. thaliana* has revealed significant cross-talk between defence pathways induced by these signalling compounds/phytohormones (Schenk et al. 2000; Adie et al. 2007) indicating a considerable network of regulatory interactions.
which have been broadly reviewed (Beckers and Spoel 2006; Robert-Seilaniantz et al. 2011).

Plants often respond to *Phytophthora* attack by the formation of cell wall appositions and restructuring of the cytoskeleton around the invading hyphae. Reactive oxygen species are then rapidly produced, as is the induction of pathogenesis related (PR) proteins to fight against the progression of the pathogen (Cahill et al. 2002; Walker et al. 2006; Smertenko and Franklin-Tong 2011). Although a range of defence genes are up-regulated in response to *Phytophthora* attack, the pathogen’s lifestyle generally dictates the type of defence response initiated by the plant and there is some evidence for the involvement of different plant defence pathways with different *Phytophthora* species (Roetschi et al. 2001; Khatib et al. 2004).

Phosphite (a salt of phosphorous acid; Phi) has been used extensively in the management of *Phytophthora* diseases in many native plant ecosystems (Guest and Grant 1991; Hardy et al. 2001) and is highly effective in controlling *P. cinnamomi* in native plant communities of Western Australia (Hardy et al. 2001; Shearer and Fairman 2007). Phi not only primes the plant for a rapid and intense response to infection involving heightened activation of a range of defence responses, but also induces some aspects of the defence response, such as the expression of defence genes involved in the SA, JA and ET pathways in the absence of a pathogen (Eshraghi et al. 2011a). Nevertheless, the importance of this up-regulation of SA and JA/ET related defence genes in the induction of resistance to *P. cinnamomi* remains unclear. The objectives of this study were to elucidate which of the known defence pathway(s) is involved in resistance to *P. cinnamomi* using *A. thaliana* as a model plant and to study the possible involvement of plant signalling pathway(s) in Phi induced resistance to *P. cinnamomi*. To this end a range of mutant lines related to defence against pathogens (Table 1) were screened for the resistance/susceptibility to *P. cinnamomi* and analysis of defence gene
expression. Results suggested the involvement of ABA related signalling in both Col-0 resistance and to a lesser degree in Phi (20 mM) mediated resistance to *P. cinnamomi* which were both independent of the common SA, JA and ET defence signalling pathways.

**Materials and methods**

**Plant and pathogen materials**

*Arabidopsis thaliana* ecotypes Landsberg erecta (Ler) and Columbia (Col-0) and several *Arabidopsis* mutants (Table 1) defective in different defence pathways were used. *Arabidopsis thaliana* ecotype Ler and Col-0 were purchased from LEHLE Seeds (Round Rock, TX, USA) and defence pathway mutants including *npr1*-2, *pad2*-1, *pad3*-1, *etr1*-3, *ein2*-1, *jar1*-1 and *aba2*-4 were obtained from the European *Arabidopsis* Stock Centre (NASC).

*Phytophthora cinnamomi* isolate MP 94.48, was obtained from the Centre for Phytophthora Science and Management (CPSM) culture collection at Murdoch University. *Phytophthora cinnamomi* zoospores were produced aseptically using the method described by Byrt and Grant (1979) and the zoospore density was determined using a bright line haemocytometer and adjusted to a concentration of $1 \times 10^5$ zoospores mL$^{-1}$ using sterile distilled water.

**Plant growth conditions and inoculation procedure for assessments of defence responses**

Wild-type ecotypes and defence pathway mutants were planted on the surface of a seed raising mix (Debco) in 5 cm free-draining polyurethane pots pre-moistened with distilled water. Seeds were stratified for three days at 4°C in the dark before being transferred to a growth cabinet with a 10-h photoperiod with 100 μmol m$^{-2}$ s$^{-1}$ at 21 ±
1°C. To study the *P. cinnamomi* and *A. thaliana* interaction, leaves of 4-week-old seedlings were inoculated using either a detached leaf assay (Eshraghi et al. 2011a) or attached leaf assay as follows.

In attached leaf assays, leaves of 4-week-old *A. thaliana* seedlings were inoculated with 3 µL of 1 x 10^5 *P. cinnamomi* zoospores mL^{-1} on the abaxial surface and pots were transferred into a box which was sealed with aluminium foil to maintain high humidity and incubated in the dark at 25°C for 12 h then transferred to a growth cabinet with a 10-h photoperiod with 100 µmol m^{-2} s^{-1} at 21 ± 1°C. Control leaves were inoculated with 3 µL of sterile distilled water. The samples were collected and assessed for lesion size, callose deposition and quantitative real time PCR (QPCR) analysis of infection.

**Phosphite treatment**

Four-week-old seedlings were treated with 20 µL (10 µL per each of two leaves) of Phi (2.5 or 20 mM) 24 h before inoculation with *P. cinnamomi* zoospores (3 µL of 1 x 10^5 zoospores mL^{-1} per each leaf) in a completely randomised design. A stock solution of filter-sterilised 100 mM potassium Phi (pH 5.5, adjusted with KOH) was freshly prepared from phosphorous acid (Aldrich Chemicals) prior to dilution with distilled water to give the concentrations used in this study. Control plants were treated with 20 µL of sterile distilled water.

**Analysis of defence responses to *P. cinnamomi* infection**

A minimum of 15 inoculated leaves in each treatment was collected randomly and assessed for lesion development 48 h and 72 h after inoculation, and the experiment was conducted twice. The percentage of leaf area affected by the *P. cinnamomi* lesion was calculated using the program Image-Pro Express (Media Cybernetics, Inc.) with photographs taken using a binocular microscope (Olympus SZ40) with a digital camera (Nikon; Cool PIX 995).
For callose visualization within the cells, attached leaves of ecotypes Ler and Col-0 and several defence pathway mutants (npr1-2, aba2-4, pad2-1 and jar1-1) were collected 24 h after inoculation and stained with Aniline blue using the method described by Eschrich and Currier (1964). Leaves were examined for callose production using an Olympus BX51 microscope fitted with a UV filter set (excitation filter 330-385 nm, emission filter 420 nm, dichromic mirror 400 nm). A minimum of 15 leaves in each line were examined for the number of callose papillae per infection area and the experiment was repeated twice.

**Quantitative PCR analysis of infection**

QPCR measurements were conducted according to Eshraghi et al. (2011b). Infected leaves were collected and snap frozen 72 h after inoculation. Five samples each containing 4 leaf discs (7 mm in diameter) from one seedling were collected per treatment.

**Cloning ABA2**

In order to restore function of ABA2 gene in aba2-4 knock out mutant, this gene was cloned and transferred to the knock out mutant as follow. Col-0 genomic DNA was used as template for cloning the ABA2 gene (AT1G52340). PCR was performed with Phusion® High-Fidelity DNA Polymerase (BioLabs) according to manufacturer’s instructions using primers containing attB recombination site (forward 5´–GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CTC GTT CGT GAA AAG CTC CTT and reverse 5´–GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC AAC CTT CGA GGA ATC TCT TGC). The PCR product was purified using QIAquick Gel Extraction Kit (QIAGEN), cloned to pDONR207 (Invitrogen) using Gateway® BP Clonase® II Enzyme Mix (Invitrogen) according to the manufacturer’s instructions and transformed into *E. coli* competent cells (DH5α). The plasmid with correct sequence was cloned into
the Gateway® compatible expression vector pGREEN0179 containing CAMV 2x35S promoter and CAMV terminator with a Gateway A cassette (Wiszniewski et al. 2009) using Gateway® LR Clonase® II enzyme mix (Invitrogen) according to manufacturer’s instructions. Positive clones were confirmed by PCR and aba2-4 mutant was transformed using Agrobacterium tumefaciens C58C1 (pCH32) (Tai et al. 1999).

**Analysis for expression of defence-related genes**

For quantitative measurement of defence gene transcription, seeds of ecotype Col-0 and A. thaliana defence pathway mutants; pad2-1, aba2-4 were germinated on half strength 0.8% (W/V) phytagar-Gamborg B-5 basal medium (Sigma) (Gamborg et al. 1968) with additional 2.5 mM MES [2-(N-morpholino)-ethanesulphonic acid]-KOH (pH 5.5) and 0.5% (w/v) sucrose. After sowing the seeds on Gamborg B-5 medium, the plates were transferred to a growth cabinet at 21 ± 1°C with a 10-h photoperiod at a photon fluorescence rate of 100 µmol m⁻² s⁻¹. The plants were grown for 3 weeks and then transferred to fresh Gamborg B-5 medium with the different Phi treatments (0, 2.5 and 20 mM) in a completely randomized design for 4 days then leaves were inoculated with 3 µL of 1 x 10⁵ P. cinnamomi zoospores mL⁻¹ on the abaxial surface. Samples were collected 72 h after inoculation, frozen in liquid nitrogen and stored at -80°C until RNA extraction and qRT-PCR.

Four biological samples per treatment were randomly chosen for RNA extraction. Each sample was a pool of three plants. Tissue samples were homogenized using Tissuelyser® (Qiagen, Inc.) and total RNA was isolated using the RNeasy plant mini kit (Qiagen, Inc.) according to the manufacturer’s instructions. Total RNA was further treated with RNase-free DNase (Qiagen, Inc.) according to the manufacturer’s instructions. Approximately, 3 µg of DNA-free RNA was used for first-strand cDNA synthesis using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Gene specific primers (Table S1) were designed using Primer Express 1.5
software (Applied Biosystems Inc.) and qRT-PCR conducted according to Eshraghi et al. (2011a).

Data analysis

ANOVA was used to compare the treatments in all measurements and the normality of residuals was tested using GenStat software (14th edition). Least significant difference (LSD) with 95 % confidence was calculated between treatments and genotypes using GenStat software (14th edition).

Results

Interaction between P. cinnamomi and A. thaliana defence pathway mutants

In order to understand the mechanism(s) of resistance to P. cinnamomi and to determine whether specific defence responses or signalling pathway(s) are influencing the Arabidopsis- P. cinnamomi interaction, several A. thaliana mutant/transgenic lines with disrupted signalling pathways were screened for their susceptibility to P. cinnamomi by assessing the lesion size, number of callose papillae and quantitative real time PCR (QPCR) analysis of pathogen biomass.

Lesion size assessments

Infection of both detached and attached A. thaliana leaves by P. cinnamomi zoospores resulted in the appearance of lesions on leaves in Ler, Col-0 and all defence pathway mutants tested in this experiment (Fig. 1a, b) which were similar in appearance to those described by Eshraghi et al. (2011a). In both attached and detached leaf assays, ecotype Col-0 showed a high level of resistance to P. cinnamomi while ecotype Ler was moderately susceptible. Assessment of lesion size (the percentage leaf area covered by lesions) showed that aba2-4, a mutant affected in ABA biosynthesis, had substantial lesion development in both attached and detached leaf assays (Fig. 1a, b). In detached
leaf assays, pad2-1, a mutant with reduced production of camalexin, glucosinolates and salicylic acid (Dubreuil-Maurizi et al. 2011), and ein2-1, a mutant insensitive to ET, showed a higher level of susceptibility to *P. cinnamomi* compared to their parent background (Col-0). However, the enhanced susceptibility of these lines was reduced in the attached leaf assays. The SA deficient lines (npr1-2, nahG and sid2-1) showed no significant differences (*P*>0.05) in their susceptibility in comparison to their parental background Col-0. The mutant jar1-1, with reduced sensitivity to JA and the mutant etr1-3, an ET-insensitive mutant were resistant to *P. cinnamomi* in both detached and attached leaf assays compared to their parental background Col-0.

*Callose production during the A. thaliana—P. cinnamomi interaction*

Callose deposition was assessed in *P. cinnamomi*-inoculated attached leaves of *A. thaliana* ecotypes Ler, Col-0 and defence pathway mutants; npr1-2, pad2-1, jar1-1 and aba2-4 at 24 h after inoculation (Fig. 2). The number of callose papillae in the resistant ecotype Col-0 was significantly (*P*<0.001) greater than that in the moderately susceptible ecotype Ler. The npr1-2 and aba2-4 mutants produced fewer callose papillae in comparison to that in the resistant background Col-0. Callose formation was not observed in non-inoculated leaves in all lines tested.

*QPCR assessments of P. cinnamomi infection*

To determine the level of infection quantitatively, the relative amount of *P. cinnamomi* biomass (DNA) in infected leaves of different lines was determined according to Eshraghi et al. (2011b). The QPCR assessment of pathogen biomass showed that aba2-4 had significantly (*P*<0.001) greater accumulation of *in planta* pathogen biomass in both attached and detached leaf assays (Fig. 3a, b). In detached leaf assays, the pathogen biomass in pad2-1 and pad3-1 was considerably greater compared to their background (Col-0); however, this enhanced pathogen biomass was
not observed in the attached leaf assays. The high level of pathogen biomass observed in the *aba2-4* mutant in both the attached and detached leaf assays indicates the ABA pathway may play a key role in resistance to *P. cinnamomi*. Furthermore, transferring the ABA2 gene into the *aba2-4* mutant restored resistance to the level observed in the parental background Col-0 (Fig. 3b) confirming its susceptibility was due to the loss of ABA2 function in the mutant. The results also showed a significant correlation between the lesion size and relative pathogen biomass in both detached leaf assays ($r = 0.986; P<0.001$) and attached leaf assays ($r = 0.897; P<0.001$).

**Pathways involved in Phi mediated resistance**

Application of Phi induced resistance in ecotypes Ler and *aba2-4* in both the attached and detached leaf assays (Fig. 3a, b). Although Phi increased the resistance in the *aba2-4* mutant to *P. cinnamomi*, it did not return to the level observed in Col-0. This suggests that ABA may be required, at least in part, for the full activity of Phi.

To investigate the effect of Phi on SA or JA/ET signalling pathways, the relative expression ratios of *PR1*, *PDF1.2*, and *THI2.1* transcripts (Table S1) in ecotype Col-0 and the defence pathways mutants (*pad2-1* and *aba2-4*) was analysed following Phi (0 and 20 mM) treatment in the presence/absence of *P. cinnamomi* inoculation (Fig. 4). To find out whether Phi mediated resistance is mediated through ABA signalling, the relative expression ratios of the ABA-responsive genes *AtMYC2*, *KIN1* and *RD22* as well as the herbivore,JA and ABA-responsive *VSP2* gene (Kurkela and Borgfranck 1992; Yamaguchi-Shinozaki and Shinozaki 1993; Anderson et al. 2004; Lorenzo et al. 2004) were analysed in Col-0, *pad2-1* and *aba2-4* following 20 mM Phi treatment in the presence/absence of *P. cinnamomi* inoculation (Fig. 5, Table S1). The transcript levels of all genes in the mutants were normalized based on expression of actin 2 (*ACT2*) measured in the same samples and presented relative to the normalized expression.
levels in non-Phi-treated non-inoculated wild ecotype (Col-0) plant (control) and as fold expression.

**SA-related defence responses**

In Col-0, the expression of the *PRI* gene increased following either *P. cinnamomi* inoculation (536-fold) or Phi treatment (71-fold) and *PRI* expression was the highest (735-fold) in *P. cinnamomi* inoculated Phi-treated samples (Fig. 4). The expression of the *PRI* gene in non-inoculated non-Phi treated *aba2-4* mutant samples was considerably (*P*<0.001) higher compared to that of its resistant background Col-0, nonetheless the levels increased (677-fold) significantly (*P*<0.001) in inoculated samples (Fig. 4). Application of Phi to *aba2-4* mutant resulted in an increase in expression of *PRI* (791-fold) compared to control (non-inoculated non-Phi treated) samples, to a comparable level to that in inoculated non-Phi treated samples. Interestingly, despite reports of SA deficiency in *pad2* mutants, expression of *PRI* was higher (52-fold) in non-inoculated non-Phi treated (control) *pad2-1* mutant samples than in Col-0 and treatment with *P. cinnamomi* or Phi induced *PRI* further (977-fold and 193-fold, respectively) (Fig. 4).

**JA/ET-related defence responses**

Expression of *PDF1.2* in both non-inoculated, non-Phi treated *aba2-4* and *pad2-1* mutant samples was higher (28-fold and 4.6-fold, respectively) compared to that in their non-inoculated non-Phi treated background Col-0. *Phytophthora cinnamomi* infection resulted in a significant (*P*<0.001) increase in expression of *PDF1.2* gene in Col-0 (173-fold), *aba2-4* mutant (107-fold) and *pad2-1* mutant (104-fold) (Fig. 4). Phi treatment also considerably (*P*<0.001) induced *PDF1.2* in Col-0 and the *aba2-4* mutant (2.9-fold and 64-fold, respectively) as well as in the *pad2-1* mutant (11.6-fold). Expression of the *THI2.1* gene was higher (4.4-fold) in the non-inoculated, non-Phi treated (control)
pad2-1 mutant compared to that in non-inoculated, non-Phi treated Col-0 and aba2-4. Phytophthora cinnamomi inoculation resulted in a significant (P<0.001) increase in the induction of THI2.1 in Col-0, aba2-4 and pad2-1 mutants (3.9-fold, 13.5-fold, and 20.5-fold, respectively) (Fig. 4). Application of Phi also induced THI2.1 in both Col-0 (7-fold) and the aba2-4 (27.4-fold) mutant, but it did not increase THI2.1 expression above the heightened basal levels present in the pad2-1 mutant (Fig. 4). Overall, these results indicated that aba2-4 and pad2-1 related signalling pathways may be involved in the maintenance of low basal levels of JA/ET responsive defence gene expression in untreated Arabidopsis but these pathways have little impact following P. cinnamomi or Phi treatment.

The effect of Phi on a positive regulator of ABA signalling

To investigate the effect of Phi and P. cinnamomi infection on the ABA signalling pathway, the relative expression ratios of AtMYC2 (Table S1) in wild ecotype Col-0 and the defence pathway mutants pad2-1 and aba2-4 were analysed following Phi treatment (20 mM) in the presence/absence of P. cinnamomi inoculation (Fig. 5). Transcript level of AtMYC2, a positive regulator of ABA (Abe et al. 2003), in non-inoculated non-Phi treated samples of both the aba2-4 and pad2-1 mutants was suppressed (6.5-fold and 3.2-fold, respectively) compared to that of the non-inoculated non-Phi treated Col-0 (Fig. 5). The lower AtMYC2 expression in aba2-4 is consistent with the ABA insensitivity of this mutant; however, the reduced AtMYC2 expression in pad2-1 suggests this pathway may also integrate with ABA signalling and is consistent with the higher levels of PDF1.2 and THI2.1 in non-Phi treated, non-inoculated samples.

In Col-0, P. cinnamomi infection down-regulated transcript levels of AtMYC2 (4.7-fold) with similar levels of suppression following Phi treatment alone or simultaneous Phi treatment and P. cinnamomi inoculation. Similar levels of suppression were observed following Phi treatment or Phi treatment and P. cinnamomi inoculation
of either *aba2-4* or *pad2-1*. Whether the down regulation of *AtMYC2* is a consequence of the up-regulation of the JA pathway, including genes such as *PDF1.2* and *THI2.1* or vice versa is difficult to determine.

**Regulation of KIN1, RD22 and VSP2 in response to *P. cinnamomi* infection and Phi treatment**

*KIN1* is a cold, dehydration and osmotic stress regulated gene of unknown function that is up-regulated by ABA (Kurkela and Borgfranck 1992; Wang and Cutler 1995). The transcriptional level of the *KIN1* gene in non-inoculated non-Phi treated *aba2-4* mutant did not differ from that of the non-inoculated non-Phi treated Col-0, while *pad2-1* showed a 2.2-fold higher expression. In Col-0, *P. cinnamomi* inoculation alone, Phi treatment alone and Phi and *P. cinnamomi* together resulted in the induction of *KIN1* (4.8-fold, 3.9-fold and 3.4-fold, respectively) (Fig. 5).

In *Arabidopsis*, the induction of *RD22*; a dehydration-responsive gene is mediated by ABA and its transcription is induced by application of exogenous ABA through *AtMYC2* (Yamaguchi-Shinozaki and Shinozaki 1993; Abe et al. 2003). In Col-0, the transcript level of *RD22* in response to *P. cinnamomi* inoculation and Phi treatment decreased (2.2-fold and 2.5-fold, respectively) which is consistent with the down-regulation of *AtMYC2* in these samples (Fig. 5). Interestingly, *RD22* was suppressed in *pad2-1* by 3 fold; however, *P. cinnamomi* inoculation and Phi-treatments did not affect its transcriptional abundance. The *aba2-4* mutant expressed *RD22* similarly to Col-0 with the exception of *P. cinnamomi* inoculated plants in which the expression of *RD22* was not suppressed as in Col-0.

*VSP2* (vegetative storage protein 2) is a JA-responsive defence gene typically associated with responses to herbivores and is positively regulated by ABA through the *AtMYC2* transcription factors (Lorenzo et al. 2004). In our study *VSP2* was highly
down-regulated (72-fold) in the aba2-4 mutant (Fig. 5) confirming a dependence on ABA synthesis for expression. Phytophthora cinnamomi inoculation of the aba2-4 mutant resulted in an even further down-regulation of this gene, while Phi appeared to remove the repression on VSP2 at the transcriptional level. In Col-0, both P. cinnamomi inoculation and Phi treatment resulted in suppression of the VSP2 gene (6-fold and 5-fold, respectively) compared to the non-inoculated non-Phi treated samples. The pad2-1 mutant showed a down-regulation (5-fold) in transcript level of the VSP2 gene and the combination of P. cinnamomi inoculation and Phi treatment caused a higher level of down-regulation (11.5-fold) for this gene (Fig. 5).

Discussion

This study investigated the P. cinnamomi-A. thaliana interaction by screening the A. thaliana ecotypes (Col-0 and Ler) and several defence-related mutant/transgenic lines deficient in SA, JA/ET, ABA signalling and the pad2-1 mutant for their responses to P. cinnamomi infection and further investigated the role of Phi mediated resistance by monitoring its effect on these defence signalling pathways.

Inoculation of A. thaliana leaves by P. cinnamomi resulted in the appearance of lesions on leaves of ecotypes Ler and Col-0 and all defence-related mutants tested. The lesion size assessments in both detached and attached leaf assays used in this study suggested a role for ABA signalling resistance to P. cinnamomi by showing a high level of infection in the aba2-4 mutant. The SA signalling deficient lines npr1-2, nahG and sid2-1 remained resistant in both attached and detached leaf assays suggesting that resistance of A. thaliana to P. cinnamomi is SA independent. Although in detached leaf assays, ein2-1, a mutant insensitive to ET and pad2-1, a mutant with reduced production of the phytoalexin camalexin, glucosinolates and SA (Dubreuil-Maurizi et al. 2011) showed a higher level of susceptibility to P. cinnamomi compared to their background
(Col-0), the susceptibility of these mutants diminished in the attached leaf assays suggesting different responses may be occurring in attached and detached leaves following *P. cinnamomi* challenge. Two possibilities for this difference may be either senescence processes, occurring in the detached leaves which have previously been associated with ethylene signalling (Liu et al. 2007), or the absence of a systemic signalling which compensates for the loss of *EIN2* and *PAD2* in attached leaves. Further studies to determine the activity of senescence associated genes such as *SAG12* in attached and detached leaves with and without *P. cinnamomi* inoculation or double mutants with *ein2/pad2-1* and systemic signalling mutants such as *dir1* may shed further light onto the processes involved in the different phenotypes observed following inoculation of attached and detached leaves.

To investigate this further, the level of *P. cinnamomi* infection (relative pathogen biomass) in attached leaves of different *Arabidopsis* wild ecotypes and defence pathway mutants was measured and compared to that in comparable detached leaves. QPCR assessment of *P. cinnamomi* infection also showed a reduction of *in planta* pathogen biomass in attached leaves versus detached leaves with the greatest difference being observed for *ein2-1* and *pad2-1*. The results also showed a high correlation between the lesion size and relative pathogen biomass measured in infected plants in both detached leaf assays and attached leaf assays. Detached leaf assays and cell suspension assays have been used extensively to study plant-pathogen interactions and particularly to screen for host plant resistance to different pathogens due to their simplicity; however, our results suggest caution in the use of detached leaves. Liu et al. (2007) who studied defence responses against hemibiotrophic *Colletotrichum* spp. also showed differences in symptom development between detached leaves and intact plants with the greatest variation on ethylene-insensitive mutants. These results suggest that when screening the
susceptibility of plants to a potential pathogen, the use of intact leaves is more reliable than detached leaves.

Together, the lesion size assessments and QPCR analysis of pathogen infection in infected detached/attached leaves suggest the role of ABA signalling in induction of resistance to \textit{P. cinnamomi} by showing a high level of infection in the \textit{aba2-4} mutant. The role of ABA signalling in the induction of resistance to \textit{P. cinnamomi} was further confirmed by cloning the \textit{ABA2} gene and transforming the \textit{aba2-4} mutant which resulted in a similar level of resistance as in Col-0. The phytohormone ABA plays an important role in developmental processes of plants and in the induction of resistance to abiotic stress (Nambara and Marion-Poll 2005). Several links between ABA and defence signalling have previously been reported (de Torres Zabala et al. 2009; Fan et al. 2009; Ton et al. 2009; Cao et al. 2011). Ward et al. (1989) reported that ABA interferes with SA-related defences in the soybean–\textit{P. sojae} pathosystem and ABA was shown to have a suppressive effect on systemic resistance in a JA/ET independent way (Yasuda et al. 2008). ABA has been shown to enhance the susceptibility of plants to biotrophic/hemibiotrophic pathogens such as \textit{Phytophthora infestans} and \textit{Cladosporium cucumerinum} (Henfling et al. 1980), and other pathogens (McDonald and Cahill 1999; Anderson et al. 2004), whilst it increases resistance to the necrotrophic pathogens \textit{Alternaria brassicicola} and \textit{Plectosphaerella cucumerina} (Ton and Mauch-Mani 2004). These different outcomes highlight the different functions of ABA in response to necrotrophic and biotrophic infections and support a role for ABA in resistance to the necrotrophic \textit{P. cinnamomi}.

ABA has also been linked to resistance against necrotrophic pathogens in which callose accumulation as well as the JA/ET signalling are implicated (Thomma et al. 1998; Ton and Mauch-Mani 2004). The assessment of callose deposition in \textit{P. cinnamomi}-inoculated leaves of \textit{A. thaliana} lines revealed that amongst all lines tested,
the resistant ecotype Col-0 had the highest number of callose papillae produced in response to pathogen infection and the highly susceptible mutant aba2-4 had the lowest number. ABA has been implicated in enabling early and efficient build up of callose papillae at the sites of infection (Flors et al. 2005) and this ABA-dependent priming of callose synthesis appears important for resistance to some pathogens (Ton and Mauch-Mani 2004). The number of callose papillae has previously been correlated with resistance to *P. cinnamomi* with the resistant ecotype Col-0 producing significantly more callose papillae in response to infection compared to the moderately susceptible ecotype Ler (Eshraghi et al. 2011a). The susceptibility of aba2-4 and the reduced number of callose papillae in this line further support the importance of this defence response in resistance to *P. cinnamomi*. Phi induced enhanced defence responses to *P. cinnamomi* in all lines screened in this study including the highly susceptible mutant aba2-4; however, Phi treatment did not restore resistance in this line to the levels observed in untreated Col-0. This suggests that Phi induced resistance may involve both ABA dependent and independent mechanisms. To examine the effect of Phi on defence signalling pathways and to find out whether Phi mediated resistance is partially through the ABA signalling pathway, the effect of Phi on the transcriptional level of several defence-related genes (*PRI, PDF1.2, THI2.1*) and ABA signalling-related genes (*AtMYC2, KIN1, VSP2* and *RD22*) in the wild ecotype Col-0, the ABA signalling mutant (aba2-4) and pad2-1 in the presence/absence of *P. cinnamomi* inoculation was quantitatively analysed.

The higher expression of *PRI* in the non-inoculated non-Phi treated aba2-4 mutant compared to that of its resistant background Col-0 indicated a negative interaction between ABA related signalling pathways and SA-related defence responses in *Arabidopsis*. This is consistent with a previous report showing higher levels of SA-induced genes in ABA-deficient *Arabidopsis* mutants (Mohr 2003). The antagonistic
interaction between SA and ABA signalling pathways has been suggested by previous studies (Yasuda et al. 2008; de Torres Zabala et al. 2009; Fan et al. 2009). Together the higher expression of PRI in the more susceptible aba2-4 mutant and the resistant phenotype observed for npr1-2, nahG and sid2-1 suggest SA is not important for resistance to P. cinnamomi in Arabidopsis. This finding is consistent with previous studies that indicated the association of SA signalling with resistance to biotrophic and hemibiotrophic pathogens, while a combination of JA and ET triggered resistance to necrotrophic pathogens (McDowell and Dangl 2000; Thomma et al. 2001; Oliver and Ipcho 2004; Glazebrook 2005).

Phi treatment and P. cinnamomi inoculation also resulted in the induction of the JA/ET-related genes PDF1.2 and THI2.1. However, screening of the JA/ET-related mutants in this study suggests that resistance to P. cinnamomi in attached leaves is independent of the JA or ET signalling pathways. Transcriptional analysis of PDF1.2 and THI2.1 in aba2-4 and pad2-1 indicated that ABA and processes attenuated in the pad2-1 mutant negatively regulate aspects of JA/ET related defence responses. The results from aba2-4 are consistent with those of Anderson et al. (2004) where exogenous application of ABA was shown to repress the transcription of the JA/ET-related genes such as PDF1.2 in a MYC2 dependent manner and over expression of AtMYC2 suppressed the expression of the PDF1.2 gene.

The transcriptional level of AtMYC2, a positive regulator of ABA signalling which is allelic to JASMONATE INSENSITIVE1 (JIN1) (Abe et al. 2003) was suppressed in both the aba2-4 and pad2-1 mutants suggesting a similar response in terms of defence gene expression between the PAD2 dependent and ABA signalling pathways. Phi treatment and P. cinnamomi infection also suppressed the transcript level of AtMYC2 gene in Col-0 and this could be due to the activation of JA-related plant defence responses by Phi application or P. cinnamomi challenge. The aba2-4 and pad2-
I mutants did not show further suppression of AtMYC2 beyond the low level of non-treated plants following Phi or P. cinnamomi inoculation. Abe et al. (2003) reported that AtMYC2 not only corresponds to an early JA-responsive gene and plays a key role in JA-regulated expression of the RD22 gene, but also increased levels of some JA and herbivore defence-related transcripts such as VSP2 while other studies have found it to be a negative regulator of other JA/ET defence genes including PDF1.2 (Anderson et al. 2004; Lorenzo et al. 2004). AtMYC2 positively regulates JA-mediated resistance to herbivores (Lorenzo et al. 2004) and is required for the suppression of SA-related defence signalling (Laurie-Berry et al. 2006). Therefore, AtMYC2 differently modulates the expression of two groups of JA-induced genes. The first group, which are the genes involved in JA/ET defence responses against pathogens is negatively regulated by AtMYC2 and the second group, which are the genes involved in JA-mediated responses to herbivory, is activated by AtMYC2 (Lorenzo et al. 2004). Whether the down-regulation of AtMYC2 by P. cinnamomi or Phi treatment in this study is a consequence of the up-regulation of the JA pathway, including genes such as PDF1.2 and THI2.1, or vice versa could be further investigated by inoculation or Phi treatment of atmyc2/jin1 and other related mutants.

The induction of RD22, a dehydration-responsive gene, is mediated by ABA and its transcription is induced by application of exogenous ABA through the AtMYC2 transcription factor (Yamaguchi-Shinozaki and Shinozaki 1993; Abe et al. 2003). In ecotype Col-0, transcriptional abundance of RD22 and VSP2 was down-regulated by either Phi treatment or P. cinnamomi inoculation. This could be associated with down-regulation of AtMYC2 in these samples from the activation of JA/ET-related defence signalling and the antagonistic relationship between aspects of the JA and ABA signalling pathways or a mechanism independent of ABA. Although the regulation of PDF1.2 in aba2-4 in response to P. cinnamomi or Phi treatment and the regulation of
VSP2 in *aba2-4* is consistent with negative regulation of *PDF1.2* and positive regulation of *VSP2* by *AtMYC2* as described previously (Anderson et al. 2004; Lorenzo et al. 2004), the role of *AtMYC2* would be further elucidated by analysis of gene expression and the resistance/susceptibility of the *atmyc2/jin1* mutant in response to *P. cinnamomi*/Phi treatment.

Overall, this study highlighted that ethylene and *PAD2* related processes appear to have a larger impact on the outcome of a plant-pathogen interaction in detached leaves. The lesion size and QPCR assessments of pathogen biomass in intact plants suggest the role of the ABA signalling pathway in the induction of resistance to *P. cinnamomi* since the *aba2-4* mutant, deficient in the synthesis of ABA, showed a significant increase in susceptibility and its role was further confirmed by complementation of the *aba2-4* mutant with the wild type gene. Phi treatment and *P. cinnamomi* inoculation increased the transcriptional abundance of defence associated genes *PR1, PDF1.2, THI2.1* in Col-0 and down regulated the expression of genes which are typically up-regulated by ABA such as *AtMYC2, RD22*, and *VSP2*. These findings are consistent with previous descriptions of a negative interaction between ABA and JA/ET pathogen defence genes such as *PDF1.2* and a positive interaction between ABA and JA herbivore induced genes such as *VSP*. However, the resistance of mutants in the SA-, JA/ET and PAD2-dependent pathways suggests these common defence pathways are not required for resistance to *P. cinnamomi* in intact plants and the ABA-mediated resistance may be through pathways not interacting with the JA/ET or SA pathways.

This possibility opens opportunities for novel roles for ABA signalling in defence against necrotrophic pathogens such as *P. cinnamomi* and in the activity of fungistatic chemicals such as Phi. Screening additional mutants with complementary phenotypes in ABA related signalling will likely help to further define the complex signalling network suggested by this study.
Acknowledgments

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a functional *PAD2* gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J 28: 293–305


Table 1: The list of *Arabidopsis* defence-related mutants/over-expressing lines used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>locus</th>
<th>Genetic alteration</th>
<th>Phenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>jar1-1</td>
<td>AT2G46370</td>
<td>EMS mutant</td>
<td>Reduced sensitivity to jasmonic acid</td>
<td>Staswick et al. (1992)</td>
</tr>
<tr>
<td>ein2-1</td>
<td>AT5G03280</td>
<td>EMS mutant</td>
<td>Insensitive to ethylene</td>
<td>Guzmán and Ecker (1990)</td>
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<tr>
<td>etr1-3</td>
<td>AT1G66340</td>
<td>EMS mutant</td>
<td>Insensitive to ethylene</td>
<td>Bleecker et al. (1988)</td>
</tr>
<tr>
<td>npr1-2</td>
<td>AT1G64280</td>
<td>EMS mutant</td>
<td>Defective in expression of pathogenesis-related (PR) genes in response to salicylic acid</td>
<td>Gaffney et al. (1993)</td>
</tr>
<tr>
<td>nahG</td>
<td>N/A</td>
<td>Introduced gene</td>
<td>No salicylic acid accumulation</td>
<td>Friedrich et al. (2001)</td>
</tr>
<tr>
<td>sid2-1</td>
<td>AT1G74710</td>
<td>T-DNA knockout</td>
<td>Defective in isochorismate-derived salicylic acid biosynthesis</td>
<td>Delaney et al. (1995)</td>
</tr>
<tr>
<td>aba2-4</td>
<td>AT1G52340</td>
<td>EMS mutant</td>
<td>Defective in abscisic acid biosynthesis, reduced sensitivity to sugar and glucose</td>
<td>Laby et al. (2000)</td>
</tr>
<tr>
<td>pad2-1</td>
<td>AT4G23100</td>
<td>EMS mutant</td>
<td>Phytoalexin (camalexin) glucosinolates and salicylic acid deficient</td>
<td>Glazebrook and Ausubel (1994); Dubreuil-Maurizi et al. (2011)</td>
</tr>
<tr>
<td>pad3-1</td>
<td>AT3G26830</td>
<td>EMS mutant</td>
<td>Defective in camalexin biosynthesis</td>
<td>Zhou et al. (1999)</td>
</tr>
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</table>

All lines mentioned are in Col-0 background.
Table S1 Sequences of the gene-specific primer pairs used in quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>AGI number</th>
<th>Forward and reverse primers (5´–3´)</th>
<th>Amplicon size (bp)</th>
<th>Amplicon Tm(^b) (°C)</th>
</tr>
</thead>
</table>
| PR1   | AT2G14610  | AGCCTATGCTCGGAGCTACG
ACCCCCAGCTAAGTTTCCC | 91                  | 82                    |
| PDF1.2| AT5G44420  | CCAAGTGGGACATGGTCAGG
TGCAATGCTCATGTTTGGCT | 91                  | 79                    |
| THI2.1| AT1G72260  | ACGCCATTCTCGAAAACCTCA
TGGAGAGTGTTGTCATGGCACC | 91                  | 79                    |
| AtMYC2| AT1G32640  | ATCAAGAACCAGCTCGAGGA
CGAAGAACACGAAGACGACA | 90                  | 78                    |
| KIN1  | AT5g15960  | CTGGCAAAGCTGAGGAGAAG
CCGCATCCGATACACTCTTT | 108                 | 82                    |
| VSP2  | AT5g24770  | AATACCACCCTTTGCGTCAC
GGCCAAGAGCAAGAGAAGTG | 105                 | 73                    |
| RD22  | AT5g25610  | CGGTAAAAGAACCACGCGTA
AAAGGGTTTGCTCTGTTT | 106                 | 81                    |
| ACT2  | AT3G18780  | CTTGCACCAAGAGCAGCATGAA
CGATCCAGACACTGTACTTCTT | 68                  | 79.8                  |

\(^a\)PR1: pathogenesis-related 1; PDF1.2: plant defensin 1.2; THI2.1: thionin 2.1; AtMYC2: JIN1; jasmonate insensitive 1; ABI: abscisic acid (ABA) insensitive 1; KIN1: ABA inducible protein kin1; VSP2: vegetative storage protein 2; RD22: responsive to dessication 22; EF-1 alpha: elongation factor 1 alpha; and ACT2: actin 2.

\(^b\)Melting temperature.
Figure Legends

**Fig. 1** The average area of lesions (expressed as the percentage of leaf area affected) on detached leaves (a) and attached leaves (b) of *Arabidopsis thaliana* ecotypes Col-0, Ler and several mutants impaired in defence pathways 48 h and 72 h after inoculation with of *Phytophthora cinnamomi* zoospores (3 µL of 1 x 10^5 zoospores/mL). The bars show the mean of 15 leaves with standard errors. ANOVA indicated a significant difference between ecotypes in both attached and detached leaf assays at both time points (P<0.001). LSD (5%; vertical bars) was 0.69 (48 h) and 0.52 (72 h) for the detached leaf experiment, and 0.85 (48 h) and 0.49 (72 h) for the attached leaf experiment.

**Fig. 2** Number of callose papillae per leaf (a) in leaves of *Arabidopsis thaliana* ecotypes Ler and Col-0 and several defence pathway mutants 24 h after inoculation with *Phytophthora cinnamomi* zoospores (3 µL of 1 x 10^5 zoospores/mL). The bars show the mean of 15 leaves per treatment with standard errors and asterisks (*) indicate a significant difference from Col-0 (wild background) according to Students T-test. (b) and (c); show callose deposition within the cells of ecotype Col-0 and *aba2-4* mutant 24 h after inoculation, respectively.

**Fig. 3** Kinetic PCR quantification of *Phytophthora cinnamomi* biomass (pg DNA per sample) in 24 h phosphite (Phi)-treated and non-treated detached (a) and attached (b) leaves of *Arabidopsis thaliana* ecotypes Ler, Col-0 and several defence pathway mutants 72 h after inoculation. Bars represent the mean and standard error from five replicates each consisting of four infected leaves. ANOVA indicated a significant (P<0.001) affect of genotype in attached and detached leaf assays. *ABA2-4* = *aba2-4* mutant complemented with ABA gene (AT1G52340), ND = not determined.
**Fig. 4** Relative expression ratios of *PR1*, *PDF1.2*, *THI2.1* transcripts in *Arabidopsis thaliana* wild ecotype (Col-0) and defence pathway mutants (*pad2-1* and *aba2-4*) following phosphite (Phi) treatments (0 and 20 mM) in the presence and absence of *Phytophthora cinnamomi* 72 hours after inoculation. Bars present the mean and standard error from four replicates each consisting of three plants. The numbers on each bar indicate the fold change in defence gene transcript levels.

**Fig. 5** Relative expression ratios of *AtMYC2*, *KIN1*, *VSP2* and *RD22* transcripts in *Arabidopsis thaliana* wild ecotype (Col-0) and defence pathway mutants (*pad2-1* and *aba2-4*) following phosphite (Phi) treatments (0 and 20 mM) in the presence and absence of *Phytophthora cinnamomi* 72 h after inoculation. Data represent the mean and standard error of four replicates of three plants each. The numbers on each bar indicate the fold increase/decrease in transcript levels.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Chapter 5

Suppression of the auxin response pathway enhances susceptibility to *Phytophthora cinnamomi* while phosphite-mediated resistance stimulates the auxin signalling pathway

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Suppression of the auxin response pathway enhances susceptibility to *Phytophthora cinnamomi* while phosphite-mediated resistance stimulates the auxin signalling pathway

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Abstract

Background: *Phytophthora cinnamomi* is a devastating necrotrophic pathogen worldwide and phosphite (Phi), an analogue of phosphate (Pi) is highly effective in the control of this pathogen. Phi also interferes with Pi starvation responses (PSR), of which auxin signalling is an integral component. In the current study, the involvement of Pi and the auxin signalling pathways in host and Phi-mediated resistance to *P. cinnamomi* was investigated by screening the *Arabidopsis thaliana* ecotype Col-0 and several mutants defective in PSR and the auxin response pathway for their susceptibility to this necrotrophic pathogen. Phi-mediated resistance was also studied by monitoring its effect on Pi- and the auxin response pathways.

Results: Here we demonstrate that *phr1-1* (phosphate starvation response 1), a mutant defective in response to Pi starvation was highly susceptible to *P. cinnamomi* compared to the parental background Col-0. The importance of the auxin signalling pathway in resistance to *P. cinnamomi* was supported by the inhibition of auxin transporters by TIBA (2,3,5-triiodobenzoic acid) which led to a significant increase in susceptibility of blue lupin (*Lupinus angustifolius*) to *P. cinnamomi*. Moreover, analysis of the *Arabidopsis tir1-1* (transport inhibitor response 1) mutant, deficient in the auxin-stimulated SCF (Skp1–Cullin–F-Box) ubiquitination pathway was also highly susceptible to *P. cinnamomi* and the susceptibility of the mutants *rpn10* and *pbe1* further supported a role for the 26S proteasome in resistance to *P. cinnamomi*. However, the mutant of SGT1B gene, which is required for SCF$^{TIR1}$ mediated auxin responses and also involved in *R*-gene-mediated resistance, remained resistant. Given the apparent involvement of auxin and PSR signalling in the resistance to *P. cinnamomi*, the possible involvement of these pathways in Phi mediated resistance was also investigated. Phi (especially at high concentrations) attenuates the response of some Pi starvation inducible genes such as *AT4*, *AtACP5* and *AtPT2* in Pi starved plants.
However, Phi enhanced the transcript levels of *PHRI* and the auxin responsive genes (*AUX1*, *AXR1* and *AXR2*), suppressed the primary root elongation, and increased root hair formation in plants with sufficient Pi.

**Conclusions:** The auxin response pathway, particularly auxin sensitivity and transport, plays an important role in resistance to *P. cinnamomi* in *Arabidopsis*, and phosphite-mediated resistance may in some part be through its effect on the stimulation of the auxin response pathway.

**Keywords:** abscisic acid, indole-3-acetic acid (IAA), phosphate starvation response (PSR), TIBA, ubiquitin proteasome pathway (UPP)

**Background**

The necrotrophic plant pathogen *Phytophthora cinnamomi* [1] causes considerable damage to agriculture, horticulture and native plant communities worldwide [2-5]. Phosphite (Phi), an analogue of phosphate (Pi) is a salt of phosphorous acid and is highly effective in controlling *P. cinnamomi* [4, 6, 7]. However, little is known about the mode of action of Phi on induction of resistance to this pathogen. Understanding the molecular mechanisms underlying plant—*Phytophthora cinnamomi* interactions and the effect of Phi on these interactions may allow the design strategies to improve disease resistance or the more effective use of Phi.

Resistance to potential pathogens depends on interaction between different plant defence signalling pathways such as those regulated by the phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and auxin [8]. Synergistic and antagonistic interactions between different signalling pathways induced
by phytohormones and their effect on induction of resistance to biotrophic or necrotrophic pathogens have been well documented [8-13].

Phi is believed to mimic Pi and interferes with the manifestation of a wide range of biochemical and developmental Pi starvation responses (PSR) in Arabidopsis thaliana and other plant species [14-17]. Pi status is very important for determining root architecture mediated through the auxin signalling pathway and auxin signalling is required for the full Pi starvation response [18-22].

Auxin is mediated largely by the SCF$^{\text{TIR1}}$ E3 ubiquitin ligase complex (UPP complex) that accelerates AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressor protein degradation in response to auxin [23, 24]. The AUX/IAA repressor proteins are recognized and ubiquitinated by a ubiquitin-conjugation cycle involving an E1 (AXR1 and ECR1), an E2 (RCE1), and the SCF$^{\text{TIR1}}$ E3, which consists of a Cullin-CUL1, the SKP1-ASK1, RBX1, and the F-Box protein TIR1 (TRANSPORT INHIBITOR RESPONSE1) [25]. Pi modulates auxin sensitivity via the auxin receptor TIR1 and Pi starvation increases the expression of the $TIR1$ gene in Arabidopsis seedlings leading to degradation of AUX/IAA repressors and activation of downstream auxin responses [23, 24]. SGT1B protein functions in SCF-TIR1 mediated degradation of AUX/IAA proteins [26-28] and interacts with $RAR1$; a component of R-gene-mediated resistance [28-30]. Both $RAR1$ and SGT1B interact with COP9 which is involved in protein degradation by the 26S proteasome [29, 31]. Furthermore, auxin has been shown to play an important role in plant–pathogen interactions and induction of resistance against necrotrophic pathogens is supported in Plectosphaerella cucumerina and Botrytis cinerea [8, 32].

In conclusion, the involvement of the auxin signalling pathway in plant defence and Pi signalling, together with interference of Phi in Pi homeostasis and PSR indicates a possible involvement of the auxin signalling pathway in resistance to P. cinnamomi and suggests that Phi mediated resistance may be, at least in part, through its effect on
Pi signalling and in particular, on the auxin signalling pathway. The objectives of this study were to examine the potential involvement of Pi and auxin signalling pathways in resistance to *P. cinnamomi* and to investigate whether Phi induces resistance to *P. cinnamomi* by manipulating the PSR and auxin signalling pathways.

**Results**

**Resistance to *P. cinnamomi* was reduced in the Pi starvation response mutant**

To investigate whether Pi signalling affects the response of *Arabidopsis* to *P. cinnamomi*, ecotype Col-0 and several PSR mutants *phrl-1, pho2-1*, and *phol-2* were screened for their susceptibility to the pathogen and the level of infection was determined quantitatively according to Eshraghi et al. [33]. The QPCR analysis of infection showed significantly greater *P. cinnamomi* biomass in the *phrl-1* mutant compared to that in its wild background Col-0 (Figure 1) suggesting a role of Pi signalling in resistance to *P. cinnamomi*. Furthermore, transferring the cloned *PHR1* gene into the susceptible *phrl-1* mutant restored resistance to the level observed in the parental background Col-0 (Figure 1) confirming that the mutant was susceptible due to loss of PHR1 function. The *PHR1* gene contributes to downstream Pi signalling by regulating the expression of Pi responsive genes [34-36] and the *phrl* mutant is defective in Pi signalling [34]. PHO1 and PHO2 both act downstream of the *PHR1* transcription factor to control the local uptake or transport of Pi [37-40]. The *pho2-1* and *pho1-2* mutants did not show significant (*P*>0.05) increase in their susceptibility to *P. cinnamomi* compared to Col-0 (Figure 1).

**The SCF<sup>TIR1</sup> complex is involved in resistance to *P. cinnamomi* infection**

The high susceptibility of the *phrl-1* mutant in current study, combined with the role of the auxin signalling pathway in the PSR and plant resistance [18, 32] suggested a
possible involvement of auxin signalling in resistance to *P. cinnamomi*. QPCR assessment of pathogen biomass showed that *tir1-1*; an auxin response mutant deficient in the auxin-stimulated SCF (Skp1–Cullin–F-Box) ubiquitination pathway [41-44] was highly susceptible to *P. cinnamomi* (Figure 1). Furthermore, transferring the cloned *TIR1* gene into the *tir1-1* mutant restored resistance to the level observed in the parental background Col-0 (Figure 1) and confirmed that susceptibility was due to loss of TIR1 function in the mutant.

Since *Arabidopsis SGT1B* contributes to the auxin response controlled by the SCF$^{TIR1}$ complex [27, 41] and functions in plant disease resistance signalling [26], we investigated whether mutations in SGT1B also affect resistance to *P. cinnamomi*. QPCR analysis showed no significant differences in susceptibility of *sgt1b-1* in comparison to its wild parental background Col-0 (Figure 1) suggesting that SGT1B does not contribute to SCF-related processes in resistance to *P. cinnamomi*.

**The 26S proteasome subunits are involved in resistance to *P. cinnamomi***

The 26S proteasome is involved in the degradation of AUX/IAA proteins and consequently activation of auxin responsive genes [45]. In the present study, several *Arabidopsis* mutants defective in 26S proteasome subunits (*pbe1, rpt2a, rpt2b, rpt5a* and *rpn10*) were screened for their susceptibility to *P. cinnamomi*. The analysis of infection revealed that the *Arabidopsis* mutant *pbe1*, a knockout mutant for 20S proteasome [46] and *rpn10* with reduced auxin sensitivity [47] were significantly (*P*<0.05) more susceptible to *P. cinnamomi* compared to their parental background Col-0 (Figure 1). Furthermore, the susceptibility of the *Arabidopsis* 26S proteasome subunit mutants *rpt5a, rpt2a, and rpt2b* (homologue of *rpt2a*) was significantly (*P*<0.05) higher compared to that in their background Col-0 (Figure 1).
Inhibition of auxin transport by TIBA treatments enhanced *P. cinnamomi* infection

The susceptibility of the *Arabidopsis* auxin response mutant *tir1-1* [32] suggested the involvement of auxin response pathway in the outcome of *A. thaliana—P. cinnamomi* resistance (Figure 1). To test this further, blue lupin seedlings were treated with an auxin transport inhibitor, TIBA, and their susceptibility determined. Infection in lupin seedling roots treated with TIBA was significantly (*P*<0.05) greater than in non-treated plants 72 h after inoculation (Figure 2).

Expression of Pi-and auxin signalling-related genes in response to Phi treatments

The relative expression ratios of the Pi responsive genes *ATPT2*, *ATAP5* and *AT4* (Table S1) in Col-0 grown under Pi sufficient or Pi deficient conditions were analysed following Phi treatments (Table 2). The transcript levels of *AT4*, *ATPT2*, and *ATAP5* increased significantly in response to Pi deficiency in wild ecotype Col-0 (Table 2). In contrast, the transcript levels of the *AT4*, *ATPT2*, and *ATAP5* genes were greatly suppressed (55.6-fold, 8.9-fold, and 4.2-fold, respectively) when the Pi starved plants were treated with 2.5 mM Phi. Furthermore, the high level of Phi (20 mM) suppressed the up-regulation of *AT4* (1.1-fold), *ATPT2* (1.1-fold), and *ATAP5* (1.07-fold) in response to Pi deficiency to the levels observed in plants grown in Pi sufficient (control) conditions demonstrating an impact of Phi on the PSR (Table 2).

Given the apparent involvement of the Pi and auxin signalling pathways in the resistance of Col-0, the interaction of these pathways with ABA signalling [38] and previous observations of the susceptibility of the *aba2-4* mutant by Eshraghi *et al.* (submitted/Chapter 4), the effects of Phi treatment on PSR gene expression was investigated in Col-0, *aba2-4* and *tir1-1* plants (Figure 3). The transcript level of the *AT4* in Pi deficient, non Phi-treated Col-0, *aba2-4* and *tir1-1* significantly (*P*<0.05)
increased 100-fold, 112-fold, and 111-fold, respectively compared to Pi sufficient Col-0. However, Phi treatments resulted in suppression of the AT4 gene in all plants tested with the level of this suppression depended on the concentration of Phi applied (Figure 3).

In non Phi-treated Col-0, the transcript level of PHRI (phosphate starvation response 1) in response to Pi deficiency increased (2.7-fold) and Phi treatments suppressed the transcript level of this gene with the highest suppression in 20 mM Phi-treated plants (Figure 3). Although Phi treatment in Pi deficient grown samples suppressed the expression of PHRI, the transcript level of this gene was induced in Col-0 samples grown in Pi sufficient media and the level of this induction depended on the concentration of Phi applied in this study (Figure 3). The expression of PHRI in response to Pi starvation was diminished in aba2-4 and tir1-1 mutants compared to that in Col-0 indicating the importance of ABA2 and TIR1 genes in induction of PHRI. Furthermore, Phi treatments had no significant (P>0.05) effect on transcript level of the PHRI gene in either the aba2-4 or tir1-1 mutants suggesting that mutation in ABA2 and TIR1 genes may disrupt the Phi effect on Pi signalling.

The high susceptibility of the Arabidopsis mutant tir1-1 (Figure 1) and the enhanced level of P. cinnamomi infection in roots of TIBA-treated lupins (Figure 2) showed that the auxin response pathway plays an important role in resistance to P. cinnamomi. In addition, the induction of PHRI gene by Phi in Col-0 samples grown in Pi sufficient media suggested that Phi induces PSR and loss of PHRI gene expression in the tir1-1 mutant highlighted the possible induction of the auxin response pathway by Phi treatments. Therefore, we hypothesised that Phi mediated resistance to P. cinnamomi may be through induction of the auxin response pathway.

To test this hypothesis, the concomitant effect of Phi and Pi on auxin signalling in Pi sufficient/Pi deficient grown Col-0, aba2-4 and tir1-1 mutants following 0, 0.5, 2.5

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and 20 mM Phi treatments was assessed by measuring the relative expression ratios of auxin responsive genes, \( AUX1, AXR1, AXR2 \) and \( SGT1B \) transcripts (Table S1). In non-Phi-treated Col-0 seedlings, Pi deficiency increased the transcript levels of \( AUX1 \) (2.3-fold), \( AXR1 \) (2.9-fold), \( AXR2 \) (2.8-fold) and \( SGT1B \) (2.3-fold) genes indicating activation of the auxin response pathway in Pi deficient plants (Figure 4). In Pi sufficient Col-0, Phi treatments (2.5 and 20 mM) induced significantly \( (P<0.05) \) the transcript levels of \( AUX1, AXR1, AXR2 \) and \( SGT1B \) genes and the level of this induction depended on the Phi concentrations used (Figure 4). Addition of Phi to Pi starved plants at 0.5 mM suppressed the enhanced transcript level of \( AUX1 \) (from 2.3-fold to 1.1-fold); while, Phi treatments at 0.5 mM had no significant \( (P>0.05) \) effect on the expression of \( AXR1, AXR2 \) and \( SGT1B \) in Pi deficient Col-0 (Fig 4). However, application of Phi at higher concentrations (2.5 and 20 mM) to Pi starved plants had no significant \( (P>0.05) \) effect on the expression of \( AUX1, AXR1, AXR2 \) and \( SGT1B \) genes (Figure 4).

In Pi deficient, non-Phi-treated \( aba2-4 \) mutant, the transcript levels of \( AUX1, AXR1, AXR2 \) and \( SGT1B \) genes did not increase significantly \( (P>0.05) \) compared to that in the Pi sufficient, non-Phi-treated \( aba2-4 \) mutant (Figure 4) suggesting that PSR-responsive expression of the auxin responsive genes is reliant on ABA signalling. Furthermore, application of Phi to the \( aba2-4 \) mutant grown under either Pi sufficient or Pi deficient conditions did not increase transcript level of auxin responsive genes \( (AUX1, AXR1, AXR2 \) and \( SGT1B \), with the exception of 0.5 mM Phi-treated plants for the \( AXR1 \) gene (Figure 4). These results suggest that the Phi-mediated activation of auxin responsive genes may involve ABA signalling. In the \( tir1-1 \) mutant, Pi starvation did not considerably change the transcription levels of the auxin responsive genes \( AUX1, AXR1, AXR2 \) and \( SGT1B \) confirming a role for TIR1 in induction of the PSR. Furthermore, application of Phi in Pi sufficient or deficient conditions to \( tir1-1 \) did not
affect the transcript levels of the auxin responsive genes tested with the exception of
AXR1 in 0.5 mM Phi-treated, Pi deficient plants (Figure 4). The results indicate that
ABA2 is to some extent required for both PSR induced auxin responsive genes and Phi
induced auxin responsive gene expression. Likewise, TIR1 is required for both PSR and
Phi mediated auxin responsive gene expression, suggesting that Phi may act through
mechanisms involving both ABA and auxin.

**Effects of Phi treatment on auxin-mediated root architecture**

Given the potential of Phi to mimic the PSR in terms of auxin responsive gene
expression, the potential for Phi to interfere with morphological responses of plant roots
to Pi starvation was investigated.

The primary root length of Pi starved Col-0 seedlings was significantly ($P<0.05$)
shorter than that in seedlings grown in Pi sufficient media (Figure 5A). Application of
Phi resulted in suppression of primary root growth in seedlings grown in Pi sufficient
media and the level of this suppression depended on the Phi concentrations used (Figure
5A). Pi starvation induced root hair formation in non-Phi treated seedlings compared to
that observed in Pi sufficient grown Col-0 seedlings (Figure 5B, C). Furthermore,
treatment of seedlings with a low concentration of Phi (2.5 mM) resulted in suppression
of root hair formation in Pi sufficient grown seedlings, while in 20 mM Phi-treated
seedlings root hair density was increased (Figure 5B, C). Phi at both 2.5 mM and 20
mM concentrations inhibited root hair formation induced by Pi starvation.

**Discussion**

The findings of this study supported the role of auxin signalling in the induction
of resistance to the necrotrophic pathogen *P. cinnamomi*. We further illustrate the effect
of Phi on Pi signalling and the importance of their concomitant effect on
activation/suppression of the auxin response pathway in relation to PSR.
Phosphate starvation response mutant showed susceptibility to *P. cinnamomi*

The involvement of Phi in resistance to *P. cinnamomi* [48] and its interference in the phosphate starvation responses [14, 49, 50] suggested a possible role of Pi signalling in the outcome of *A. thaliana—P. cinnamomi* interactions. QPCR analysis of infection revealed that *phr1*-1, a mutant defective in response to Pi starvation was highly susceptible to *P. cinnamomi*; while, the mutants *pho2*-1 and *pho1*-2 remained resistant. The MYB-like transcription factor encoded by the *PHOSPHATE STARVATION RESPONSE 1 (PHR1)* is vital for adaptation to phosphate deficiency in *Arabidopsis* [51] and this gene contributes to downstream Pi signalling by regulating the expression of Pi-responsive genes [34-36]. The PHO2 in *Arabidopsis* is a sub-component of the Pi-signalling network that functions downstream of *PHR1* and regulates a subset of Pi-dependent responses, including Pi allocation between the shoot and the root [39, 40]. Thus, mutation in the *PHR1* gene impairs many Pi signalling-related functions [34], while *pho1* and *pho2* mutations individually attenuate Pi uptake and distribution within tissues [52, 53]. Up-regulation of *PHO1* is shown to be dependent on the *PHR1* transcription factor [37, 38]. Furthermore, the cross talk between Pi, ABA and auxin signal transduction pathways have been suggested by Ribot et al. [38] demonstrating that application of exogenous ABA and auxin down-regulates the expression of *PHO1* independent of the plants’ Pi status [38]. Therefore, mutation of *PHR1* is likely to affect other Pi responses in addition to those dependent on PHO1 and PHO2 and the regulation of these responses, and resistance to *P. cinnamomi*, may be associated with ABA and/or auxin signalling.

**Auxin response pathway is involved in *P. cinnamomi* resistance**

The phytohormone auxin, in addition to being involved in many aspects of development and growth in healthy plants [54-57], plays an important role in plant–pathogen interactions [8, 58]. Together the role of the auxin (IAA) signalling pathway
in the PSR [18], plant disease resistance [32] and the high susceptibility of \textit{phrl-1} observed in the present study suggested a possible involvement of auxin signalling in resistance to \textit{P. cinnamomi}. The QPCR analysis of \textit{P. cinnamomi} infection in inoculated leaves of Col-0 and auxin-related mutants showed that \textit{tir1-1}, an auxin receptor mutant was highly susceptible to \textit{P. cinnamomi}. An effective auxin response in \textit{Arabidopsis} depends on the removal of AUX/IAA family of transcriptional factor (TF) repressors through auxin-stimulated binding by the SCF\textsuperscript{TIR1} complex [42, 44] and the TIR1 protein acts as an auxin receptor which directly links auxin perception to degradation of the AUX/IAA repressor proteins. In \textit{Arabidopsis} auxin response mutants, the defective degradation of AUX/IAA transcriptional repressor proteins affect the induction of Auxin Response Factors (ARFs) and consequently the expression of auxin responsive genes [59]. Therefore, the susceptibility of the \textit{tir1-1} mutant which is defective in the F-box TIR1 protein and AUX/IAA degradation [41-43] suggested that ubiquitin-mediated AUX/IAA protein degradation is important in plant resistance to the necrotrophiic pathogen \textit{P. cinnamomi}.

TIBA (a polar auxin transport inhibitor) treatment also led to the enhanced susceptibility of lupin seedlings to \textit{P. cinnamomi} suggesting that the suppression of auxin transporters and consequently disruption of auxinsignalling is important in plant resistance to \textit{P. cinnamomi}. Llorente et al. [32] also suggested the involvement of the auxin signalling pathway in resistance to necrotrophic pathogens by demonstrating that the suppression of the auxin response pathway enhanced the susceptibility of \textit{Arabidopsis} to \textit{Botrytis cinerea} and \textit{Plectosphaerella cucumerina}.

In \textit{Arabidopsis}, SGT1B contributes to the auxin response controlled by the SCF\textsuperscript{TIR1} complex [27, 41], through SCF-TIR1 mediated degradation of AUX/IAA repressor proteins [27, 28, 60]. SGT1B also functions in \textit{R} gene mediated plant disease resistance signalling and in this regard interacts with RAR1 [30, 60]. When challenged
with *P. cinnamomi* the *sgt1b-1* mutant showed no significant difference to its parental background Col-0, suggesting that SGT1B contributes a redundant role to resistance to *P. cinnamomi*. Together the susceptibility of *tir1-1*, the enhanced susceptibility of TIBA-treated plants and the resistance of *sgt1b-1* indicated that auxin plays a substantial role in resistance to *P. cinnamomi* through a SCF<sup>TIR1</sup>-mediated ubiquitination mechanism that is independent to SGT1B function.

**Involvement of 26S proteasome in *A. thaliana*–*P. cinnamomi* interaction**

The 26S proteasome is involved in the ubiquitination of AUX/IAA proteins and consequently activation of auxin responsive genes [45], and mutants that are compromised in 26S proteasome activity attenuate auxin sensitivity and other plant processes such as root apical meristems maintenance, leaf organ size and gametophyte developments [25, 61-64]. In the present study, several *Arabidopsis* mutants defective in 26S proteasome subunits (*pbe1, rpt2a, rpt2b, rpt5a* and *rpn10*) were screened for their susceptibility to *P. cinnamomi*. The analysis of pathogen infection revealed that *pbe1*, a 20S proteasome knockout mutant was highly susceptible to *P. cinnamomi* in comparison to its parental background Col-0. In addition, the *rpn10* mutant, defective in ubiquitin/26S proteasome-mediated proteolysis in auxin and ABA signalling, was susceptible to *P. cinnamomi*. RPN10 is a subunit of the 26S proteasome pathway which affects several regulatory processes in *Arabidopsis* by directing the unwanted proteins to the 26S proteasome for degradation [47]. The *Arabidopsis rpn10* mutant shows a decreased sensitivity to auxin and is highly sensitive to exogenous application of ABA [47]. The reduction in auxin sensitivity in *rpn10* may relate to its susceptibility to *P. cinnamomi* and further supports a role for TIR1/26S proteasome in resistance to *P. cinnamomi*.

Furthermore, *Arabidopsis* 26S proteasome subunit mutants *rpt5a, rpt2a* and *rpt2b* (homologue of *rpt2a*) also showed a higher level of susceptibility compared to that
observed in their parental background Col-0. The susceptibility of the mutants defective in 26S proteasome subunits to *P. cinnamomi* suggested the role of 26S proteasome subunits in resistance to *P. cinnamomi*, possibly through degradation of auxin inhibitor proteins following their ubiquitination by TIR1.

**Concomitant effect of Phi and Pi is important in the activation/suppression of PSR- and auxin-related genes**

Eshraghi et al. [48] found that Phi mediated resistance to *P. cinnamomi* in the susceptible *Arabidopsis* ecotype Ler resembled the response of the resistant ecotype Col-0 in terms of timing and the defence responses induced. Phi has also been shown to interfere with a broad range of biochemical and developmental responses including PSR in plants [14-17] many of which have been shown to rely on auxin signalling involving the SCF^{TIR1} UPP complex [18-20]. The susceptibility of *Arabidopsis* auxin response pathway mutants and the Pi response mutant *phr-1* to *P. cinnamomi* in this study together with the interference of Phi in Pi homeostasis and its role in the induction of plant defence responses against *P. cinnamomi* [48] suggested that Phi mediated resistance could be through its’ effect on Pi signalling, and in particular, on the auxin signalling pathway.

The transcript levels of the PSR responsive genes *AtPT2*, *AtACP5*, and *AT4* increased in response to Pi deficiency. However, Phi applications at all levels suppressed their enhanced expression similar to that observed in plants grown in Pi sufficient media, demonstrating the effect of Phi in suppression of PSR. These results are supported by Ticconi *et al.* [49] who reported a similar effect of Phi on suppression of PSR genes.

Perez-Torres *et al.* [24] demonstrated that auxin sensitivity was enhanced in Pi deficient *Arabidopsis* plants by an increased expression of *TIR1*, which accelerated the
degradation of AUX/IAA proteins. Eshraghi et al. (submitted/Chapter 4) suggested that Phi acts partially through an ABA2 dependent mechanism. Therefore, to investigate whether Phi acts through TIR1 or ABA2 and whether mutations in these two genes are affecting the impact of Phi on PSR, the effect of Phi on expression of AT4 and PHRI at the transcriptional level was further tested in Pi sufficient and Pi deficient grown Col-0, aba2-4 and tir1-1. Although in Col-0 Phi treatments suppressed the enhanced transcript levels of PHRI induced by Pi deficiency, Phi enhanced the transcript levels of PHRI in Pi sufficient grown samples and the level of this induction depended on the Phi concentrations used. These results suggested that although Phi suppressed the PSR in Pi starved plants, application of Phi to Pi sufficient plants resulted in activation of PSR. One explanation for this may be competition between Pi and Phi for uptake or transport. It has been shown in Brassica spp. that high Phi concentrations inhibit plant development by competing with Pi absorption [65, 66]. Our results showed that the expression of PHRI in response to Pi starvation was affected in the aba2-4 and tir1-1 mutants suggesting the importance of ABA2 and TIR1 genes in the induction of PHRI and PSR. Furthermore, Phi treatments had no considerable effect on transcript level of the PHRI gene in either aba2-4 or tir1-1 mutants suggesting that mutation in the ABA2 and TIR1 genes may disrupt the Phi effect on Pi signalling.

The induction of the PHRI gene by Phi in Col-0 samples grown in Pi sufficient media suggested that Phi induces PSR and the loss of PHRI gene expression in the tir1-1 mutant highlighted the possible induction of the auxin response pathway by Phi treatments. To test this further, the concomitant effect of Phi and Pi on auxin signalling in Pi sufficient/Pi deficient grown Col-0, aba2-4 and tir1-1 mutants was assessed by measuring the relative expression ratios of the auxin responsive genes, AUX1, AXR1, AXR2 and SGT1B transcripts. Pi deficiency increased the transcript levels of all genes tested suggesting the induction of the auxin response pathway in Pi starved plants. In Pi
sufficient Col-0, Phi treatments (2.5 and 20 mM) induced the transcript levels of \textit{AUX1}, \textit{AXR1}, \textit{AXR2} and \textit{SGT1B} and the level of this induction depended on the Phi concentrations used. Moreover, Pi starvation did not considerably change the transcription levels of the auxin responsive gene \textit{AUX1}, \textit{AXR1}, \textit{AXR2} and \textit{SGT1B} in the \textit{aba2-4} and \textit{tir1-1} mutants confirming a role for \textit{ABA2} and \textit{TIR1} in the induction of the PSR. Overall, the results suggested that \textit{ABA2} and \textit{TIR1} genes are required for both PSR and Phi mediated auxin responsive gene expression, indicating the Phi may act through both the ABA and auxin pathways.

**Effect of Phi on root morphology**

Considering the potential of Phi to mimic the PSR in terms of auxin responsive gene expression, the effect on morphological responses to Pi starvation was investigated, as Pi status acting through auxin signalling is important for determining root architecture [18-20]. Pi deficiency suppressed the primary root length and induced root hair formation in roots of ecotype Col-0 and the application of Phi resulted in suppression of primary root growth in seedlings grown in either Pi sufficient or Pi deficient media in a dose-response manner. The morphological responses of Pi starved roots were consistent with those previously described [19, 20, 67-69]. Phi at lower concentration (≤ 2.5 mM) inhibited root hair formation induced under phosphate starvation; however, 20 mM Phi induced root hair formation in Pi sufficient plants. Gilbert et al. [70] also showed that Phi dramatically increased the number of proteoid root segments (a phosphate starvation response) in Pi sufficient lupin seedlings. Overall, both, morphological and gene expression data suggested the involvement of the auxin signaling pathway and phosphate signalling in responses to Phi treatment.
Conclusions

This study highlighted the importance of Pi signalling in plant resistance to *P. cinnamomi* by illustrating the susceptibility of *phr1-1* (a mutant defective in Pi signalling) and linked this role with the auxin response pathway through the susceptibility of *tir1-1* and TIBA-treated plants to *P. cinnamomi*. A role for the 26S proteasome, which is required for auxin signalling [25, 45], was further supported by the susceptibility of lines with mutations in various components. Moreover, the link between Phi treatment and PSR, as demonstrated by morphological PSR responses and analysis of Pi starvation gene expression following Phi treatment under Pi sufficient and deficient conditions and in auxin and ABA response mutants, suggested that the mechanism of action of Phi may include modulation of Pi signalling involving auxin. The activity of Phi also appears to rely on ABA synthesis relating to ABA2. Further studies, including Phi treatment of susceptible *Arabidopsis* PSR, auxin and ABA mutants are required to assess whether the responses observed in this study are directly related to the enhanced resistance to *P. cinnamomi* following Phi treatment.

Methods

**Plant and pathogen materials**

*Arabidopsis thaliana* accession Columbia (Col-0) and several *A. thaliana* mutant/transgenic lines (Table 1) in the Col-0 background that are defective in different signalling pathways were used in this study. *Arabidopsis thaliana* genotype Col-0 was purchased from LEHLE Seeds (Round Rock, TX), and the mutants *rpt2a, rpt5a, rpn10,* and *cni1* were provided by Dr Derek Gotto and Prof. Junji Yamaguchi (Hokkaido University, Japan). The remaining mutants were obtained from the *Arabidopsis* Biological Resource Centre (ABRC, Ohio State University); [https://abrc.osu.edu/](https://abrc.osu.edu/). Blue
lupin (*L. angustifolius* L., cv. Mandalup) seeds were obtained from Department of Agriculture and Food, Western Australia (DAFWA).

*Phytophthora cinnamomi* (isolate MP 94.48) was obtained from the Centre for Phytophthora Science and Management (CPSM) at Murdoch University. *Phytophthora cinnamomi* zoospores were produced aseptically according to the method described by Byrt & Grant [71], and the zoospores density was determined using a bright line haemocytometer and adjusted to a concentration of $1 \times 10^5$ zoospores mL$^{-1}$ using sterile distilled water.

**Plant growth conditions and inoculation procedure**

*Arabidopsis thaliana* ecotype Col-0 and mutants were germinated on half strength Gamborg's B-5 Basal medium with 0.8% (w/v) phytagar [72]. In addition, 2.5 mM MES [2-(N-morpholino)-ethanesulphonic acid]-KOH (pH 5.7) and 0.5% (w/v) sucrose were included (pH 5.7, adjusted with KOH). After sowing the seed on the medium, seeds were stratified for 3 days at 4°C in the dark before being transferred to a growth cabinet at 21°C ± 1°C with a 10-h photoperiod at a photon fluorescence rate of 100 µmol m$^{-2}$s$^{-1}$. To study *A. thaliana*—*P. cinnamomi* interactions, attached leaves of four-week-old seedlings were inoculated either with 3 µL of $1 \times 10^5$ *P. cinnamomi* zoospores mL$^{-1}$ or 3 µL of sterile distilled water (control) on the abaxial surface. Five samples per genotype were collected 72 h after inoculation for quantitative PCR (QPCR) analysis of infection and the experiment was conducted twice.

The lupin (*Lupinus angustifolius* L., cv. Mandalup) seeds were surface-sterilized in 70% ethanol for 2 min followed by immersion in 50% bleach solution (6.25% available chlorine) for 5 min. The sterilized seeds were germinated on sterile filter paper pre-moistened with distilled water at 25°C in the dark for 3 days. The seedlings were placed on a bed of damp absorbent paper (24 x 38.5 cm), placed between two layers of
clear plastic, rolled up and placed in 200 mL beakers filled with 50 mL half-strength hydroponic Hoagland medium [73] and grown for a further five days in a growth cabinet with a 10-h photoperiod (100 µmol m⁻² s⁻¹ at 21 ± 1°C) until treatments. Lupin seedlings were inoculated by placing a 4 mm diameter plug of *P. cinnamomi* mycelium at the tips of roots. Root tissue samples were collected for lesion size assessments at 48 h and 72 h after inoculation.

**Quantitative PCR analysis of infection**

To determine the level of infection quantitatively, QPCR analysis was conducted and the relative amount of *P. cinnamomi* biomass (DNA) in infected *Arabidopsis* leaf samples was measured and normalized based on plasmid DNA (internal control) according to [33]. Samples were collected and snap frozen 72 h after inoculation. Five samples per treatment each containing four leaf discs (7 mm in diameter) from one seedling were collected.

**TIBA treatments and lesion size assessments in lupin**

The auxin transport inhibitor TIBA (2, 3, 5-triiodobenzoic acid; Sigma Aldrich) was dissolved in absolute ethanol and filter sterilised TIBA was added to sterilized half-strength liquid Hoagland medium to give a final concentration of 10 µM. TIBA treatment was conducted on five-day-old lupin seedlings by transferring the seedlings to half-strength hydroponic Hoagland medium containing 10 µM TIBA (+TIBA). For controls (-TIBA), the seedlings were transferred to the half-strength hydroponic Hoagland medium with the same amount of ethanol as in +TIBA medium. 48 h after treatments, the roots were inoculated by placing a 4 mm diameter plug of *P. cinnamomi* mycelium at the root tips and seedlings were kept in half-strength hydroponic Hoagland medium until harvested.
A minimum of 10 lupin roots per treatment was assessed for lesion development and the experiment was repeated twice. The level of infection was measured 48 and 72 h after *P. cinnamomi* inoculation and the data were presented as mean percentage infected root area. The digital images of the roots were captured with an Epson Expression 1680 scanner and the area of lesions formed by *P. cinnamomi* infection were calculated using the program WinRHIZO™ (Régents Instruments, Inc.).

**Cloning PHR1 and TIR1**

Col-0 genomic DNA was used as template for cloning *PHR1* (AT4G28610) and *TIR1* (AT3G62980). PCR was performed with Phusion® High-Fidelity DNA Polymerase (BioLabs) according to manufacturer’s instructions using primers containing the attB recombination sites (**PHR1**–forward 5’–GGG GAC AAG TTT GTA CAA AAA AGC AGG CT T CTC TTC TCT GGT CCT GGA TTG and **PHR1**–reverse 5’–GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCT TCC TTG GGG ATC TGT TG, **TIR1**–forward 5’–GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CCG CTG TCC AAC TTC TTC CTC and **TIR1**–reverse 5’–GG G GAC CAC TTT GTA CAA GAA AGC TGG GTC GTT CCT AAA CCG GAA CAC GA. The PCR products were cloned to pDONR207 (Invitrogen) using Gateway® BP Clonase® II Enzyme Mix (Invitrogen) according to the manufacturer’s instructions and transformed into *E. coli* competent cells (DH5α). After confirmation by sequencing, the insert was cloned into the Gateway® compatible expression vector pGREEN0179 containing CAMV 2x35S promoter and CAMV terminator with a Gateway A cassette [74] using Gateway® LR Clonase® II enzyme mix (Invitrogen) according to manufacturer’s instructions. Positive clones were confirmed by PCR and *phr1-1*, and *tir1-1* mutants were transformed using *Agrobacterium tumefaciens* C58C1 (pCH32) [75].
Quantitative measurements of gene transcription using qRT-PCR

For quantitative measurement of gene transcription, seeds of ecotype Col-0 and the *A. thaliana* mutants *aba2-4* and *tir1-1* were germinated on half strength 0.8% (W/V) phytagar-Gamborg B-5 basal medium (pH 5.7) as previously described. The seedlings were grown in a growth cabinet at 21 ± 1°C with a 10-h photoperiod at a photon fluorescence rate of 100 µmol m⁻² s⁻¹ for three weeks and then transferred to half strength 0.8% (W/V) phytagar—Hoagland medium [73] (pH 5.7, adjusted with KOH) with different concentrations of Pi (0 and 1.25 mM) and Phi (0, 0.5, 2.5 and 20 mM) in a completely randomized design and grown for a further five days. Samples were collected, frozen in liquid nitrogen and stored at -80°C until RNA extraction and qRT-PCR.

Four biological samples per treatment were randomly collected for gene expression analysis and each sample was a pool of three plants. Tissue samples were homogenized using Tissuelyser® (Qiagen, Inc.) and the RNeasy plant mini kit (Qiagen, Inc.) was used to isolate RNA according to the manufacturer’s instructions. Approximately, 3 µg of DNA-free RNA was used for first-strand cDNA synthesis using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Gene specific primers (Table S1) were designed using Primer Express 1.5 software (Applied Biosystems Inc.) and qRT-PCR conducted according to [48]. The transcript levels of all genes in the mutants were normalized based on expression of actin 2 (*ACT2*) measured in the same samples and presented relative to the normalized expression levels in corresponding Pi sufficient, non Phi-treated plants as fold expression.

Root morphology assessments

For morphological assessments of *A. thaliana* roots, seeds of ecotype Col-0 were grown on half strength Gamborg B-5 basal medium with 0.8% (W/V) phytagar (pH
5.7) as previously described. One-week-old seedlings were transferred to half strength Hoagland medium with 0.8% (W/V) phytagar [73] (pH 5.7) with different concentrations of Pi (0 and 1.25 mM) and Phi (0, 2.5 and 20 mM) in a completely randomized design and grown for further seven days. A minimum of 10 seedlings per treatment was assessed for their primary root growth using the photographs were taken by a digital camera (Nikon; Cool PIX 995) and the experiment was repeated twice. A minimum of six seedlings per treatment was assessed for their root hair density using the photographs taken by a binocular microscope (Olympus SZ40) with an attached digital camera (Nikon; Cool PIX 995) and the experiment was repeated twice. Root hair density was determined as the number of hairs in a 5 mm root segment (from the root tip).

Data analysis

ANOVA was used in all measurements to compare the treatments and the normality of residuals was tested using GenStat software (14th edition). 5% least significant difference (LSD) was calculated for the mean comparisons of treatments and genotypes using GenStat software (14th edition).

Abbreviations

PSR, Phosphate starvation response; ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; IAA, indole-3-acetic acid; TIBA, 2, 3, 5-triiodobenzoic acid; UPP, ubiquitin proteasome pathway; qRT-PCR, quantitative reverse transcription polymerase chain reaction; QPCR, quantitative real-time polymerase chain reaction.

Acknowledgements

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Research Scholarship (MURS). We also thank the ABRC (Columbus, OH) for providing the majority of the mutant lines tested in this study.

References


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**Additional material**

**Table S1** Sequences of the gene-specific primer pairs used in quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) experiments
<table>
<thead>
<tr>
<th>Name</th>
<th>locus</th>
<th>Genetic alteration</th>
<th>Phenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>phr1-1</td>
<td>AT4G28610</td>
<td>T-DNA-insertion</td>
<td>Defective in response to phosphate starvation</td>
<td>[76, 77]</td>
</tr>
<tr>
<td>pho1-2</td>
<td>AT3G23430</td>
<td>EMS mutant</td>
<td>Decreased Pi level in shoot, but normal Pi level in root</td>
<td>[78]</td>
</tr>
<tr>
<td>pho2-1</td>
<td>AT2G33770</td>
<td>EMS mutant</td>
<td>Pi over-accumulator and exhibits increased levels of Pi in the shoots</td>
<td>[79]</td>
</tr>
<tr>
<td>tir1-1</td>
<td>AT3G62980</td>
<td>EMS mutant</td>
<td>Defective in auxin response</td>
<td>[80]</td>
</tr>
<tr>
<td>sgt1b-1</td>
<td>AT4G11260</td>
<td>EMS mutant</td>
<td>Defective in SCF&lt;sup&gt;TRI&lt;/sup&gt; mediated auxin response</td>
<td>[27]</td>
</tr>
<tr>
<td>pbe1</td>
<td>At1G13060</td>
<td>T-DNA-insertion</td>
<td>A knockout mutant for 20S proteasome</td>
<td>[46]</td>
</tr>
<tr>
<td>rpt2a</td>
<td>At4g29040</td>
<td>T-DNA-insertion</td>
<td>Defective in 26S proteasome subunit</td>
<td>[61, 64, 81]</td>
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<tr>
<td>rpt2b</td>
<td>At2g20140</td>
<td>T-DNA-insertion</td>
<td>Defective in 26S Proteasome Subunit</td>
<td>[61, 81]</td>
</tr>
<tr>
<td>rpt5a</td>
<td>At3g05530</td>
<td>T-DNA-insertion</td>
<td>Defective in 26S Proteasome Subunits</td>
<td>[64]</td>
</tr>
<tr>
<td>rpn10</td>
<td>At4G38630</td>
<td>T-DNA-insertion</td>
<td>Defective in ubiquitin/26S proteasome-mediated proteolysis (UPP)</td>
<td>[47, 82]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>substrate recognition and in abscisic acid signalling</td>
<td></td>
</tr>
</tbody>
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All lines mentioned are in the Col-0 background. Pi = phosphate
Table S1 Sequences of the gene-specific primer pairs used in quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>AGI number</th>
<th>Forward and reverse primers (5´–3´)</th>
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<tr>
<td>AT4</td>
<td>AT5G03545</td>
<td>GTGTGTGAATGGGAGCGATGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATCGAAGTTGCCCCCCAAACGA</td>
</tr>
<tr>
<td>ATACP5</td>
<td>AT3G17790</td>
<td>GGTGACGCAGAAGCTCAGCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCAACTCTGATCAACGACAA</td>
</tr>
<tr>
<td>ATPT2</td>
<td>AT2G38940</td>
<td>GTTTTGCTTGGATTGGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAAAGCTCCGGAGTCTTCT</td>
</tr>
<tr>
<td>PHR1</td>
<td>AT4G28610</td>
<td>CAAATTCCGCAACCTCAGAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGTTCCGTTATTGCTTIT</td>
</tr>
<tr>
<td>AUX1</td>
<td>AT2G38120</td>
<td>CGCTGTATATCCATGCTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCATCCCAATCCTCTTCC</td>
</tr>
<tr>
<td>AXR1</td>
<td>AT1G05180</td>
<td>ATCGTCAGTCAGGATTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAAAGCTCCGAAACCAGTAG</td>
</tr>
<tr>
<td>AXR2</td>
<td>AT3G23050</td>
<td>TCTCCGAAACCCTGGATCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTTCCTCCTGGGAACAGCAG</td>
</tr>
<tr>
<td>SGT1B</td>
<td>AT4G11260</td>
<td>TGCTCCTCCTGTTCCAATTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATTGTACCAACCCTCTTTC</td>
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<tr>
<td>ACT2</td>
<td>AT3G18780</td>
<td>CTTGACCAAGCAGCATGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGATCCAGACACTGTACTTCTT</td>
</tr>
</tbody>
</table>

AT4: AtIPS2 (induced by Pi starvation 2); ATACP5: Arabidopsis thaliana acid phosphatase 5; ATPT2: Arabidopsis thaliana phosphate transporter 2; PHR1: phosphate starvation response 1; ABA3: abscisic acid (ABA) deficient 3; AUX1: auxin resistant 1; AXR1: auxin resistant 1; AXR2: auxin resistant 2; SGT1B: enhancer of tir1-1 auxin resistance 3; and ACT2: actin 2.
Table 2 Expression analysis of phosphate starvation response (PSR) genes in *Arabidopsis thaliana* ecotype Columbia (Col-0) grown under phosphate (Pi) sufficient (+Pi; 1.25 mM) and Pi deficient (-Pi; 0 mM) conditions and subjected to different phosphite (Phi) treatments. The transcript level of genes was determined using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and results are expressed as both normalized relative transcript level (mean ± SE) and the factor of increase in transcription compared with the control (+Pi/0 mM Phi). Data are means of four biological samples per treatment.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normalized Relative transcript level</th>
<th>Relative fold difference to control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Pi (0 mM Phi)</td>
<td>-Pi (0 mM Phi)</td>
</tr>
<tr>
<td></td>
<td>0 mM Phi (control)</td>
<td>2.5 mM Phi</td>
</tr>
<tr>
<td>AT4</td>
<td>0.261 ± 0.005</td>
<td>0.175 ± 0.013</td>
</tr>
<tr>
<td>ATPT2</td>
<td>0.449 ± 0.011</td>
<td>0.662 ± 0.112</td>
</tr>
<tr>
<td>ATAP5</td>
<td>0.429 ± 0.008</td>
<td>0.560 ± 0.032</td>
</tr>
</tbody>
</table>

\*See Table 1
Figure Legends

Figure 1 Phytophthora cinnamomi response in several mutants of Arabidopsis thaliana defective in auxin signalling, phosphate signalling and 26S proteasome subunits. Quantitative PCR (QPCR) assessment of Phytophthora cinnamomi biomass (pg DNA per sample) 72 h after inoculation of attached leaves of Arabidopsis thaliana ecotype Colombia (Col-0) and several Arabidopsis mutants defective in Pi signalling (pho1-1, pho2-1, and phr1-1), the auxin signalling pathway (tir1-1 and sgt1b-1), and 26S proteasome subunits (pbe1, rpt2a, rpt2b, rpt5a and rpn10). Bars represent the mean and standard error from five replicates each consisting of four infected leaves. ANOVA indicated a significant (P<0.001) difference between genotypes. LSD (5%) was 8.92. phr1-1* = phr1-1 mutant complemented with PHR1 gene (AT4G28610) and tir1-1* = tir1-1 mutant complemented with TIR1 gene (AT3G62980).

Figure 2 Negative effect of auxin transporter inhibitor (TIBA) on Phytophthora cinnamomi resistance in lupin. The effect of TIBA; an auxin transport inhibitor on lesion development in Lupinus angustifolius roots inoculated with Phytophthora cinnamomi mycelial plugs. (A) Percentage infected root area in TIBA-treated (+TIBA) and non TIBA-treated (-TIBA) lupin roots 48 h and 72 h after inoculation. (B) and (C) show disease symptoms caused by P. cinnamomi infection in non-TIBA-treated (B) and TIBA-treated (C). ANOVA indicated a significant (P<0.001) difference between treatments.

Figure 3 Effect of phosphite on phosphate starvation responsive genes in Col-0, aba2-4 and tir1-1. Relative expression ratios of AT4 and PHR1 transcripts in Arabidopsis thaliana wild ecotype (Col-0) and Arabidopsis mutants aba2-4 and tir1-1 grown in phosphate (Pi) sufficient media (1.25 mM) for three weeks followed by a further five days growth in different Pi (1.25 mM Pi; +Pi and 0 mM Pi) conditions.
Pi; -Pi) and phosphite (Phi) (0, 0.5, 2.5 and 20 mM) levels. Bars present the mean and standard error from four replicates each consisting of three plants. The numbers on each bar indicate the fold change in gene transcript levels relative to corresponding Pi sufficient (+Pi), non Phi-treated plants. ANOVA indicated a significant ($P<0.001$) difference between genotypes. LSD (5%) for $AT4$ and $PHR1$ were 23.96 and 1.01, respectively.

**Figure 4 Effect of phosphite on induction of auxin responsive genes.** Relative expression ratios of $AUX1$, $AXR1$, $AXR2$ and $SGT1B$ transcripts in Arabidopsis thaliana wild ecotype (Col-0) and mutants ($aba2-4$ and $tir1-1$) grown in a phosphate (Pi) sufficient medium (1.25 mM) for three weeks following further five days growth in different Pi (1.25 mM Pi; +Pi and 0 mM Pi; -Pi) and phosphite (Phi) (0, 0.5, 2.5 and 20 mM) levels. Data represent the mean and standard error of four replicates of three plants each. The numbers on each bar indicate the fold increase/decrease in transcript levels relative to corresponding Pi sufficient (+Pi), non Phi-treated plants. ANOVA indicated a significant ($P<0.001$) difference between treatments for all genotypes. LSD (5%) for $SGT1B$, $AXR1$, $AXR2$, and $AUX1$ were 1.06, 1.25, 1.16, and 0.82 respectively.

**Figure 5 Inhibitory effect of phosphite on primary root length and root hair density under phosphate deficiency.** The effect of phosphite (Phi) treatments on primary root length (A) and root hair density (B) of Arabidopsis thaliana ecotype Columbia (Col-0) grown under different phosphate (+Pi and -Pi) regimes. (A) Primary root length measured 7 days after transferring the seedlings to different Pi (+Pi; 1.25 mM and –Pi; 0 mM) and Phi media. Data are the means of 10 roots assessed per treatment with standard errors and bars with the same letter are not significantly different according to Tukey HSD test. (B) Root hair density (number) 7 days after transferring the seedlings to different Pi (+Pi and –Pi) and
Phi medium. Root hair density was determined as the number of hairs in a 5 mm root segment, 2.5 mm from the root tip, and each bar represents the mean of six plants with standard error bars and bars with the same letter are not significantly (P>0.05) different according to Tukey HSD test. (C) Shows the effect of different Phi concentration on root hair formation in seedlings grown in +Pi or –Pi media.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Chapter 6

General Discussion
Introduction

This study has made a significant contribution to our understanding of host and phosphite (Phi)-mediated resistance to Phytophthora cinnamomi in Arabidopsis thaliana and these include:

1. Comparison of resistant and susceptible Arabidopsis lines showed that the early production of hydrogen peroxide (H$_2$O$_2$), callose and systemic PR (pathogenesis-related) gene expression was associated with the resistant response and these occurred later and to a lesser degree in susceptible plants (Chapter 2).

2. Phi treatment of A. thaliana resulted in a more rapid and intense induction of defence responses following P. cinnamomi infection in a manner reminiscent of that induced in resistant plants (Chapter 2).

3. Phi enhanced the transcript levels of defence genes relating to the salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) signalling pathways even in non-inoculated plants indicating that phosphite-mediated resistance is not only through greater release of elicitors from the invading pathogen, but that Phi itself modulates plant defence responses by up-regulating a range of defence pathways (Chapter 2).

4. A new quantitative PCR (QPCR) assay was developed that enables rapid and accurate assessment of pathogen infection by measuring the relative and total amount of P. cinnamomi biomass (DNA) in infected plants through normalization based on plasmid DNA as an internal control (Chapter 3).

5. Phi treatment together with P. cinnamomi inoculation enhanced the transcript levels of the SA-related gene PRI and the JA/ET-related genes PDF1.2 and THI2.1; however, screening the SA-, JA- and ET-related mutants for their susceptibility to P. cinnamomi indicated that resistance to this necrotrophic
pathogen in intact plants is independent of the SA, JA or ET signalling pathways (Chapter 4).

6. ABA is involved in the outcome of *A. thaliana—P. cinnamomi* interactions since the ABA synthesis mutant *aba2-4* is susceptible. The role of ABA appears to be independent of interaction with the JA/ET pathway since mutants in this pathway remain resistant (Chapter 4).

7. In addition to ABA, the auxin response pathway and SCF$^{TIR1}$-mediated ubiquitination are important in resistance to *P. cinnamomi* and Phi-mediated defence gene expression and this is independent of SGT1B (Chapter 5).

8. The *phrl-1* mutant which is defective in response to Pi starvation was highly susceptible to *P. cinnamomi* suggesting the role of Pi signalling in resistance to this pathogen (Chapter 5).

9. In ecotype Col-0, the application of Phi on Pi sufficient plants enhanced the transcript level of *PHR1* and the auxin-related genes *AUX1, AXR1, AXR2* and *SGT1B*. However, application of Phi had no effect on the transcript level of these genes in either *aba2-4* or *tir1-1* mutants suggesting that mutation in *ABA2* and *TIR1* genes may disrupt the Phi effect on Pi signalling (Chapter 5).

10. The expression of *PHR1* and auxin-related genes *AUX1, AXR1, AXR2* and *SGT1B* in response to Pi starvation was diminished in both the *aba2-4* and *tir1-1* mutants indicating the importance of the *ABA2* and *TIR1* genes in the induction of *PHR1* and PSR (Chapter 5).

**Effect of Phi on Arabidopsis defence responses**

Phi primed the host for more rapid and intense defence responses such as $\text{H}_2\text{O}_2$ production and callose deposition following *P. cinnamomi* infection, than those in plants not treated with Phi (Chapter 2). Daniel & Guest, (2006) who studied host defence responses in Phi-treated *P. palmivora*-infected *A. thaliana* also observed a
similar stimulation of ROS (superoxide; $O_2^-$ and $H_2O_2$). However, they did not observe any $O_2^-$ release in the non-Phi-treated plants after infection, while in the present study, production of $H_2O_2$ occurred in response to *P. cinnamomi* infection, including in non-Phi-treated plants. Similarly, Daniel & Guest (2006) found that Phi did not stimulate callose production in *P. palmivora*–challenged *A. thaliana* whereas challenge with *P. cinnamomi* in this study did result in the production of callose. The differences between the two studies could be associated with differing responses to different *Phytophthora* species and that the mechanism of action of Phi may be largely dependent on amplification of the plant’s natural response.

The heightened defence responses in Phi-treated, *P. cinnamomi*-inoculated Ler closely resembled the responses observed in the resistant line Col-0 in the absence of Phi treatment suggesting that the enhanced resistance induced by Phi treatment may at least in part be caused by the augmentation of the defence responses that would otherwise be induced in the plant, but at a later stage and to a lesser degree.

**Development of a quantitative PCR assay for accurate assessment of *P. cinnamomi* infection**

*Arabidopsis* has been used to study interactions with various *Phytophthora* species (Khatib et al., 2004, Senchou et al., 2004, Rookes et al., 2008, Wang et al., 2011b); however, *Arabidopsis* shows a strong resistance to most *Phytophthora* spp. (Kamoun et al., 1999, Kamoun, 2001, Takemoto et al., 2003). Therefore, screening within the *Arabidopsis* germplasm for determination of resistance to *P. cinnamomi* or for isolate virulence based only on symptomology, lesion measurements or sporangial counts is not efficacious especially in the early stages of infection where the extent of lesion development is very limited or for identifying small differences in the degree of quantitative resistance.
A reliable method for quantification of pathogen biomass (based on DNA) is likely to be highly beneficial for the accurate determination of the level of infection. To date, several QPCR assays have been developed to detect and quantify different Phytophthora spp. in planta, however, in these QPCR assays the pathogen biomass was normalized either to host plant DNA or the sample surface or weight of the collected sample. The current study found the use of plant DNA, sample surface area or sample weight for normalisation of P. cinnamomi DNA led to the overestimation of the pathogen DNA in both resistant (A. thaliana—P. cinnamomi) and susceptible interactions (lupin—P. cinnamomi) (Chapter 3). Moreover, DNA yield was shown to vary considerably in samples for which the equivalent amount of starting tissue was used indicating the potential for inaccuracy when normalising to the sample input in this way. These problems led to development of a novel QPCR assay (Chapter 3) that enabled the accurate assessments of pathogen infection by measuring the relative and total amount of P. cinnamomi biomass (DNA) in infected plants and normalizing this based on plasmid DNA as an internal standard. This QPCR technique should be applicable to a wide range of plant–pathogen systems, as it is reliable and accurately quantifies pathogen DNA in samples with a wide range of infection and/or tissue necrosis.
Effect of Phi on defence-related genes

Phi treatments enhanced the transcript levels of defence genes relating to the SA (PR1, PR5) and JA/ET (PDF1.2 and THI2.1) signalling pathways even in non-inoculated plants suggesting that Phi not only primes the defence responses for a rapid response following pathogen challenge, but also induces defence gene expression in the absence of the pathogen (Chapter 2).

Several studies have shown that Phi enhanced the secretion of elicitors by P. capsici and P. cryptogea from the hyphal cell wall (Rouhier et al., 1993, Perez et al., 1995, Wilkinson et al., 2001) and suggested that this enhanced secretion could lead to a more rapid elicitation of plant defence responses and thus contribute to the enhanced resistance of Phi-treated plants. However, in the present study, the enhanced defence gene expression in non-inoculated plants suggested that the effect of Phi on the plant’s defence system is not only mediated through greater release of elicitors from the invading pathogen, but that Phi itself also modulates plant defence responses and up-regulates a range of defence pathways in a coordinated way.

Concomitant effect of Phi and Pi is important in activation/suppression of phosphate starvation responses (PSR)

Phi, an analogue of Pi, has been shown to interfere with a broad range of biochemical and developmental Pi starvation responses in Arabidopsis and other plants (Ticconi et al., 2004, Lee et al., 2005, Fang et al., 2009, Li et al., 2010).

The transcript levels of the Pi starvation responsive genes ATPT2, ATACP5 and AT4 increased in response to Pi deficiency and the application of Phi to Pi deficient plants suppressed their induction, indicating Phi suppresses the PSR. Ticconi et al. (2001) reported a similar effect of Phi on suppression of PSR genes. Application of Phi also suppressed the enhanced transcript levels of PHRI induced by Pi deficiency;
however, it enhanced the transcript level of the PHR1 gene in Pi sufficient plants. These results showed that although Phi suppressed gene expression relating to the PSR in Pi deficient plants, application of Phi on Pi sufficient plants resulted in activation of some phosphate starvation responsive genes. Moreover, application of Phi to Pi sufficient plants led to Pi starvation-like changes to root morphology. The impacts of high Phi (20 mM) on PHR1 expression and root morphology support the role of Phi in Pi processes, possibly due to competition between Pi and Phi for uptake or transport. High Phi concentrations have been shown to inhibit the development of Brassica spp. by competing with Pi absorption (Carswell et al., 1996, Carswell et al., 1997). Gilbert et al. (2000) showed that Phi dramatically increased the number of proteoid root segments (a phosphate starvation response) in Pi sufficient lupin. Together these results suggested that Phi act differently on Pi deficient and Pi sufficient plants in terms of suppression or activation of PSR.

Pathways involved in P. cinnamomi resistance

The resistance of several mutants (npr1-2, nahG, sid2-1, jar1-1, ein2-1 and etr1-3) in the common defence signalling pathways, SA, JA or ET suggested that the high level of resistance to P. cinnamomi in the Arabidopsis ecotype Col-0 was independent of these pathways (Chapter 4). However, a role for ABA signalling in the induction of resistance to P. cinnamomi was indicated by a high level of infection in the aba2-4 mutant. ABA has been shown to enhance plant resistance to the necrotrophic pathogens Plectosphaerella cucumerina and Alternaria brassicicola (Ton & Mauch-Mani, 2004), while it enhances the susceptibility of plants to biotrophic/hemibiotrophic pathogens (Henfling et al., 1980, Anderson et al., 2004).

The involvement of Phi in resistance to P. cinnamomi (Chapter 2) and its interference in the phosphate starvation response (Chapter 5) suggested a possible role
of Pi signalling in the outcome of *A. thaliana—P. cinnamomi* interactions and the susceptibility of the *phr1-l* mutant further supported this link. However, *pho1-2* and *pho2-1* mutants were resistant to *P. cinnamomi*. Mutation in the *PHR1* gene impairs Pi signalling (Rubio et al., 2001); while, PHO1 and PHO2 both act downstream of the *PHRI* transcription factor to control the local uptake or transport of Pi (Miura et al., 2005, Chiou, 2007, Ribot et al., 2008, Rouached et al., 2011). Therefore, mutation of PHR1 is likely to affect other Pi responses in addition to those dependent on PHO1 and PHO2 and this may relate to the susceptibility of the *phr1-l* mutant compared to the resistance observed in the *pho1-2* and *pho2-1* mutants.

High susceptibility of the *phr1-l* mutant to *P. cinnamomi* and the role of the auxin signalling pathway in both Pi signalling (Lo´pez-Bucio et al., 2002, Al-Ghazi et al., 2003, Rietz et al., 2010) and plant resistance to other pathogens (Llorente et al., 2008) suggested a possible involvement of auxin signalling in *P. cinnamomi* resistance. The susceptibility of *tir1-l* (Chapter 5), an auxin receptor mutant defective in AUX/IAA repressor degradation (Gray et al., 2001, Dharmasiri et al., 2005a, Dharmasiri et al., 2005b) indicated that ubiquitin-mediated AUX/IAA protein degradation is important in plant resistance to *P. cinnamomi* and supported a role for the auxin signalling pathway in resistance to this necrotrophic pathogen. However, the resistance of the *sgt1b-l* mutant (defective in the SCF^{TIR1} mediated auxin response pathway and in *R* gene mediated resistance to some pathogens) indicated that while SCF^{TIR1}-mediated ubiquitination is required for resistance to *P. cinnamomi*, it does not act exclusively through the common pathway involving *SGT1B*. Furthermore, the *rpm10-l* mutant, defective in the 26S proteosome and shown to affect auxin and ABA signalling (Smalle et al., 2003), was susceptible to *P. cinnamomi* and supported a role of the TIR1/26S proteosome in resistance to *P. cinnamomi*. 

The susceptibility of \textit{aba2-4} (defective in ABA synthesis) and \textit{tir1-1} (defective in auxin response) to \textit{P. cinnamomi} suggested the concomitant involvement of the ABA and auxin signalling pathways in the induction of resistance to \textit{P. cinnamomi}. Furthermore, application of TIBA (a polar auxin transport inhibitor) also enhanced the susceptibility of lupin seedlings to \textit{P. cinnamomi} indicating that the suppression of auxin transporters and subsequent disruption of auxin signalling is important in resistance to \textit{P. cinnamomi}. In \textit{Arabidopsis} auxin response mutants, the defective degradation of AUX/IAA transcriptional repressor proteins affect the induction of Auxin Response Factors (ARFs) and consequently the expression of auxin responsive genes (Hagen & Guilfoyle, 2002). Wang \textit{et al.} (2011a) reported that the expression of \textit{ARF2} (\textit{AUXIN RESPONSE FACTOR 2}) was induced by ABA treatment and indicated \textit{ARF2} as a novel regulator of the ABA signalling pathway. Therefore, the susceptibility of the \textit{aba2-4} mutant (defective in ABA synthesis) could be through the suppression of ARF with consequent suppression of auxin responsive genes (Chapter 5).

\textbf{Effect of Phi on ABA and auxin signalling pathways and their link to \textit{P. cinnamomi} resistance}

Phi mediated resistance to \textit{P. cinnamomi} in the susceptible \textit{Arabidopsis} ecotype Ler resembled the response of the resistant ecotype Col-0 in terms of timing and the defence responses induced (Chapter 2). Given the apparent involvement of the auxin and ABA signalling pathways in the resistance of Col-0 to \textit{P. cinnamomi}, the possible involvement of these pathways in Phi-mediated resistance was also investigated (Chapters 4 and 5).

The higher expression of \textit{PRI} in the non-inoculated non-Phi treated \textit{aba2-4} mutant compared to that of its resistant background Col-0 suggested a negative interaction between the ABA-related signalling pathway and SA-related defence
responses in *Arabidopsis*. This is consistent with previous reports showing higher levels of SA-induced genes in ABA-deficient *Arabidopsis* mutants (Mohr, 2003) and the antagonistic interaction between the SA and ABA signalling pathways (Yasuda et al., 2008, de Torres Zabala et al., 2009, Fan et al., 2009). Together, the higher expression of PR1 in the more susceptible *aba2-4* mutant and the resistance of the *npr1-2*, *nahG* and *sid2-1* mutants suggested that SA is not important for resistance to *P. cinnamomi* in *Arabidopsis* (Chapter 4).

The enhanced transcript levels of *PDF1.2* in the non-inoculated non-Phi-treated *aba2-4* mutant indicated that endogenously synthesized levels of ABA negatively regulate aspects of JA/ET related defence responses. In agreement with this, Anderson et al. (2004) reported that exogenous application of ABA repressed the transcription of *PDF1.2* in a *MYC2* dependent manner. *PDF1.2* is negatively regulated by *AtMYC2* (a positive regulator of ABA signalling) and ABA while other JA responsive defence genes including *VSP* and *PI* are positively regulated by both JA and *AtMYC2/ABA* (Lorenzo et al., 2004). Furthermore, Phi treatment together with *P. cinnamomi* inoculation resulted in the induction of the JA/ET-related genes *PDF1.2* and *THI2.1* in Col-0; however, screening the JA/ET-related mutants indicated that the resistance of Col-0 to *P. cinnamomi* is independent of the JA or ET signalling pathways (Chapter 4) and thus the interaction between ABA and JA/ET may not be playing a major role in host and Phi mediated resistance.

Application of ABA and auxin suppressed the expression of *PHO1* independently of the plant Pi status (Ribot et al., 2008) and ABA treatment has been shown to repress the induction of *At4*, a Pi starvation responsive gene (Shin et al., 2006). Given the apparent involvement of the auxin signalling pathway in the resistance of Col-0 (Chapter 5) and its’ link to the Pi and ABA signalling pathways (Ribot et al., 2008), the
involvement of these pathways in Phi mediated resistance was also investigated (Chapter 5).

In ecotype Col-0, the application of Phi on Pi sufficient plants enhanced the transcript level of PHR1 in a dose responsive manner. However, application of Phi had no significant effect on transcript level of the PHR1 gene in either the aba2-4 or tir1-1 mutants suggesting that mutation in ABA2 and TIR1 genes may disrupt the Phi effect on Pi signalling. The expression of PHR1 in response to Pi starvation was also diminished in both the aba2-4 and tir1-1 mutants indicating the importance of the ABA2 and TIR1 genes in the induction of PHR1 and PSR (Chapter 5).

Pi deficiency enhanced the transcript levels of the auxin responsive genes AUX1, AXR1, AXR2 and SGT1B indicating the induction of the auxin response pathway in Pi deficient plants; however, in Pi sufficient Col-0, Phi treatments induced the transcript levels of these genes in a dose-responsive manner. Moreover, Pi deficiency did not considerably change the transcription levels of the auxin responsive genes AUX1, AXR1, AXR2 and SGT1B in the aba2-4 and tir1-1 mutants confirming a role for ABA2 and TIR1 in the induction of the PSR. In conclusion, the results suggested that both ABA2 and TIR1 are required for both PSR and Phi-mediated auxin responsive gene expression, indicating that Phi may act through both the ABA and auxin pathways.

Conclusions

In conclusion, Phi primed the host for more rapid and more intense defence responses to P. cinnamomi and Phi treatments together with P. cinnamomi inoculation increased the transcriptional abundance of the defence associated genes PRI, PDF1.2, THI2.1 in Col-0. However, the resistance of mutants in the SA and JA/ET dependent pathways suggested that these common defence pathways are not required for resistance to P. cinnamomi. It was demonstrated that the suppression of the auxin response
pathway in the *tir1-1* mutant and in TIBA-treated lupins together with the disruption of Pi signalling in the *phr1-1* mutant resulted in the susceptibility of plants to *P. cinnamomi*. Furthermore, Phi stimulated the expression of *PHR1* and auxin responsive genes suggesting that phosphite-mediated resistance may in some part be through its effect on stimulation of the auxin response. The analysis of gene transcriptions in the *aba2-4* and *tir1-1* mutants revealed that mutation in *ABA2* and *TIR1* genes may disrupt the Phi effect on Pi signalling and highlighted the importance of *ABA2* and *TIR1* in PSR responses of *Arabidopsis*. Overall, the involvement of the ABA and auxin signalling pathways in resistance to *P. cinnamomi* and the requirement of *ABA2* and *TIR1* for both PSR and Phi-mediated auxin responsive gene expression suggested the concomitant involvement of these pathways in resistance to *P. cinnamomi* and Phi-mediated resistance.

**Conceptual Model and Future Directions**

Figure 1 is a conceptual model that summarizes the links between the different sections in this study and proposes the future research directions on the following aspects of Phi mediated defence responses to *P. cinnamomi*.

1. The wild type resistance of *jar1-1, ein2-1* and *etr1-3* showed that resistance to *P. cinnamomi* is independent to the JA and ET signalling pathways. However, it is possible that a synergistic effect of JA and ET may impact on *P. cinnamomi*, not JA or ET alone; therefore screening double mutants with genes in both of these pathways as well as additional mutants including *coi1-1*, will help to examine this possibility in more detail.

2. Screening additional ABA mutants such as *abi1, abi2* and *abi5* for their response to *P. cinnamomi* will further confirm the involvement of the ABA
pathway and help to elucidate how this pathway is involved in resistance to *P. cinnamomi*.

3. Whether down regulation of *AtMYC2* by *P. cinnamomi* or Phi treatment in this study is a consequence of the up-regulation of the JA pathway including genes such as *PDF1.2* and *THI2.1* or vice versa could be investigated by inoculation with *P. cinnamomi* or Phi treatment of double mutants of *atmyc2/jin1* with other ABA/JA related genes.

4. Screening the *axr1-1*, *axr2-1* and *aux1-1* mutants for their response to *P. cinnamomi* and Phi treatment will reveal whether induction of the auxin responsive genes *AXR1*, *AXR2* and *AUX1* by Phi are directly related to the enhanced resistance to *P. cinnamomi*.

5. To investigate whether a combination of Phi and auxin treatments enhances the effectiveness of Phi and improves the control of *P. cinnamomi* infection. This might enhance plant resistance against *P. cinnamomi* and open up mechanisms for *Phytophthora* management in agriculture and natural ecosystems.

6. Phi treatment in the *aba2-4* mutant did not affect the expression of the auxin responsive genes *AXR1*, *AXR2* and *AUX1* (Chapter 5). It has been demonstrated that ABA treatment enhanced the expression of *ARF2* leading to expression of auxin responsive genes (Wang et al., 2011a). Therefore, the susceptibility of the *aba2-4* mutant (defective in ABA synthesis) could be through the suppression of *ARF2* and in turn in the suppression of auxin responsive genes (Chapter 5). Therefore, it will be very interesting to dissect the interaction between ABA and auxin responsive genes and vice versa by screening *arf2* mutants for Phi-mediated resistance and their response to *P. cinnamomi*. 
7. SIZ1 is involved in the negative regulation of auxin patterning to modulate root architecture in response to Pi starvation (Miura et al., 2011) and it negatively regulates PHR1 and the expression of Pi starvation-responsive genes (Miura et al., 2005, Lin et al., 2009). This study showed that PHR1 and TIR1 are both involved in resistance to P. cinnamomi and the expression of PHR1 and the auxin-related genes AUX1, AXR1, AXR2 in response to Pi starvation was diminished in the tir1-1 mutant. Screening the siz1-1 mutant for its response to P. cinnamomi infection and Phi treatments will help to elucidate a role of the PSR in host and Phi-mediated resistance to P. cinnamomi.
Figure 1 A Conceptual model for *Arabidopsis thaliana—Phytophthora cinnamomi* interactions and the effect of phosphite (Phi) and phosphate (Pi) on this pathosystem. The blue font represents the genes studied in this thesis. The red font represents the mutants screened for their response to *P. cinnamomi*. “R” means resistant and “S” means susceptible to *P. cinnamomi* (Pc). The red “X” represents the absence of involvement of the pathway in resistance to *P. cinnamomi*; whilst, the red “√” represents the involvement of the pathway. The dashed arrows and the “?” represent pathways which are presently unclear and require future research.
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