Investigation of induced resistance against *Fusarium circinatum* in *Pinus patula*

by

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Supplementary Data

DECLARATION

I, Katrin Fitza, hereby declare that the dissertation submitted herewith for the degree *Magister Scientiae* at the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other university.

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Katrin Fitza

April 2012

PREFACE

Pines represent over 50% of the trees planted by forestry companies in South Africa. They are valued not only for their oils and biofuels, but for their solid timber, pulp and paper. Diseases pose a threat to pine trees. In particular the necrotrophic pathogen *Fusarium circinatum* causes pitch canker disease on *Pinus patula*. The pathogen causes significant losses to *P. patula* production in the forestry sector. Due to the economic impact of this problem, means to reduce disease incidence are being investigated, including the prospect of improving resistance in *P. patula*.

One of the means of improving resistance is the enhancement of defences using chemical or biologically derived products to elicit an array of signalling pathways which lead to the expression of defence related genes, a phenomenon known as induced resistance. Induced resistance includes systemic acquired resistance (SAR) and induced systemic resistance (ISR). Each of these responses is associated with specific plant hormones, activation of specific defence genes and is effective against different pathogens. Due to the uniqueness of each pathway the defence proteins can be used as markers to identify the onset of each respective pathway.

The **aim of this MSc** study was to identify and select inducers which effectively trigger induced resistance against *F. circinatum* in *P. patula* seedlings and to determine whether the treatments were accompanied with an induction of marker genes associated with induced defence.

Chapter 1 is a review of the literature concerning the *P. patula* - *F. circinatum* plant pathogen model. This chapter begins with information on the importance of pine for the forestry industry and the losses caused by this fungal pathogen. It further describes the function of induced resistance and its use in agriculture to combat diseases. Finally a few examples of inducers are provided, focusing on their function in induced resistance and their successful application in agriculture.

Chapter 2 of this dissertation describes the evaluation of candidate inducers against the pitch canker pathogen and the characterization of the expression patterns of the putative defence genes for the chitosan treatment in *P. patula*. The inducers were evaluated by performing both a nursery and greenhouse trial, and in the latter, manual mechanical inoculation was used, ensuring the presence of the fungus in the

seedlings. Putative defence gene orthologs were identified using the EST database (ConiferGDB) which consisted of primarily data generated from *P. taeda*. This data was used in a phylogenetic approach to elucidate *P. patula* orthologs. The putative candidates were investigated for their role in *P. patula* defence mechanisms against *F. circinatum*. In future the use of the genome becoming available will provide more insight into the defence employed by pine trees.

Final conclusions, implications and future prospects are summarized and discussed at the end of this dissertation in the **Concluding Remarks** section.

The research findings presented in this study represent the outcomes of the study undertaken from March 2009 to March 2011 in the Department of Genetics, University of Pretoria under the supervision of Dr. S. Naidoo and co-supervision of Prof. A.A. Myburg and Prof. Emma Steenkamp. The following posters and congress presentations were generated based on the results obtained from this study:

Fitza K., Myburg A.A., Steenkamp E. and Naidoo S. 2011. Expression profiling of defence genes during induced resistance in *Pinus patula*. Southern African Society for Plant Pathology conference (SASPP). January 23-26. Berg-en-Dal, Kruger Park, South Africa. (Poster Presentation).

Fitza K., Myburg A.A., Steenkamp E., Payn K. and Naidoo S. 2011. Expression profiling of defence genes during induced resistance in *Pinus patula.* IUFRO Tree Biotechnology Conference S5 P18. June 26 – July 2. Arraial d'Ajuda, Bahia, Brazil. (Poster presentation) (Published online in BMC proceedings)

Fitza K., Myburg A.A., Steenkamp E., Payn K. and Naidoo S. 2012. Elucidation of defence responses associated with inducer application in *Pinus patula.* South African Association of Botanists conference (SAAB). January 15-18. Pretoria, South Africa. (Oral Presentation).

Chapter 2 has been written up as a publishable unit and will be submitted for publication shortly.

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- To the rest of my **family and friends**. Thank you for all the support and encouragement throughout these years.

ABBREVIATIONS

26s	26s proteosome subunit
ai	Active ingredient
AOS	Allene oxide synthase
ARF	ADP-ribosylation factor
BLAST	Basic Local Alignment Search Tool2
bp	Base pair
cDNA	Complementary DNA
Cq	Quantification cycle
DAD	Defective in anther dehiscence
DAMPS	Damage-associated molecular patterns
DMAPP	Dimethylallyl pyrophosphate
dNTP	Deoxynucleoside triphosphate
DXS	1-deoxy-D-xylulose 5-phosphate synthase
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector triggered immunity
FMO	Flavin-dependent monooxygenase
GDP	Guanosine diphosphate
GGPP	Geranylgeranyl phosphasphate
GM	Genemark
GPP	Geranyl pyrophosphate
GS	Genscan
Ha	hectare
HR	Hypersensitive response
hrs	Hours

IPP	Isopentenyl pyrophosphate
ISR	Induced systemic resistance
JA	Jasmonic acid
JA	Jasmonic acid
JAZ	Jasmonate ZIM-domain
LOX	Lipoxygenase
MAMP/PAMP	Microbial/Pathogen associated molecular patterns
Mbp	Megabase pair
MeJA	Methyl jasmonate
MEP	Methylerythritol phosphate
ML	Maximum likelihood
NBS-LRR	Nucleotide-binding site leucine rich repeats
NCBI	National Center for Biotechnology Information
NJ	Neighbour joining
NPR1	Nonexpressor of the pathogenesis related protein 1
OPR3	12-oxophytodienoate reductase 3
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
Pdens	*Pinus densiflora*
PDF	Plant defensins
PGPR	Plant growth promoting rhizobacteria
Ppa	*Pinus patula*
Ppin	*Pinus pinaster*
PR	Pathogenesis related
PRR	Pattern recognition receptors
Pt	*Pinus taeda*
PTI	PAMP triggered immunity

RNA	Ribonucleic acid
RT-qPCR	Reverse Transcription Quantitative PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
SIR	Systemic induced resistance
yr	year

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Chapter 1

Literature review

Induced resistance: From model plants to pine trees.

1.1 Introduction

Pine plantations are an important resource for paper and pulp production as well as solid wood products sold worldwide. The South African industry focuses predominately on *Pinus* (51%, FSA, 2009) and *Eucalyptus* (40.4%). Pine is extensively planted because it is one of the faster growing coniferous trees. Planting these exotic tree species in non-native countries such as South Africa is done to fulfil the needs on timber and other products for the forestry industry. Each year South Africa produces timber valued at R4 billion (FSA, 2009), and the forestry industry is a vital contributor to national employment. In addition, the industry has a strong export branch, making yearly profits of approximately R12.5 billion (FSA, 2009). Nineteen of the world's 111 pine species have been found to be suitable for afforestation i.e. the establishment of a forest by planting trees or their seeds, (Farjon and Gardens, 2001; Price *et al.*, 1998). *Pinus patula, P. taeda, P. radiata* and *P. elliotti* are commonly planted pine species in South Africa.

Pinus patula is a pine species that originated from Mexico and has since been planted in many countries, including South Africa. The majority of the countries planting pine make use of monoculturing, the presence of even-aged trees of one species, as the economically viable intensive management (Evans, 1992). From an industry perspective, monoculturing allows greater yield and more efficient farming can be conducted reducing the overall costs. However, single species planting increases the risk of rapid spreads of diseases. Pine trees, non-native to South Africa, are confronted with different pathogens compared to their native environment, making the plantation of pine species risky.

The serious losses caused by the fungus *Fusarium circinatum* have great impact on the economy (Wingfield *et al*., 2008; Wingfield *et al.*, 2002b). *Fusarium circinatum* is a necrotrophic fungus responsible for the disease known as pitch canker. The global spread of the fungus makes it of great concern for the forestry sector. Pitch canker was first identified in South Africa in the 1990s, causing oozing lesions and cankers in nursery seedlings (Viljoen *et al.*, 1994). Once the fungus is established on a tree, the timber quality drastically reduces due to deformed stems and reduced growth (Barrows-Broaddus and Dwinell, 1985). Pine species have been tested for tolerance against the disease and it was found that *P. patula* and *P. radiata* are two of the most susceptible pine species (Hodge and Dvorak, 2000). The susceptibility of the *Pinus* species and the use of monoculture give reason for the rise of the destructive epidemics caused by the pitch canker fungus (Wingfield *et al.*, 1998).

Efforts have been made to identify or breed more resistant hybrids. It was shown that hybrids *P. patula x P. oocarpa* and *P. patula x P. radiata* were most tolerant to *F. circinatum* (Roux *et al.*, 2007). The selection process to generate more resistant trees will need to continue, however, this is a difficult approach, as the pathogen undergoes sexual reproduction, opening an arms race whereby the pathogen is able to overcome the plants' defences and the plant has to find alternative protective measures (Britz *et al.*, 2005; Viljoen *et al.*, 1997). This arms race therefore allows adaptation of the pathogen to the tolerant plants (Giraud *et al.*, 2010). Currently, nurseries control the disease using good hygiene and management practices. The use of toxic chemicals such as fungicides and pesticides is increasingly avoided due to environmental concerns associated with those chemicals. More importantly the increased resistance of the pathogens to the fungicides have reduced their usage (Gerhardson, 2002) and the control of outbreaks of root pathogens (e.g. *Fusarium* species) with fungicides has become less successful (Yan *et al.*, 1993). Incorporation of biological control methods such as priming the plants' resistance and activating induced resistance, have recently received more attention (Ton *et al.*, 2009). The presence of induced resistance in woody perennial plants is seen as a possibility to sustain populations (Hammerschmidt, 2006; Ton *et al.*, 2009).

Induced resistance refers to an array of signalling cascades triggered by various pathogens and inducing agents with the main purpose to prime the plant against subsequent challenges by a pathogen (Reglinski and Dick, 2005). This phenomenon encompasses systemic acquired resistance (SAR), induced systemic resistance (ISR) and systemic induced resistance (SIR). SAR signalling is associated with salicylic acid (SA) dependent signalling and is effective against biotrophic pathogens, whereas ISR activates the jasmonic acid and ethylene dependent pathway against necrotrophic pathogens (Ton *et al.*, 2009). SIR is a wounding response also leading to the increase in the hormones ethylene and jasmonic acid. All these pathways are characterised by different activational pathways and are induced by different pathogens. As resistance is an integrated response, it includes anatomical reactions such as lignification of the cell wall, as well as chemical defence responses, such as the production of secondary metabolites and activation of pathogenesis-related proteins which have antimicrobial and antifungal properties (Demain and Fang, 2000).

The phenomenon of induced resistance through the application of biological elicitors has been investigated for agricultural purposes (Boller and Felix, 2009; Bruinsma *et al.*, 2010; Tamm *et al.*, 2011). The elicitors range from bacterial (harpin) and fungal (chitosan)

compounds to non-pathogenic agents (*Fusarium oxysporum*) (Schreiber and Desveaux, 2008). Studies have been performed on model organisms, which provide a better understanding of the complex networks involved in effective resistance. It is of interest to identify differences in resistance between annual (plants completing their life-cycle in a year) and perennial plants (shrubs or woody plants), and through comparison find unique resistance aspects in trees. Furthermore, exploiting the knowledge from model organisms such as *Arabidopsis* assists in understanding of resistance in other plants, such as trees. This review introduces *Pinus patula* and the pitch canker pathogen, which poses a threat to the pine forestry industry. A brief summary on what is currently known on induced resistance in the context of model organisms is provided, as well as a section on how this could be adapted to the *Pinus patula-Fusarium circinatum* pathosystem.

1.2 *Pinus patula*

1.2.1 Biology of *Pinus* species

Pinus (Pinaceae) represents one of the largest genera of conifers with 111 species identified in the group (Farjon and Styles, 1997; Price *et al.*, 1998). Conifers, like pine, spruce and cycad, are soft wooded trees that form the gymnosperms (Figure 1.1, Farjon and Styles, 1997). The gymnosperms are referred to as the naked seed-bearing plants where the seeds are found on the scales of cones and furthermore in this group usually the trees have needles. This stands in contrast to the angiosperms, which are flowering plants where the seeds are protected by the surrounding ovule and the leaves are broad (reviewed by Sundaresan and Alandete-Saez, 2010). One of the 111 species found in the *Pinus* genus is *Pinus patula* which is commonly known as Mexican weeping pine (Dvorak *et al.*, 2000). The tree can be found in the subgenus *Pinus*, section *Trifoliae* and subsection *Australes*, together with other species such as *P. taeda* and *P. radiata*, seen in Figure 1.2 (Gernandt *et al.*, 2005).

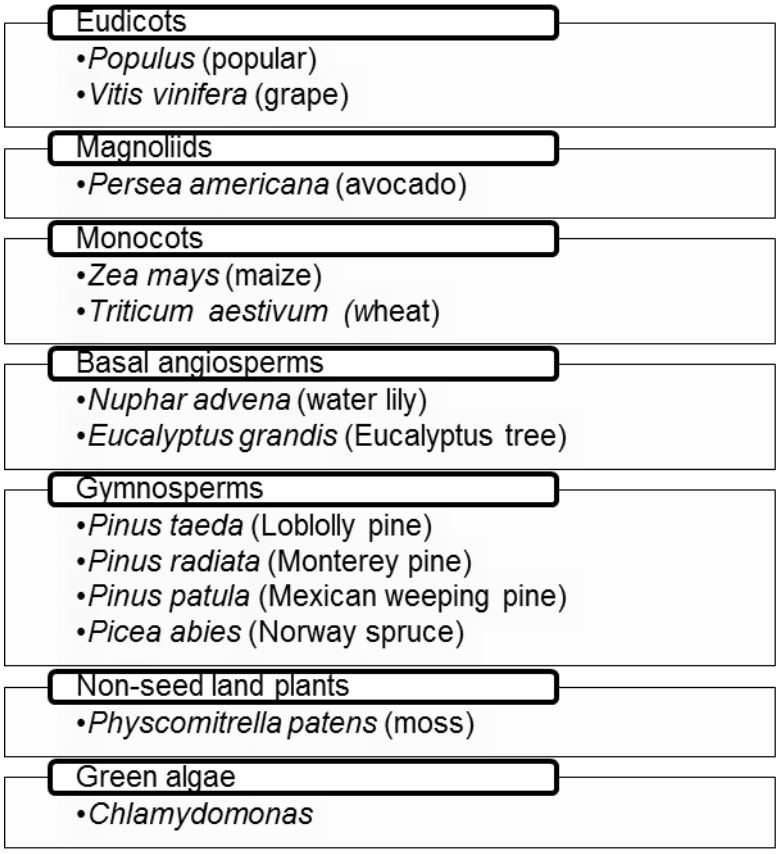

Figure 1.1: Diagram identifying the different green plant groups. The plant group names (adapted from http://smallrna.udel.edu/libraries.php) are given in the thicker framed boxes. Examples of plant species are then mention in the box below. For all the given plant species the genome sequences are available, except for both gymnosperms for which only RNA sequences are currently provided.

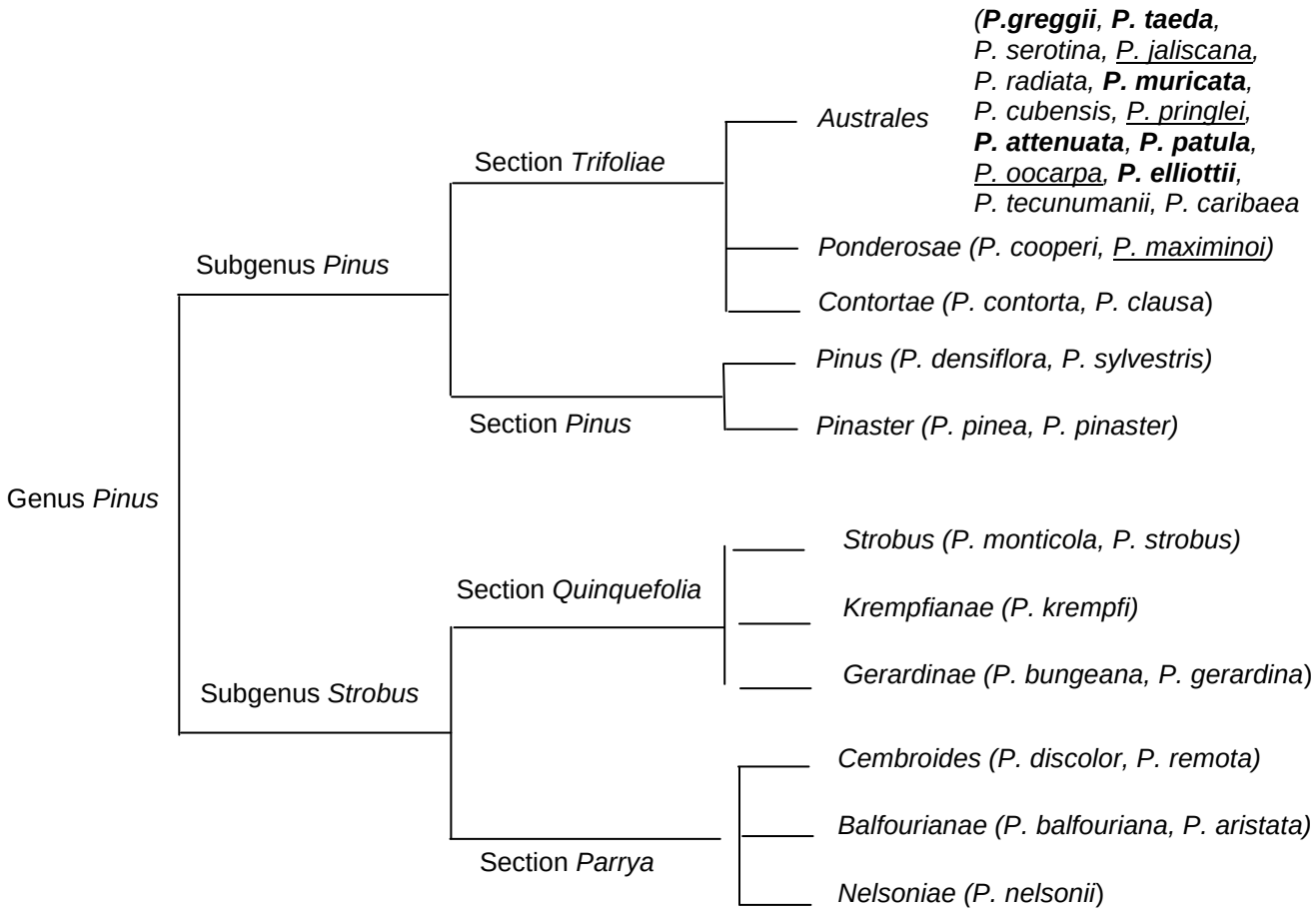

Figure 1.2: Synopsis of *Pinus* classification recognising four different sections. Pines include two subgenera *Pinus* and *Strobus*. Under the subgenus *Pinus* two sections have been identified. *Pinus patula* is found in the section *Trifoliae*, subsection *Australes*, together with *P. taeda* (Gernandt *et al.*, 2005). The pine species which are highly susceptible to the pitch canker pathogen are presented in bold. The underlined *Pinus* species reprensent more resistant species to the fungus *Fusarium circinatum*. Two sections fall under the subgenus *Strobus*, each having three subsections.

1.2.1.1 Morphological characteristic features of conifers

Conifers are woody, evergreen trees with characteristics such as cone bearing, seed production and needles rather than leaves. They can reach up to 40 m in height (a single straight trunk with side branches) and have bark which is red-brown to grey-brown in colour. The needles are thin, pointy and arranged in groups of two, three or five. Furthermore, the needles have a wax coating limiting water loss. Pines have separate female and male cones. The female cone can be identified through woody scales and presence of seeds (Flores-Renteria *et al.*, 2011). The male cones, holding the pollen, are found on the upper branches, whereas the female cones are located in the lower part of the tree. Male cones have microsporophylls that are arranged in a spirally manner, centred towards an axis and

they differ only to a small extent among the different conifer species (Walden *et al.*, 1999). Female cones carry the ovules and are variable in structure among the conifer families, allowing identification of many species based on the female cones. Once the male cones are mature, taking from four months up to three years, the scales open, allowing the pollen to be spread via wind. All these features enable the conifers to survive harsher conditions and become old, the oldest conifer being 4,700 years old (Reimer *et al.,* 2007) .

1.2.1.2 Genomic resources available for pine

Long generation times and huge genome sizes, which make the sequencing of the entire genome of conifers a challenging task, as well as limited funding, have held back advances in genomic research of coniferous forest trees. Pine species have a large genome size of around 20,000 Mbp (Kirst *et al.*, 2003) and spruce of around 15,000 Mbp (Scotti *et al.*, 2005) compared to *Eucalyptus* with 640 Mbp (Myburg *et al.*, 2011). The larger genome size of pine can be attributed to highly repeated DNA sequences (Kovach *et al.*, 2010). The 20,000 Mbp of DNA is divided into 12 chromosomes. Due to recent rapid development in technology, making sequencing less costly and quicker, genomic research programmes have been initiated in spruce (*Picae abies*) in 2010 and pine (*Pinus taeda*) in 2011 (Neale and Kremer, 2011). An established conifer database is currently available with EST sequences from *P. taeda* (Liang *et al., 2007*). The database contains around 450,000 ESTs, 41,310 unigenes, and 22,700 SNPs are accessible for pine (Neale and Kramer, 2011).

In the current age, where genomes are being released more rapidly, the evolutionary relationship of those genomes is of interest and allows for comparative analysis. Highlighting areas of homology between proteins or DNA sequences enable researchers to assign putative functions to unknown genes. Two key concepts of homology of sequence are orthology and paralogy. Orthologs refer to genes in different species that occur through a speciation event and originated from a single ancestral gene (Fitch, 2000; Chen *et al*., 2007). Through a gene duplication event in an organism, the same gene may occur at different positions in the genome and causes sequence divergence. These genes are referred to as paralogs (Fitch, 2000). Mostly, orthologous genes retain similar functions as well as high sequence similarity, whereas paralogs may undergo divergence resulting in new functions. These homologous relationships between sequences can be determined by means of phylogenetic analysis.

Many genes have been well described in model organisms such as *Arabidopsis* or *Poplar* which allows the identification of orthologs between the model species and the species of interest (Brunner *et al.*, 2004). Comparative sequence analysis of the three *Arabidopsis CesA* genes identified three *Pinus taeda* orthologs. This was achieved using a phylogenetic approach (Nairn and Haselkorn, 2005). Extensive conservation of defence genes between the known *Arabidopsis* genes and the orthologs in rice was found. One putative ortholog in rice was found for each of the *NPR1*, *PAD4* and *EDS1 Arabidopsis* genes. Brunner *et al*. (2004) concluded a high similarity between monocots (eg. rice) and dicots (eg. *Arabidopsis*) exists. Applying the approach of phylogenetics forms the starting point to elucidate possible orthologs. To further narrow down the candidates and identify the true ortholog/s, functional experimentation, protein analysis and complementation studies will need to be conducted. In combination these methods provide a holistic means to identify functionally equivalent genes in other species (Fang *et al.*, 2010).

1.2.2 Ecological and economic importance of pine

Pines are ecologically the dominant component of the forests, accounting for the largest terrestrial carbon sink. Their natural habitat is the northern hemisphere. Pines are able to exist in temperate, tropical forests and arid and boreal conditions, where they are considered exotic plants (Mirov, 1967; Richardson and Rundel, 1998). Growth requirements for the conifers are deep, well-drained soils, preferably at altitudes above 1,000 m. Pine trees grow in moist temperate to subhumid temperate climates, with an average temperature ranging from 10°C to 18°C. All of these factors contribute to cultivation of many pine species such as *Pinus radiata, Pinus patula* and *Pinus pinaster* among others (Le Maitre, 1998). The majority of pine plantations which exist in North, Central and South America, Africa, Asia and Oceania (FAO, 2001)

Considering all the morphological and ecological attributes of *Pinus* it is understandable that it is one of the most important genera economically, seeing that 31% of the world's land area is used for forestry. The forestry sector makes up 0.4% of the total labour force in the world and accounts for 1% of the GDP (Lebedys, 2004). Pines are used for wood, paper, resins, charcoal, ornaments and the seeds have nutritional value. The rapid growth of *Pinus*, compared to other slower growers, makes it ideal for wood production. The current Global Conifer Sawlog Price Index (GSPI) lies at R658.8/m^3 (www.forestry.co.za). For paper and pulp production, the removal of lignin is essential and the presence of high levels of α–cellulose and hemicelluloses is required. Pine is a softwood and has around 40% of α–

cellulose and 30% of both hemicelluloses and lignin, making it a valuable tree species (Panshin and De Zeeuw, 1980). Furthermore, the chemical composition of pine trees allows turpentine to be produced from the resin. Recently, more interest has been put into developing biofuels from wood pellets. These pellets are derived from compacted saw dust or pine logging residues (Lehtikangas, 2001; Samuelsson *et al.*, 2009), emphasising that everything from the tree can be processed into economically valuable products.

1.2.2.1 The South African Forestry industry

Plantation areas in South Africa are dominated by pine trees; 1% of South Africa's land area is used for tree plantations, which is 1.2 million hectares. South Africa is in 11th place in comparison to the plantation area of other countries in the world. Of the 1% plantation area, 51% is used for pine, 40.4% for *Eucalyptus*, 8.2% for wattle and 0.4% for other species (FSA, 2009). These 0.65 million ha (pine) can be further broken down into production objectives whereby 64.2% are used for sawlogs, 33.5% for pulpwood and 1.7% for poles; the remaining 0.6% is used for other management objectives (www.forestry.co.za). Average productivity values in commercial plantations vary from 12 m^3/ha/yr to around 25 m^3/h/yr at rotation ages of 15 to 25 years for pine. The forestry industry hence forms one of the major pillars of South Africa´s economy, ensuring employment for more than 100 000 people and contributing 1% to the total GDP (www.fao.org).

Pinus patula was introduced into South Africa in 1907 (Kotze and Eckbo, 1926). Globally, 4.5 million ha are used for *P. patula* plantations, 1 million ha of which can be found in southern and eastern Africa (Dvorak *et al.*, 2000). In South Africa roughly 337,000 ha of *P. patula* plantations are being managed in the Eastern Cape (www.fao.org), in the Natal midlands and Mpumalanga areas, where preferred temperature conditions are met (van Wilgen and Richardson, 2006).

As with most tree species grown for wood production, pathogens pose a threat. Examples are the dwarf mistletoe pathogen *Arceuthobium americanum* (Epp and Tardif, 2010), *Sphaeropsis sapinea* causing blue stain and canker development, *Leptographium serpens* causing the blue stain and root rot disease (Santini *et al., 2008), Phytophthora pinifolia* associated with needle disease (Durán *et al.*, 2008) and the most destructive, *Fusarium circinatum,* which causes pitch canker. *Pinus patula,* one of the most commonly planted species, was identified to be one of the most susceptible species, together with *P. radiata* to the pitch canker fungus. Other pine species, such as *P. elliottii* and *P. taeda* are more

tolerant to *F. circinatum* (Lyon and Sinclair, 2005). Elucidating the interaction of this pathogen with *P. patula* and understanding the complex defence network and the associated genes is therefore of great interest to the forestry industry. From a research perspective, as most of the work done on pathogen defence has been performed on herbaceous angiosperms, of interest is to elucidate to what extent the knowledge can be applied to gymnosperms. How much of the plant defence is conserved in woody gymnosperms? Are there specific aspects of defence that are unique in trees?

1.3 Pitch canker caused by *Fusarium circinatum* in pine

1.3.1 Taxonomy of the pathogen

The genus *Fusarium* forms part of the filamentous fungi (Booth, 1971) and are known to cause a variety of diseases such as vascular wilts, root, stalk and cob rots, collar rots of seedlings, and rots of tubers, bulbs and corns. Within the species complex *Gibberella fujikuroi,* to which *F. circinatum* belongs, other *Fusarium* species such as *F. thapsinum*, *F. nygamei* and *F. proliferatum* can be found (Steenkamp *et al.*, 1999). The fungus of interest, *F. circinatum,* is a member of the section *Liseola* (Hepting and Roth, 1946).

1.3.2 Symptoms associated with pitch canker

Fusarium circinatum is a pathogen that specifically infects trees of the genus *Pinus* and is the causal agent of pitch canker (PCF). The areas of infection are branches, shoots, cones, exposed roots and stems. Importantly, the fungus is able to infect seeds and this can only be identified once germination has occurred (Gordon *et al.*, 2001; Viljoen *et al.*, 1994). Reports showed the isolation of the pitch canker fungus from apparently healthy seeds which gave rise to infected seedlings (Carey *et al.*, 2005; Dwinell, 1998a). The symptoms displayed by the fungus are characterised by canker formation, resin secretion and discolouration of the wood at infected areas (Dwinell *et al.*, 1985). This disease is recognized in seedlings in the form of damping off, discolouration of the needles, which turn from green to reddish brown, and both root and collar rot (Hammerbacher, 2005; Viljoen *et al.*, 1994). As early as three weeks after infection, the seedlings begin to show severe wilting symptoms and by six weeks these seedlings die (Figure 1.3). In mature trees, the symptoms start off with the discolouration of newly developed tips and shoots, leading to the death of the new growth and ending in large canker development on stems and branches, as well as tree death (Barnard and Blakeslee, 1980; Dreaden and Smith, 2010; Gordon, 2006).

Figure 1.3: Visual presentation of pitch canker disease symptoms. Seedling on the far left is a healthy one, seedling in the middle shows early PCF symptoms and seedling on the right is a dying seedling showing severe symptoms. (Photo by Dr. K. Payn)

1.3.3 Genetic variation in resistance against *F. circinatum* in the pine species

Resistance against the pitch canker pathogen is important and various studies have been undertaken to identify more resistant pine tree species. In 1985 Dwindell *et al*. already identified varying degrees of symptom severity in different pine species. In 2000, Hodge and Dvorak conducted a more in depth study by artificially inoculating different pine species seedlings with *F. circinatum*. In this study, pine species native to Mexico and Central America were evaluated for their resistance. These species are of most interest to South Africa as these are the ones used by the forestry sector. A trend that was detected was that pine species from lower elevation such as *P. oocarpa, P. jaliscana* and *P. tecunumanii* seemed to be less affected by the pathogen (Figure 1.2). Furthermore, a link between the closed-cone pine species and a high survival rate against the pathogen was found. Pine species such as *P. radiata, P. patula, P. elliottii, P. muricata, P. attenuata, P. greggii* and *P. herrerae* were identified to be highly susceptible to *F. circinatum* (Figure 1.2). Understanding of genetic variation will be of benefit for breeding of desirable traits. Additionally, this knowledge may aid investigation of differences between resistant and susceptible species found on a genetic level, such as identifying genes important for resistance.

1.3.4 Reproduction of *F. circinatum*

Fusarium is a soil borne pathogen belonging to the group of Ascomycetes (Agrios, 2005). The fungus survives in the soil as mycelia or as spores in the absence of its host. In the presence of the host, the mycelium, developing from germinating spores, enters the roots of the tree. Penetration is successfully achieved through the penetration of germ tubes, entering via micro-cracks (Thoungchaleun *et al.*, 2008). Upon gaining access, the pathogen enters the vascular system, specifically the xylem, where the fungus moves and multiplies. The vessels of the xylem are invaded through the xylem pits and act as the transport system for the pathogen to reach the stem and the crown of the plant. Microconidia occur through the growth of the mycelium (Agrios, 2005) and are transported by means of the sap stream. Germination of the microconidia leads to the penetration of the upper wall of the xylem. Water is important for the plant, and the growth of the fungus in the xylem reduces the water supply within the plant. Reduced water closes the stomata and induces typical wilting symptoms, eventually resulting in death. At this stage of the fungus life-cycle, the fungus invades the parenchymal tissue until it reaches the surface of the dead tissue, thereafter sporulation occurs (Agrios, 2005). Initiation of the pitch canker disease is caused by the presence of the conidia. These condia are produced on dead or dying branches, as well as in needle fascide scars or on stems. The spores have also been identified on cocoons constructed by pine weevils (*Pissodes nemorensis*) beneath the bark (Coutinho *et al.*, 2007; Wingfield *et al.*, 2008). The spread of the disease is dependent on precipitation and turbulent air as the spores of the fungus are airborne.

Fusarium circinatum makes use of both sexual and asexual reproductive strategies. Sexual reproduction means that new genotypes develop, making the adaptation of the fungus to tolerant planting stock more likely, whereas asexual reproduction refers to clonal propagation. Until now, no successful sampling in nature of the sexual structures has been done, but the presence of the structures have been recorded under laboratory conditions (Britz *et al.*, 2005). The fungus falls under the heterothallic group of fungi which means that the single spore isolates are self-sterile species. A single gene defining the mating type showed the presence of two mating type alleles (MAT-1 & MAT-2). For successful mating, two individuals of opposite mating types need to reproduce (Whitehouse, 1949). To identify different mating populations in the *Gibberella fujikuroi* complex, fertility is used as a criterion, placing the populations into seven mating populations (A-G). *Fusarium circinatum* represented a new mating population named H, implying that they are seen as a new biological species in the *G. fujikuroi* complex (Britz *et al*., 2005). The vegetative compatibility

groups (VCG), used as markers to identify diversity of fungal populations (Croft and Jinks, 1977), showed that a high number of VCGs are recognised in South Africa's population and this diversity may be explained through sexual reproduction (Viljoen *et al.*, 1997). Yet, the high level of diversity found could also be explained through mutation events. More research still needs to be done to understand the establishment of further genetic diversity of the disease.

1.3.5 Pathogenicity and environmental factors causing infection

The pathogenicity of fungi is due in part to the production of secondary metabolites that are harmful to plants and animals (reviewed by Collemare and Lebrun, 2011). Their functions range from growth rate control, affecting individual biosynthetic pathways to acting as weapons against pathogens or even favouring symbiosis (Demain and Fang., 2000). Such phytotoxic products functioning as protective shields, are enniatin and fusaric acid that are important for infection and virulence. In the genus *Fusarium,* both avirulent and virulent fungi are found. Studies have revealed that virulent *Fusarium* produce higher amounts of cutinases compared to the avirulent isolates. Cutinases aid the pathogen in overcoming the physical barrier represented in the form of cutin, the major structural element of the cuticle layer in the plant (Morid *et al*., 2010). Once these barriers are breached, the vulnerability of pine to become infected with *F. circinatum* is dramatically increased due to the presence of open wounds (Schweigkofler *et al.*, 2004). These could be wounds located anywhere in the aerial part of the plants, as well as the root system. Any damage caused by bad handling of seedlings, water logging, hail storms or other environmental conditions permits access of the pathogen into the plant. Insects, such as pine weevils (*Pissodes nemorensis*) inflict wounds allowing transmission of the disease (reviewed by Hammerbacher, 2005). The insects are transmitters of the pathogen, as they are attracted to oozing sap from the developed canker. Sap contains spores and once the insects have been in contact with the contaminated sap, there is transmission to the next plant. Another important aspect with regards to aiding infection is fertilization, which with increased nitrogen (N) levels has specifically been shown to actually enhance the disease severity (Lopez-Zamora *et al.*, 2007; Solel and Bruck, 1989). A positive correlation between high levels of N and pitch canker severity has been observed (Solel and Bruck, 1989). Results of high levels of N in the plants are the development of more succulent tissue and decreased production of plant metabolites with antifungal properties which aid fungal entry (Hesterberg and Jurgensen, 1972). On the other hand high levels are not required for the development of the disease.

1.3.6 Distribution of the fungus

The pitch canker fungus originates from Mexico and from there has been established in many states of the USA (Wikler and Gordon, 2000). In some areas of California the mortality rate in mature pine trees can reach 80%. Subsequently, the disease has caused devastating effects in New Zealand, Australia and South Africa. The first recognition of the exotic fungus in South Africa occurred in the 1990s, mainly in nurseries, sometimes with 100% mortality in seedlings (Viljoen *et al.*, 1994).

The disease has mostly been reported in areas with warmer average annual temperatures. The first symptoms were seen in plantations located in the south-eastern part of the United States in North Carolina in 1946 (Hepting and Roth, 1946). The spread of the disease was difficult to prevent because *Pinus radiata* has been widely planted in the USA and this species is highly susceptible to the fungus. The disease then spread throughout the United States and attacked different species of pine (Correll *et al.*, 1991; Dwinell, 1998b; Storer *et al.*, 1994). From an epidemic in the USA it then reached other countries, such as Mexico (Santos and Towar, 1991), Japan (Kobayashi and Kawabe, 1992), South Africa (Viljoen *et al.*, 1994), South Korea (Lee *et al.*, 2000), Chile (Wingfield *et al.*, 2002a), Spain (Dwinell, 1998b; Landeras *et al.*, 2005), Italy (Carlucci *et al.*, 2007) and more recently Portugal and Uruguay in 2009 (Alonso and Bettucci, 2009; Bragança *et al.*, 2009).

In 1994, Viljoen *et al.* described the presence of a pathogen that caused seedling losses and discolouration in a nursery located in Mpumalanga province. It was identified to be the pitch canker pathogen, possibly brought into the country through infected material. From then, the spread of the fungus was unavoidable, yet it seemed to be limited to nurseries, specifically, as the fungus was not detectable in the natural environment and plantations. In 2007, Coutinho *et al.* discovered known symptoms of pitch canker in mature trees planted in the Western Cape that revealed that *F. circinatum* had become present in the natural environment. *Pinus radiata* trees, five to nine years old, developed large cankers from which large amounts of resin were oozing and die-back of various tree parts, such as main stem and branches, was observed.

1.4 Current status on the management of the disease

To understand the pathogen and its mode of action is one aspect, whereas looking at the plant's resistance is another vital aspect when devising proper strategies to curb the

disease. As previously mentioned, the pathogen is able to survive in seeds, however, the treatment of seeds with hydrogen dioxide was shown to greatly reduce fungal growth (Allen *et al.*, 2004) and has since been implemented in nurseries. The nursery stage may be regarded as the basic level in which changes can have an impact through proper handling strategies to avoid injuries on the young seedling. The daily protocol in nurseries includes general inspection of the plants. Any seedling suspected of carrying the disease will be discarded to avoid further spread. Trays that had infected seedlings would be washed in a strong chlorine solution and the floor kept clean (Porter, 2010). If a potential outbreak is suspected, some fungicides may be sprayed as a preventative measure, but only as a last resort. Pathogens have become resistant to these chemical weapons, and the environmental hazard of fungicides makes it less feasible. Applying fertiliser that is low in nitrogen also aids in protecting the plant, as nitrogen provides favourable conditions for the pathogen (Lopez-Zamora *et al.*, 2007). Furthermore, protecting the seedlings from harsh environmental conditions contributes to greater seedling survival. Although these are minimal precautionary steps, these steps seem to have limited fungal load in the plants and reduced the death of seedlings in the nursery to less than 3% (A. Morris, Sappi Forests (Pty.) Ltd. Sappi, Howick. pers. comm.)

While good hygiene practices have reduced the losses of the seedlings in the nursery, the major concern lies with the planting stock. As an increased number of seedlings that get dispatched to plantations seem to be asymptomatic, once planted in the field, 42% losses are caused due to post planting mortality. Therefore, the second approach to improve the situation with respect to combating the disease is tree breeding. Efforts have been made to identify or breed more resistant hybrids. It was shown that hybrids *P. patula x oocarpa* and *P. elliottii x caribaea* were most tolerant to infections (Roux *et al.*, 2007). The selection process to generate more resistant trees will need to continue, however, this is a difficult approach as the pathogen undergoes sexual reproduction, allowing for further genetic diversity to occur (Britz *et al.*, 2005; Viljoen *et al.*, 1997). The presence of new genotypes means the ability of the fungus to adapt to those selected, more tolerant plants. Hence an understanding of plant defence signalling involved in resistance is important.

1.5 Induced resistance

Plants frequently have to cope with challenges such as biotic and abiotic stresses, herbivore attack and infection by pathogens. Plants are able to survive and combat invasions by making use of an immune system that relies on existing physical and chemical barriers, and

induced defence pathways (Pieterse and van Loon, 2007). Two forms of immunity have been described in plants, namely horizontal defence, also known as basal resistance, and resistance (R) gene-based, or vertical defence (Göhre and Robatzek, 2008). The interactions between the two is a continuum described by the Jones and Dangl ZIG-ZAG model (Dangl and Jones, 2001).

1.5.1 Pattern-triggered immunity (PTI)

The first line of defence is a broad spectrum resistance against all members of a specific pathogen species, due to non-host recognition (Heath, 2000). This form of innate immunity is associated with pattern recognition receptors (PRRs) that are able to detect signal molecules associated with microbes (Figure 1.4). Once the PRRs recognise signalling molecules, an immune response called Pattern- Triggered Immunity (PTI) is activated. PTI is induced through either Microbe-Associated Molecular Patterns (MAMPS), also called Pathogen-Associated Molecular Patterns (PAMPS), or Damage-Associated Molecular Patterns (DAMPS) (Schwessinger and Zipfel, 2008). Molecular patterns, such as flagellin from bacteria, peptidoglycans and chitin, a major fungal cell wall component, are examples of molecules activating PTI. Several of these PRRs and their associated molecular patterns have been studied. One of the better understood examples is the bacterial flagellin that is recognised by its leucine rich repeat domains by the flagellin-sensing 2 (FLS2) receptor in plants. In the *Arabidopsis thaliana* - *Pseudomonas syringae* pathosystem, it was found that a 22-amino acid peptide sequence, in a conserved region of flagellin (flg22), is the trigger for plant defence (Chinchilla *et al.*, 2006; Gómez-Gómez and Boller, 2000; Zipfel *et al.*, 2004). Zipfel *et al.* (2004) identified 246 genes that responded to the recognition of flagellin by the FLS2 receptor. These genes included transcription factors, protein kinases and flg22-rapidly elicited (*FLARE*) genes.

1.5.2 Effector-triggered immunity (ETI)

Through evolution the plant is able to adapt to the pathogen and in return, the pathogen devises new strategies to overcome the plants defence, this process is known as the arms race. Successful pathogens are able to breach the PTI defences whereby they release effector molecules into the host, also termed avirulent proteins, repressing PTI. Bacteria deliver the effectors by the type three section system and exocytosis is the mechanism employed by fungi and oomycete to release their effectors (Göhre and Robatzek, 2008).

The plant in return has hence developed Effector-Triggered Immunity (ETI, vertical response) which is the direct or indirect response to effector molecules and the gene-for-gene recognition of the pathogen (Glazebrook, 2005). This is the host-specific recognition where the effector molecules from the pathogen interacts with the host's R protein (Flor, 1971). Hence, a plant that possesses a corresponding *R* resistance gene (*R*) is resistant to the pathogen (Figure 1.4). In the indirect recognition, the R protein monitors or guards a protein/product (guardee) that is the actual target of the effector molecule. Once changes in the guardee are observed, resistance is activated This model is called "the Guard hypothesis" (Jones and Dangl, 2006).

1.5.2.1 The functions of *R* genes

R genes are broadly expressed in the genome. The protein structure is composed of NOD-like and Toll-like receptors. The majority of the *R* genes belong to a class of nucleotide-binding site leucine rich repeats (NBS-LRR) represented by 150 genes in *Arabidopsis* (reviewed by Dangl and Jones., 2001). The interaction between avirulent genes and *R* genes causes immediate activation of defence pathways and restricts growth of the pathogen. Associated with *R* gene recognition is an oxidative burst, initiated through reactive oxygen species (ROS), which is a group of defence signalling molecules, and the activation of systemic acquired resistance (Lamb and Dixon, 1997; Mehdy, 1994). Following the accumulation of ROS is the hypersensitive response (HR), a rapid programmed cell death, occurring at the site of pathogen invasion, that restricts the pathogen (reviewed by Boller and Felix, 2009; Jones and Dangl., 2006). HR is one of the first steps in the signalling pathway of ETI and the rising toxic intermediates result in the death of the plant cell and the pathogen. It is speculated that HR perhaps also functions in long-range signalling (Coll *et al.*, 2011).

Apart from responses to the local detection of a pathogen, the plant is able to activate signalling pathways that establish enhanced defences in organs distant from the primary attack site. The perception of MAMPS/PAMPS or DAMPS, therefore, results in a systemic response (Liu *et al.*, 2010). Systemic response sustains long-lasting resistance to pathogens, a state known alternatively as systemic acquired resistance (SAR) triggered by abiotic and biotic elicitors, induced systemic resistance (ISR) associated with rhizobacteria and systemic induced resistance (SIR, Figure 1.5). All three pathways are forms of induced resistance in plants that prime the plant against subsequent challenges by pathogens.

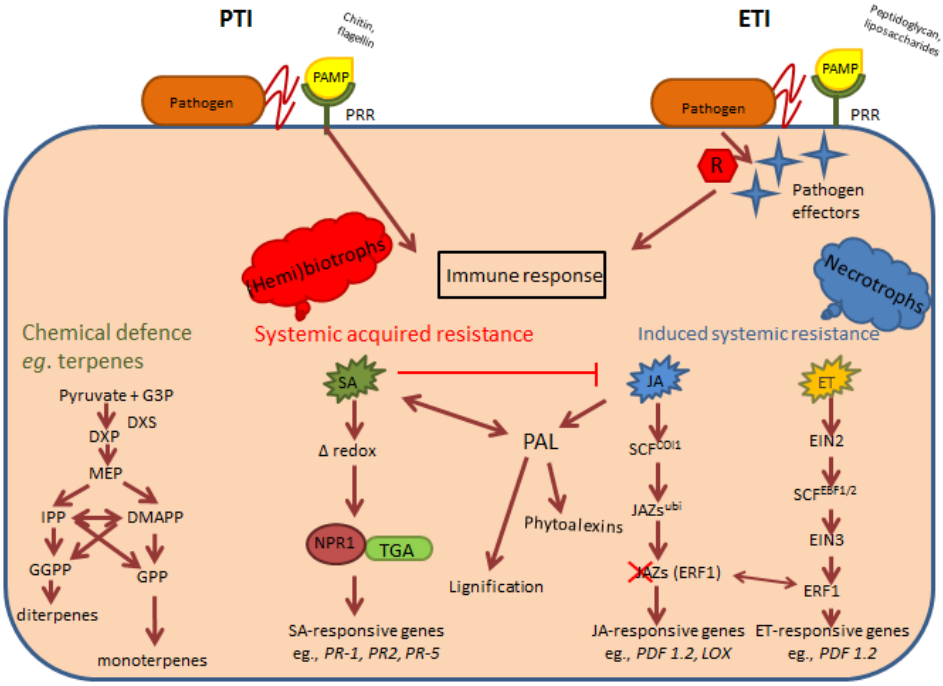

Figure 1.4: Schematic representation of induced resistance (adapted from Vallad and Goodman, 2004 and Pieterse *et al.*, 2009) In this figure, PAMP-triggered immunity (PTI) is activated through the pathogen-associated molecular patterns such as chitin and flagellin. These are then recognized by the pattern recognition receptors (PRRs). Through evolution the pathogen has developed mechanisms, whereby they release effectors to breach the host defence systems. Due to the arms race the host has evolved R-proteins which are able to recognize and suppress the effectors secreted by various pathogens. This interaction between the effector molecules and the R-proteins is known as Effector-triggered immunity (ETI) represented on the right of the figure. Shown here are the specific pathways for (Hemi)biotrophs involving the salicylic acid (SA) pathway activating the nonexpressor of the pathogenesis related protein 1 (NPR1). The binding of the monomeric NPR1 to TGA transcription factor leads to the activation of SA-responsive genes. In the Jasmonic acid (JA) signalling the SCF^{COI1} complex and the jasmonate ZIM-domain (JAZ) form a complex that gets ubiquitinated and degraded upon JA accumulation. This is followed by the activation of JA-responsive genes. In the ethylene (ET) signalling cascade, the hormone gets recognised by the negatively regulated receptor ETR1. The perception of ET then repressed the positive regulator EIN2. The degradation of the $SCF^{EBF1/2}$-dependent 26S proteosome is stopped and EIN3-like transcription factors are activated. Downstream the ERF1 transcription factors leading to the expression of ET-responsive genes, are being positively regulated. Secondary metabolites, representing the chemical induced response, like terpenes, are derived from the methylerythritol phosphate pathway. Pyruvate and guanosine diphosphate (GDP) in combination with 1-deoxy-D-xylulose-synthase (DXS) leads to the activation of methylerythritol phosphate (MEP). Its function is to produce isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The geranylgeranyl phosphasphate (GGPP) and geranyl pyrophosphate (GPP) are intermediates. Both pathways are required for the production of mono- and diterpenes.

1.5.3 Systemic acquired resistance (SAR)

The plant's response to local attack by pathogens occurs both in the originally infected plant organ and in distantly located, yet uninfected parts. The systemic response provides the plant with long-lasting resistance to invaders (Figure 1.5). SAR and ISR can be differentiated on the basis of the elicitors and regulatory pathways involved. By challenging the plant with virulent, avirulent or non-pathogenic microbes, or with chemicals, such as salicylic acid, 2,6-dichloro-isonicotinic acid (INA) or benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), the SAR pathway is activated (Ryals *et al.*, 1996).

The synthesized plant hormone salicylic acid (SA) is responsible for various functions in the plant such as flowering and thermogenesis but also plays a major role in resistance (Wildermuth, 2006, Vlot et al., 2009). The latter function has been well studied in plants such as *Arabidopsis thaliana*. This hormone is closely associated with SAR. Two pathways are responsible for synthesizing SA namely phenylalanine ammonia lyase (PAL) and isochorismate (ICS). The leading signal to activate SAR seems to be methyl salicylate (MeSA), whereby SA is converted to MeSA and this then gets transported to distal parts of the plant (Mauch-Mani and Metraux, 1998; Ryals *et al.*, 1994).

NPR1 (*Nonexpressor of PR genes 1*) is a key regulator of the SA-pathway downstream of MeSA and therefore results in the activation of SAR. SA, the key hormone associated with SAR, facilitates changes in the cellular redox potential (Figure 1.5), causing the reduction of disulfide bonds in cysteine residues of *NPR1* in the cytoplasm. This results in the release of *NPR1* monomers from the oligomer. Once these activated monomers are localised in the nucleus they interact with TGA sequence specific transcription factors thereby resulting in the expression of pathogenesis-related (PR) proteins (Koornneef and Pieterse, 2008). The high molecular weight oligomers remains in the cytoplasm (Beckers and Spoel, 2006). It is suggested that the nuclear NPR1 causes the activation of the PR-genes but in contrast the cytosolic NPR1 monomer functions in inhibiting the JA signalling (Pieterse *et al.*, 2009).

The expression of the *PR* genes contributes to the enhanced state of resistance. These PR proteins have antimicrobial, as well as antifungal properties. To date, 17 families of PR proteins have been described (van Loon *et al.*, 2006). *PR* genes can be used as markers to identify the activation of SAR defences. Well-known *PR* genes include *PR-1*, *β-1,3-glucanases* (*PR-2*), *chitinases* (*PR-3*), *hevein-like* (*PR-4)*, *thaumatin-like* (*PR-5)* and *osmotin* (Stricher *et al.*, 1997). In conjunction with the activation of SAR the genes *PR-1, PR-2* and

PR-5 specifically serve as markers to verify the onset of SAR (Cao *et al.*, 1994; Delaney *et al.*, 1994; Uknes *et al.*, 1992).

1.5.3.1 The agricultural perspective on SAR

The exciting aspect for the agricultural sector lies in the induction of SAR via exogenous application of inducing compounds providing the plant with a form of protection to subsequent pathogen attacks (Sticher *et al.*, 1997). Sticher *et al* (1997) gave three criteria that identify a SAR inducer. Firstly, the agent should only have indirect antimicrobial activity, secondly, the agent must be able to build resistance against a broad range of pathogens and thirdly, one must be able to detect the up-regulation of defence marker genes expressed during SAR pathway. There are a number of compounds that conform to these three criteria. The well-known and studied inducers specifically for the SAR pathway are salicylic acid, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH, Durrant and Dong, 2004). Phosphate salts and silicon are inorganic compounds shown to activate SAR in cucumber, bean and maize (Datnoff *et al*., 2005). Another agent includes the bacterial *hrp* gene that induces resistance in non-host plants. Hrp has been used on cucumbers where it activated *PR*-genes (Smith *et al.*, 1991). Propenazole and β-aminobutyric acid (BABA) have also been used as inducers to enhance resistance (Sticher *et al.*, 1997). Although these chemicals have benefits over the use of fungicides, they are subject to critical limitations. These chemicals are phytotoxic when applied at high amounts to the plant. The concentration of application is a factor which will be one of the hurdles to overcome.

Resistance provided by SAR is defined as broad spectrum and long lasting but it does not necessarily ensure complete control of the infection. SAR inducers may provide effectives in reducing the disease between 20% to 90%. For example a reduction of 89% of powdery mildew was achieved in barley treated with phosphate (Mitchell and Walters, 2004). Such decrease in disease severity correlates to a decrease in mortality as more plants are able to survive. In most studies a reduction in lesion number as well in lesion size is observed in the *Pinus-F. circinatum* interaction (Bonello *et al*., 2001; Reglinski *et al*., 2004). This variation in effectiveness may vary even more once these inducers are applied in the field as there are environmental factors, genotypic variance as well as nutritional aspects which influence the effect of the induced resistance (Walters *et al.*, 2005). To maximize the efficiency of induced resistance better understanding of defence is required and understanding how to best identify optimal disease control strategies.

1.5.4 Induced systemic resistance (ISR)

In addition to the SA-dependent defence response, there are other signalling molecules associated with the second type of systemic resistance (Figure 1.5). The signalling molecules that activate induced systemic resistance (ISR) are jasmonic acid and ethylene. Induction of this pathway typically occurs by the plant growth promoting rhizobacteria (PGPRs, Latha *et al.*, 2009; van Wees *et al.*, 1997; Yan *et al.*, 2002). Examples of such PGPRs are *Bacillus* spp. and *Pseudomonas* spp. that are associated with improved plant growth and health. PGPRs can be referred to as biocontrol agents, minimizing plant disease by competing for nutrients and space with the pathogens, as well as initiating plant defence. Compared to systemic acquired resistance, this type of resistance does not show the onset of the SAR *PR* marker genes, but instead is characterized by induction of cysteine-rich plant defensins (PDFs) and thionins. As with SAR, there are marker genes such as *PR-3, PR-4, Thi2.1 (PR-13)* and *PDF1.2 (PR-12*) for the ISR pathway.

The plant hormone jasmonic acid is synthesized from linoleic acid (Vallad and Goodman, 2004). The signal is perceived in distal part of the plant by jasmonoyl-isoleucine (JA-Ile). This amino acid is recognised by the COI1 protein. The association of COI1 and the *Arabidopsis* Skp1-like1, Skp1-like2, cullin 1 and a ring box protein 1 lead to the formation of the SCF^{COI1} complex. The complex together with the jasmonate ZIM-domain (JAZ) form a complex that gets ubiquitinated and degraded upon JA accumulation. This is followed by the activation of JA-responsive genes like *PR-3, PR-4, LOX (*Vallad and Goodman, 2004; Pieterse *et al.*, 2009).

The second plant hormone associated with ISR is ethylene. Ethylene has been shown to be involved in germination, flowering and plant defence. For the latter function the hormone gets recognised by the negatively regulated receptor ETR1. The perception of ET then represses the positive regulator EIN2. The degradation of the $SCF^{EBF1/2}$-dependent 26S proteosome is stopped and ethylene insentive (EIN3)-like transcription factors are activated. Downstream the ERF1 transcription factors leading to the expression of ET-responsive genes, are being positively regulated. This $SCF^{EBF1/2}$ complex is very similar to the JA SCF^{COI1} complex showing close association between the two hormones *(*Vallad and Goodman, 2004; Pieterse *et al.*, 2009).

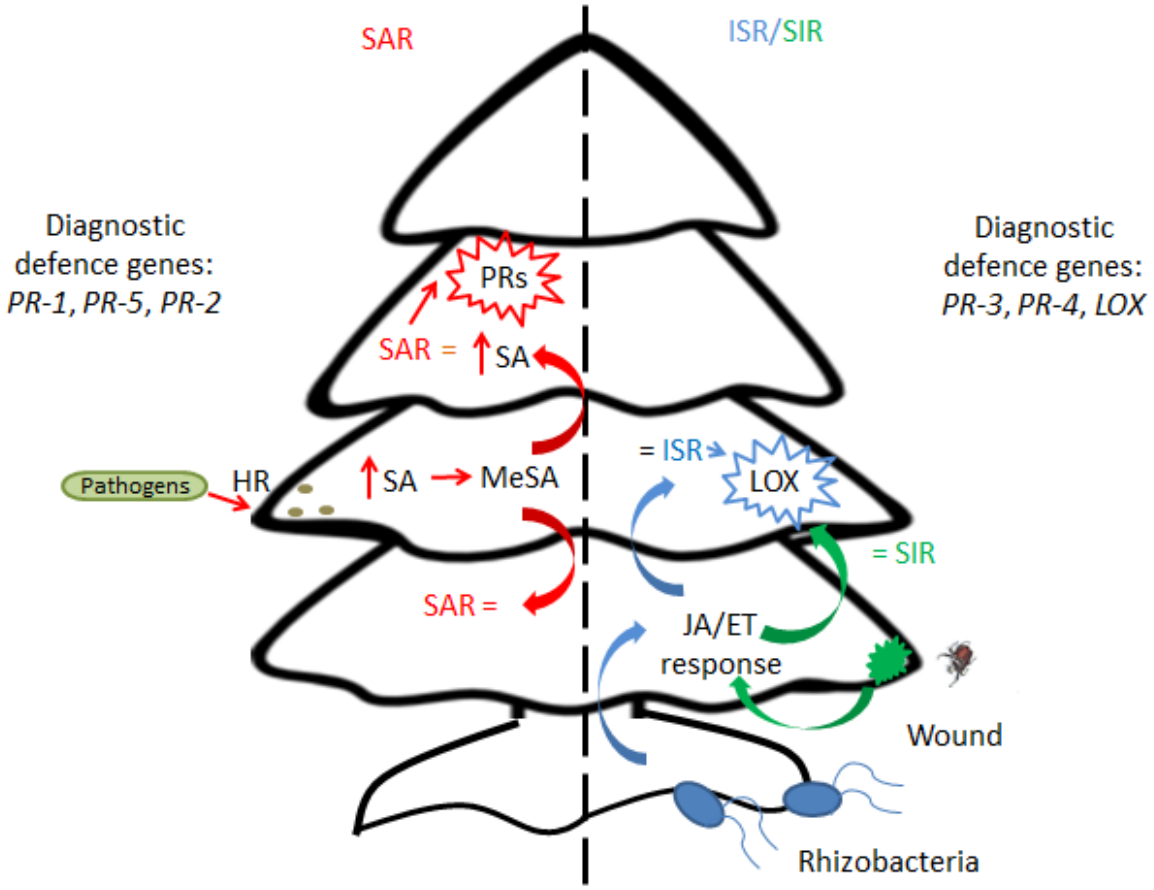

Figure 1.5: Representation of systemically induced resistance (adapted from Pieterse *et al.*, 2009). Systemic acquired resistance is activated locally the hypersensitive response (HR) caused by (hemi)biotrophs and leading to an increase in salicylic acid. This hormone gets converted to the signal MeSA. Transport of the signal to distal tissue leads to the onset of SAR, which is required for the activation PR proteins. Colonization of the roots by rhizobacteria causes the onset of induced systemic resistance (ISR). The long distance signalling molecules for ISR are jasmonic acid (JA) and ethylene (ET). This pathway activates defence genes such as *LOX*, *PR-3* or *PR-4*. SIR is triggered by a wounding response and leads to an increase in the hormone levels of ET and JA.

1.5.4.1 Function of NPR1 in the JA signalling

For the plant to distinguish between the two pathways SAR/ISR, NPR1 functions in the cross-talk between the two pathways. The cytosolic NPR1 monomers inhibit JA signalling and the model identified suggests that the monomers repress the transcription of JA biosynthesis genes (*Defective in anther dehiscence1 (DAD1), lipoxygenase 2* (*LOX2)*, allene oxide synthase (*AOS), 12-oxophytodienoate reductase 3* (*OPR3),* Beckers and Spoel, 2006; Devoto and Turner, 2003). The NPR1 prevents the targeting of the SCF^{COI1} complex

to repressors of the JA-defence genes. Inhibition of those genes occurs through ubiquination followed by degradation by the 26s proteasome. Normally once biosynthesis of JA has occurred, the hormone is perceived by receptors of the SCF^{COI1} complex. The complex consists of proteins, SKP1, COI1 and cullin, which function in targeting repressor proteins for the degradation of JA genes (Beckers and Spoel, 2006). If JA responsive genes (*PR-3, PR-4, Thi2.1 (PR-13)* and *PDF1.2 (PR-12*) are activated the up-regulation of the genes can be used to identify the onset of ISR (Penninckx *et al.*, 1998; Thomma *et al.*, 1998).

1.5.5 Systemic induced resistance (SIR)

SIR, known as a wounding response or systemic induced resistance is activated through mechanical wounding or herbivores. It is closely linked to induced systemic resistance as it also leads to the increase in the hormones ethylene and jasmonic acid (Gurr and Rushton, 2005). SIR is elicited through biotic and abiotic responses. In contrast, ISR is triggered through plant growth-promoting rhizobacteria (van Loon, 2007).

1.5.6 Mechanism of defence against biotrophs and necrotrophs

Plant defence responses are tailor-made specifically for the different types of pathogens. The question now arises as to where SAR and ISR fit in connection with the different pathogens. Pathogens can be categorised into biotrophs, hemibiotrophs and necrotrophs according to their lifestyle. Biotrophs live and multiply on other living organisms (Agrios, 2005). This lifestyle is dependent on living tissue, making the *R* gene resistance and the SA signalling the suited strategies for the plant to defend itself (Glazebrook, 2005). Due to the occurrence of the hypersensitive response, the plant cells that are attacked by the pathogen die off and consequently, the pathogen has no food source. An example given in the literature for biotrophs is the oomycte, *Hyaloperonospora parasitica* in *Arabidopsis thaliana.* The defence in *Arabidopsis* was boosted through external application of a SA derivative compound and was successful against *H. parasitica* in *Arabidopsis* (Thomma *et al.*, 1998).

Necrotrophs, like the *F. circinatum* fungus, are microorganisms that feed on dead organic tissue. To employ the latter strategy against necrotrophs would not be successful as they are not dependent on living tissue, instead this strategy would provide the pathogen with more favourable conditions. The defence pathways that are activated associated with necrotrophs are the ET/JA pathways. It was shown while studying the defences against *Alternaria brassiciola*, a known necrotroph in *Arabidopsis*, that resistance was enhanced by the JA

pathway and camalexin, a major phytoalexin. *Arabidopsis* mutants with defects in the SA-pathway (*npr1, pad4, sid2*) showed no enhanced susceptibility against *A. brassiciola*, and likewise for the *ein2* mutant, representing the ethylene pathway (Thomma *et al.*, 1998; van Wees *et al.*, 2003).

Hemibiotrophs are pathogens that live parasitically on a host for a time and then become necrotrophic for the rest of its life-cycle. The focus for strategies to combat the pathogen is during the biotrophic phase as it is in this stage that infection occurs (Bailey *et al.*, 1992). It is important to remember that sometimes it is not always as simple as just applying one defence strategy as there is an array of defence pathways involved. Literature has shown that there is, for example, an antagonistic behaviour between JA and SA at higher concentrations, but both hormones present at low concentrations at the same time actually displayed a synergistic behaviour (Mur *et al.*, 2006).

1.5.7 What is known about pathogen defence in conifers

Most of the information established regarding plant resistance has been gathered from model plants such as *Arabidopsis* and tobacco. Extrapolating from one plant type such as annuals to another plant type (perennials) is not always easy as each of them have different characteristics. Annuals have a one year life-cycle and utilize most of their energy for growth and fecundity (Herms and Mattson, 1992). These high costs affect the energy that is put into resistance. Perennial plants such as shrubs and trees live longer and face more challenges than annuals. Because of that perennials have higher defence levels compared to annuals (Zangerl and Bazzaz, 1992). Little information exists as to the comparison between defence in angiosperms and gymnosperms. Perhaps as the study from Germain *et al*. (2012) suggests, we may not expect that much of a difference. It was found that *Arabidopsis* transformed with *Picea glauca defensin 1* (*PgD1*) showed stable expression. This indicated that the promoter possessed the same elements as in *Arabidopsis* therefore suggesting a degree of conservation in terms of plant defence between angiosperms and gymnosperms (Germain *et al*., 2012).

It is known that trees have constitutive and inducible defence mechanisms which prevent access into the plant and either kill or limit pathogens. The characteristic of constitutive defence is that it responds at all times. It has been found that tissue attacked by herbivores have increased constitutive defences compared to less attacked tissue (Eyles *et al.*, 2009). However, if these defences are overcome by the pathogen, inducible defence mechanisms

are triggered. The latter defence mechanisms can be broken down into various categories according to their function. The production of secondary metabolites, defined by their pathways from which they are synthesised, is the inducible chemical defence. These secondary metabolites have antifungal as well as antimicrobial properties and examples are phenolic compounds and terpenoids e.g. resin (Eyles *et al.*, 2009). The second category is called the protein-based defence. Under this category the well known PR proteins, which also display antifungal and antimicrobial activities, are induced. Mechanical and structural barriers achieved through processes such as lignification, callose deposition and isolating the infected tissue from health tissue are defined as inducible anatomical defence (Eyles *et al.*, 2009). Lastly the plant has the ability to redirect biological processes to healthy tissue in order to compensate for negative fitness caused by tissue damage. This is referred to as induced civilian defence (Eyles *et al.*, 2009).

In 2000, a study revealed that the application of non-pathogenic rhizobacteria acted as an inducing agent and ISR was established in loblolly pine (*P. taeda*) against fusiform rust (*Cronartium quercuum* f. sp. *fusiforme*) (Enebak and Carey, 2000). Bonello *et al.* (2001) reported for the first time the presence of systemic induced resistance in conifers, specifically pine. It was shown that repeated mechanical inoculation of *P. radiata* with *F. circinatum* results in smaller lesions. Furthermore, it was identified that pine stands, which had been naturally infected with *F. circinatum* were less severely affected by disease development and subsequent challenges with the pathogen revealed that the pine trees were able to survive the pathogen (Gordon, 2006; Gordon *et al.*, 2001). Repeated inoculation was not only an effective approach against *F. circinatum,* but also against *Diplodia pinae* and *D. scrobiculata* in Austrian pine (*P. nigra*) (Blodgett *et al.*, 2007; Wallis *et al.*, 2008). Chemical treatment with salicylic acid derivatives, which would trigger the activation of SAR, enhanced resistance against the fungus *Sphaeropsis sapinea*. This pathogen is responsible for shoot blight, crown wilt; canker and sap stain on a number of conifer species. This resistance remained active for up to a month (Bonello and Blodgett, 2003; Reglinski *et al.*, 1998). In 2004, Reglinski *et al*. conducted a study where spraying *P. radiata* seedlings with chitosan showed improved resistance to *F. circinatum,* the disease incidence was curbed by 60%, but this was dosage dependent, and the effectiveness of chitosan was evident for four years following application. Long-term effects of methyl jasmonate on Scots pine (*P. sylvestris*) suggest that the production of terpenoids reduces the performance of sawflies, in reducing their growth rate (Heijari *et al.*, 2008).

Conifers are able to contain pathogens through the production of secondary products such as terpenoids, phenolics and lignin. It is important to understand that the process of defence

induced by biochemical agents is highly co-ordinated and therefore involves not only the production of PR proteins, but also lignification, terpenoids and other protein defences (Bonello *et al.*, 2006; Keeling and Bohlmann, 2006; Vallad and Goodman, 2004). Increased terpenoid production and lignification were observed either through methyl jasmonate treatment or in response to insect attack (Heijari *et al.*, 2008; Hudgins and Franceschi, 2004; Wallis *et al.*, 2008). Zulak and Bohlmann (2010) described the accumulation of terpenoids in cortical resin ducts and in developed xylem trauma-associated resin ducts as being the constitutive defence of pines. In light of the recent events in respect to induced resistance as a means to control diseases in conifers and the understanding of the host-pathogen interaction more appropriate disease control strategies can be implemented.

1.5.8 The host-pathogen interaction (*Pinus* – *Fusarium*)

Several studies have been done to investigate pine genes induced through infection by the necrotrophic pathogen *F. circinatum*. Construction of cDNA arrays were used to investigate which tree genes are influenced by the pathogen attack (Morse *et al.*, 2004). The study performed by Morse *et al.* (2004), revealed 29 cDNAs that responded to the pathogen. It was postulated that necrotrophic pathogens cause up-regulation of the jasmonic acid and ethylene pathway in pine trees. It was suggested that the up-regulated genes could be associated with defence responses.

When studying primary defence responses, specifically in *P. patula* after inoculation with *F. circinatum,* no levels of chitinase activity were detected up to seven days (Venter, 2004). Chitinase breaks down the cell wall structures of the fungus by hydrolysing chitin, as the cell walls of fungi are composed of chitin. The increased production of chitinase in plants is a sign of defence against pathogens. These chitinase enzymes can be induced through a variety of environmental factors or even through pathogens. The lack of induction of chitinase may be one explanation for the susceptibility of *P. patula* to pitch canker, especially in seedlings. However, the genes PSCH14 and LP6 (chitinase homologs) have been proven to be present in the *Pinus* species (Venter, 2004; Davis *et al.*, 2002; Wu *et al.*, 1997). Furthermore, 80% - 99% similarity in sequence of those two genes was found by comparing orthologs in *P. patula*, *P. taeda* and *P. strobes* (Venter, 2004). Venter (2004) came to the suggestion that the tree is more dependent on the β-1,3-glucanase induction for defence, since chitinase is not produced. β-1,3-glucanases are hydrolytic enzymes which inhibit the growth of the fungi and degrade the cell wall of the pathogen, resulting in the release of elicitors and activation of defence pathways (Sela-Buurlage *et al.*, 1993).

Other genes related to stress were up-regulated in response to *Fusarium circinatum* in *P. patula* (Morse et al., 2004; Venter, 2004). One of those stress related genes was UDP-glucose pyrophosphorylase, which functions in the formation and deposition of new parenchyma after wounding. It was suggested that wood formation, such as lignification of parenchyma cells contributes to resistance in *Pinus* (Barrows-Broaddus and Dwinell, 1983; Krokene *et al.*, 1999). Other studies have led to the same conclusion (Barrows-Broaddus and Dwinell, 1984). Another gene that was up-regulated was the blue copper protein (BCP). This protein belongs to the redox-active proteins which are only found in plants (Nersissian *et al.*, 1998). Their function lies in defence responses, oxidative stress responses (Nersissian *et al.*, 1998) and lignin formation in cell walls (Zhang *et al.*, 2000). Furthermore, two genes connected to metal detoxification were found to be induced by infection with *F. circinatum* (Venter, 2004). In the study performed by Venter two genes were isolated that could be encoded by the fungus (Venter, 2004). One of the genes is the glucose repressible gene 1 (*GRG1*) known to be involved in switching on genes important for fungal survival. The other, folic acid helps in the transfer, reduction and oxidation of one carbon units, and it is required for the production of thymidine, methionine and purines (Ouellette *et al.*, 2002). When *Pinus* resistance genes were compared, it was found that several of the conserved regions shared high similarity with other plants NBS motifs. NBS, nucleotide binding site leucine-rich repeat domains, form the largest group of resistance genes and have been used to identify homologs. Slight differences were identified in the P-loop and the GLPL motifs compared to the analogs of *Arabidopsis thaliana* indicating differences between angiosperms and gymnosperms (Venter, 2004).

1.6 Inducer application as a means to improve resistance

Knowledge of the life-cycle of the pathogens, their host preference and their preferred environmental conditions aid in developing an appropriate control system. Thus far the emphasis has been on developing resistant plant varieties and the use of proper nursery hygiene. The use of fungicides through sprays or other types of applications on seeds or soil is another control method (Agrios, 2005). However, pathogens have become resistant to these chemical weapons and the environmental hazard of fungicides makes it less feasible. Disease management strategies have been redirected towards looking for alternative ways to curb the pitch canker disease (Obradovic *et al.*, 2005). One such approach is to induce disease resistance by using abiotic and biotic elicitors. Table 1 is a list of the plant resistance activators applied on various plants to curb diseases.

Table 1.1: Inducers previously identified to activate SAR and ISR pathways

Inducer	Pathway activation	Host	References
Messenger® (harpin protein)	SAR	Vegetables, *Citrus paradise* (grapefruit)	(Graham and Leite Jr., 2004)
Chitin (Crab shell)	SAR	*Malus domestica* (apple), *Lycopersicon esculentum* (tomato)	(Dietrich *et al.*, 2004; Kombrink *et al.*, 2011)
Bion (Benzodiathiazole derivative)	SAR	Leafy vegetables, *Nicotiana benthamiana* (tobacco), *L. esculentum* (tomato), *Lactuca savita* (lettuce), *Spinacia oleracea* (spinach), *P. radiata*	(Dietrich *et al.*, 2004; Hofgaard *et al.*, 2005; Reglinski and Dick, 2005)
Salicylic acid	SAR	*Arabidopsis thaliana*, *Prunus avivum* (cherry fruit), *P. radiata*	(Reglinski and Dick, 2005; Yao and Tian, 2005)
Monobasic potassium phosphate	SAR	*P. radiata*	(Ali *et al.*, 1999; Reglinski and Dick, 2005)
***Fusarium oxysporum* (non-pathogenic)**	ISR	*Ocimum basilicum* (basil), *L. Esculentum* (tomato)	(Belgrove, 2008)
Methyl jasmonate	ISR	*Picea abies* (Norway spruce), *P. radiata*	(Erbilgin *et al.*, 2006; Mikkelsen *et al.*, 2003; Wen Hui *et al.*, 2004; Heijari *et al.*, 2008; Reglinski *et al.*, 2008)
Flagellin	ISR	*A. thaliana*, *L. esculentum* (tomato)	(Felix *et al.*, 1999; Gómez-Gómez *et al.*, 2000; Zipfel *et al.*, 2004)
***P. fluorescens* (non-pathogenic)**	ISR	*Pseudotsuga menziesii* (douglas fir)	(Reglinski and Dick, 2005)

1.6.1 Biologically and chemically derived inducers known from literature

Resistance towards the tobacco mosaic virus was achieved through the activation of SAR by treating the plants with salicylic acid (White, 1979). Another application of SA was done on pre-harvested and post-harvested sweet cherry fruits. Results gathered indicated that the pre-harvest treatment against *Monilinia fructicola* was more successful than post-harvest treatment (Yao and Tian, 2005). Cross-talk occurs between the SA - and JA – pathway. This observed cross-talk results in an inhibition of the JA-pathway. NPR1, the key regulator of the SA-pathway (Koornneef and Pieterse, 2008), hinders the JA/SA cross talk by negative regulation of SA production. The study performed by van Wees *et al.* (2000) has given evidence for an additive effect occurring by simultaneously activating both pathways. For SAR activation Col-O *Arabidopsis* was challenged with the avirulent *Pseudomonas syringae* bacteria. ISR was activated using *P. fluorescens*. Plants treated with a combination of both inducers showed a high reduction of disease severity. It did not seem to matter which inducer was applied first. Further analysis showed that no elevated NPR1 occurred when both types of resistances were activated (van Wees *et al.*, 2000). There are two hypothesised explanations for the mechanism underlying the additive effect. Model 1 shows that both pathways produce different defensive compounds. The other possibility, model 2, is that the simultaneous activation of SAR and ISR causes the production of identical antibacterial compounds. In the separate induction of the pathways these antibacterial compounds did not reach that maximum level. This highlights the usage and effectiveness of the inducers. Resistance of plants can be induced in several ways by using nematodes, insects, endophytes, PGPR, plant-associated microbes and exogenous application of inducers (Schreiber and Desveaux, 2008). There are many inducers which have been tested in previous research, however, only a selected number will be discussed further.

Chitosan

The walls of fungi are made of components such as glucan, chitin and chitosan (Reglinski and Dick, 2005). Poly-D-glucosamine, also known as chitosan, binds to fungal receptor sites and causes the activation of defence response. This process is similar to the recognition of fungal spores that bind to the receptor, inducing resistance. This initiates the defence signalling cascade. Further down this cascade are the phytoalexins, which are antimicrobial compounds, that inhibit fungal infection (Copping and Duke, 2007; Hadwiger and Beckman, 1980). Foliar application of chitosan to *P. radiata* showed the effectiveness of the inducer against *F. circinatum (Reglinski et al., 2004)*. The observation was made that protection of

the seedlings was insured by 100 spores per plant and 86% reduction of lesion length was identified. Challenges of 500 spores or more produced no disease control. Chitosan efficiency seemed to be inoculum-dose dependent. This proves the ability of chitosan to induce systemic disease resistance (Reglinski *et al.*, 2004). In *Arabidopsis,* chitosan treatment has been studied by performing a microarray analysis, whereby it was shown that several defence response genes and the camalexin biosynthesis genes were up-regulated (Povero *et al.*, 2011). Camalexin is one of the phytoalexins produced against abiotic stresses. Also chitosan has proven to inhibit the growth of the hyphae and inhibit fungal spores to germinate (Amborabé *et al.*, 2008; Benhamou and Thériault, 1992; El Ghaouth *et al.*, 1994). In terms of physical defence, chitosan aids in strengthening plant cell walls through lignification and increasing callose formation (Conrath *et al.*, 1989; El Hadrami *et al.*, 2010; Pearce and Ride, 1982). These key functions of chitosan attributed to the wide range of usage and interest it has received as an inducer.

Bion® (benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester)

Bion® is a commercially available product selected to activate plant resistance. It is a functional analogue of SA and therefore is translocated systemically and induces the SAR defence signalling pathway. In crops it has been shown to be effective against a wide range of pathogens such as fungi, bacteria and viruses. Interestingly, it seems that when applied on monocots Bion® has a longer lasting effect compared to dicots (Oostendorp *et al.*, 2001). Bion® not only directly induces elicitor-induced genes but also enhances their expression (Conrath *et al.*, 2001; Métrauxs, 2001; Thulke and Conrath, 1998). Thulke and Conrath (1998) treated parsley cells with SA analogues and found the potentiated elicitation of phenylpropanoid genes and PR-10 compounds that promoted plant defence resistance. Bion® showed positive effects against root rot infection caused by *Phytophthora cinnamomi* in *P. radiata* and in combination with potassium phosphate (1 g/L) it was even more effective in enhancing defence *(Ali et al., 2000).*

Messenger® (bacterial harpin protein)

Messenger® is another commercially available product, containing 3% harpin protein. These proteins originally derived from *Erwinia amylovora* and are acid heat-stable, cell envelope-associated proteins (Eyles *et al.*, 2009). Harpins are associated with the type three secretion system in pathogens, eliciting a hypersensitive response, followed by systemic defence responses. The type three secretion system is a means for the pathogens, specifically bacteria to move effectors through the plant intercellular space into the plant cell. It is

hypothesised that the harpins may aid in effector delivery (Alfano and Collmer, 2004). Messenger® further improves yield and quality of treated plants (Jones, 2001). Crops sprayed with Messenger® showed improvements in biomass, photosynthesis, nutrient uptake and root development, in general improving the plants' fitness (Wei and Betz, 2007). Harpin has an associated pattern recognition receptor called HrBP1 found in *Arabidopsis,* through which it is recognised (Chinchilla *et al.*, 2006; Oh and Beer, 2005). Studies revealed that Messenger® works even more effectively in combination with either potassium phosphonate or agriphage, when curbing disease severity of *Xanthomonas campestris pv. vesicatoria,* for example (Obradovic *et al.*, 2005). In 2001, Messenger® was awarded the Presidential Green Chemistry Award by the United States Environmental Protection Agency, recognising its potential benefits.

Flagellin

Flagellin comes from gram-negative bacteria and is part of the flagellum. It is one of the most typical PTI triggering agents. Together with the harpin protein and chitosan, flagellin plays a key role in PAMP/MAMP immunity (Jones and Dangl, 2006). Flagellin induces callose deposition, another type of plant defence response (Verma and Hong, 2001). In *Arabidopsis,* the LRR receptor-like kinase FLS2 was identified to be important for recognising flagellin. When flagellin was applied to *Arabidopsis*, production of active oxygen species and ethylene were observed, indicating the activation of defence responses (Gómez-Gómez *et al.*, 2000; Zipfel *et al.*, 2004).

Pseudomonas fluorescens

Fungicides and other pesticides bring side effects such as copper residues in the soil and scrutinize the usage of such products. Therefore, biological control agents are alternatives suggested to controlling diseases. By providing a wide range of antibiotic metabolites *Pseudomonas fluorescens* is effective against a broad range of pathogens. Examples of metabolites produced by *P. fluorescens* include pyoluteorin, toxic against oomycetes, 2,4-diacetylphlororglucinol, which protects against fungi and bacteria, and pyrrolnitrin which has affects against the ascomycetes (Howell and Stipanovic, 1980; Shanahan *et al.*, 1992). *Pseudomonas fluorescens* is one of the biocontrol agents proven to moderately improve the resistance against *Fusarium* crown and root rot of tomato (Duffy and Défago, 1997). In combination with zinc and copper, the activity of the biocontrol agent was significantly increased. Another example is the use of *P. fluorescens* for infection of Douglas fir seedlings

with *F. oxysporum*. The treatments were performed by either soaking the seeds or the roots of the seedlings in a bacterial suspension (Reddy *et al.*, 1997). Increased activity of *phenylalanine ammonia lyase, peroxidase, chitinase, β-1,3-glucanase* and the accumulation of phenolics in banana after treatment with *P. fluorescens* suggest enhanced bioprotection against *F. oxysporum* (Thangavelu *et al.*, 2003).

Methyl jasmonate

Methyl jasmonate is a volatile chemical which functions as an airborne signal (Kessler and Baldwin, 2001). It is a plant hormone, produced in the chloroplast, peroxisome and the cytosol, and is important for signalling during development, reproductive processes and for alerting distal parts about stress responses and pathogen attacks (Matthes *et al.*, 2008; Schaller *et al.*, 2004). For signalling to occur, jasmonates are recognised by the COI1 (Coronatine-insensitive 1) gene, a key regulator in the JA signalling pathway, which then causes an interaction with JAZ (jasmonate ZIM-domain) proteins. The JAZ proteins get degraded, allowing for expression of JA-inducible genes (Chini *et al.*, 2007; Staswick, 2008; Thines *et al.*, 2007). Thorpe *et al.* (2007) studied the transport of jasmonates in tobacco plants and found that the transport occurs in the phloem and xylem. The volatility of methyl jasmonate seems to further increase the exchange between the phloem and the xylem. Investigations were done on the long-term effects of exogenous application of methyl jasmonate. It was studied whether the number of sawflies was reduced and if the long-term application had any effect on the growth of *Pinus sylvestris*. In the experiment, 14 - year - old pine trees were used. Observations were performed over a period of three years. In the first year, methyl jasmonate was applied twice. In the second and third year three booster treatments were performed. High concentrations of terpene or high resin acid were found. A low-concentration of the exogenous inducer seemed to affect the production of terpernoids and did not influence growth of the pine trees (Heijari *et al.*, 2008). Conifers seem to use terpene compounds for defence against pathogen attack. This was also emphasised in an experiment performed to reduce bark beetles in Norway spruce (Erbilgin *et al.*, 2006). The methyl jasmonate treated bark of spruce showed increased terpenoid resin which was linked to the observed decreased colonization of the bark beetles.

Monobasic potassium phosphate

Potassium phosphate directly affects the various stages of a pathogen and also promotes wound healing. The most important functions it fulfils are the maintenance of the electrical gradient across cell membranes, generating turgor pressure and activating enzymes (Britto

and Kronzucker, 2008). Phosphorus speeds up the growth of the plant but also ensures the balance of nutrients in the plant. The ability of potassium phosphate to act as an inducer was shown with root rot (*Phytophthora cinnamomi*) of *Pinus radiata*. Foliar spray application of potassium phosphate suppressed dieback symptoms in the pine trees (Zahid *et al.*, 1999). Induction of systemic resistance was caused by potassium phosphate application and resulted in decreased rust infection in broad beans (Walters and Murray, 1992). Potassium phosphate seems to *activate phenylalanine ammonia lyase*, *peroxidase* and *lipoxygenase* in barley (Mitchell and Walters, 2004).

Salicylic acid (SA)

Salicylic acid is a plant hormone like jasmonic acid important for the activation of broad defence responses. It can either be produced from phenylalanine (PAL) or isochorismate synthase (ICS) , both originating from the metabolite chorismate from the shikimate pathway (Vlot *et al.*, 2009). The SAR signal moved to other parts of the plant (systemic) through the conversion of SA to methyl salicylate (MeSA). The function of MeSA was shown in tobacco plants that had the salicylic-acid-binding protein 2 mutant, which compromised systemic acquired resistance (Park *et al.*, 2007). Repeated exogenous application of SA in nine month intervals significantly improved the growth of *P. patula*, specifically root growth, stem diameter and height (San-Miguel *et al.*, 2003).

Fusarium oxysporum

Fusarium oxysporum (*Fox*) belongs to the group of filamentous fungi like *F. circinatum*. Pathogenic strains of *F. oxysporum* are the causal agent of root or other wilting diseases in for example tomatoes, bananas and asparagus (Berrocal-Lobo and Molina, 2008). On the other hand non-pathogenic strains of *F. oxysporum* can actually act as a biocontrol agents against fungi as they compete with the other pathogens for nutrients provided in the soil which hinders the germination of the chlamydospores of the pathogen. Furthermore, they do not only compete for nutrients, but also for gaining access to infection sites and then cause the activation of plant defence. Another advantage is also that large quantities of the non-pathogenic strains can be easily produced. *Fox* has been successfully used as a biocontrol agent to curb *Pythium* infection in cucumbers. The application of *Fox* leads to the onset of plant defence causing a reduction in pathogen viability (Benhamou *et al.*, 2002). Treatment was also effective against *Fusarium* wilt in tomatoes and carnations (Larkin and Fravel, 1999; Lemanceau *et al.*, 1992).

1.7 Conclusion

Pine trees are important worldwide for the forestry industry due to their vast usage ranging from paper to turpine production. One million hectares worldwide are used to plant *P. patula* and *P. taeda*, exotic resinous evergreen conifers. Yet, a plethora of pathogens affect these trees annually, therefore causing significant damage. *Fusarium circinatum* is one of the fungi that causes severe losses in pine plantations. The symptoms that are associated with the pitch canker pathogen are formation of an oozing canker leading to die-back of the trees. Reports of the pathogen devastation initiated in California (1946), then Japan (1992), South Africa (1994), Chile (2002), Spain (2005), Italy (2007) and most recently in Portugal and Uruguay (2009). In all these countries the pathogen seemed to thrive in cultured stands rather than in naturally occurring stands. Further, specifically in South Africa, the pathogen was known to be only present in nurseries, which changed in 2006 when five and nine year old pine trees were identified to be infected with *F. circinatum*. This caused a change in not only the level of awareness but also concern regarding an epidemic outbreak (Coutinho *et al.*, 2007).

Due to their preferable characteristics for paper production and solid wood products the two most commonly planted pine species in SA are highly susceptible to this pathogen. Investigations into the disease tolerance of pine species have revealed a degree of tolerance in some species and hybrids, and have shown evidence for genetic resistance. One of the aspects that researchers are looking into is breeding for resistance. This, coupled with good hygiene practices and biochemical treatments, are important strategies to curb the disease.

Disease resistance is associated with an array of pathways that get activated. In plant biology, many plant hormones (SA, MeJA), bacterial extracts (harpin) or fungal components (chitin, chitosan) have been shown to trigger responses that aid the plant in building resistance, allowing the plant to protect itself when subsequently challenged with a pathogen. It is important for the plant to not only have local resistance at the point of infection, but also systemically at parts of the plant away from the centre of infection (Verhage *et al.*, 2010). In model systems such as *Arabidopsis* and *Pseudomonas syringae* these biochemical agents (e.g. SA, MeJA, harpin) have been successful in activating induced systemic resistance. In conifers they too, seem to be effective in reducing disease severity (Bakker *et al.*, 2007; Reglinski and Dick, 2005).

1.8 Aim of the study

Considering the evidence provided regarding induced resistance as a means to manage diseases occurring in coniferous trees, the aim of this project was to examine the effectiveness of induced resistance against *F. circinatum* in *P. patula*. The first objective entailed evaluating the potential of a range of inducers, both chemical and biological, in triggering an induced resistance response and consequently, reducing disease symptoms in *P. patula* infected with *F. circinatum*. A second objective was to determine whether the initiation of induced resistance in *P. patula* could be linked to differential regulation of defence marker genes. Various inducers were tested by spraying young seedlings and the effectiveness of the inducers in boosting resistance was observed. By utilizing the available sequences from *P. taeda,* putative orthologous defence genes were identified to profile the response of those selected putative defence genes to inducer application. This assisted in elucidating the defence responses triggered by the inducer and establishing which defence mechanisms were effective against *F. circinatum*. In future, the effective inducers may contribute to a disease management strategy for pitch canker. Also, the results obtained will aid future studies that aim to reveal the molecular characteristics of induced resistance in *P. patula* and other *Pinus* species.

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CHAPTER 2

Elucidation of defence responses associated with inducer application in *Pinus patula*

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2.1 Abstract

Commercial forestry incurs large economic losses from the pathogen *Fusarium circinatum*, mostly due to post planting stress which makes pine trees more susceptible. Between 20%-30% of planting stock is lost annually due to infection. Hence it is of great interest to the industry that seedlings and mature trees have a high survival rate. The importance of conifers in South Africa is evident since more than 51% of its forestry relies on pine trees. Knowledge of defence signalling is still very limited in conifers, even though this group is essential to forestry worldwide. Plant defence is critical for plants to overcome pathogen attack and environmental stresses. By enhancing defence through pre-treatment of plants with chemicals or biologically derived treatments, resistance can be stimulated. This process is known collectively as induced resistance. Various categories like chemical defences, protein defences and anatomical defences fall under induced resistance, which can be identified through unique expression profiles of genes associated with the different defence pathways. The aim of this study was to evaluate the efficiency of inducers in activating induced resistance in *P. patula*. *Pinus. patula* is one of the highly susceptible pine species to *F. circinatum*, the pitch canker fungus. Seedlings were treated with a panel of ten selected inducers. The application of chitosan at a concentration of 10 mg/ml significantly reduced lesion length after inoculation with a cocktail of *F. circinatum* isolates, compared to the control over a period of six weeks. Chitosan has various functions that range from hindering fungal growth to the activation of peroxidases, phytoalexins and pathogenesis related (PR) proteins. The expression levels of genes encoding for chemical defences [*DXS1* (1-deoxy-D-xylulose 5-phosphate synthase), *PAL (phenylalanine ammonia lyase)*], protein based defences [*PR-3 (chitinase), PR-5 (thaumatin-like), LOX (lipoxygenase), FMO (flavin-dependent monooxygenase*)] and anatomical defences (*PAL*) were investigated by performing real time-quantitative PCR (RT-qPCR). Bioinformatics tools (BLAST, Neighbour joining analysis and Maximum Likelihood analysis) and available expressed sequence tag (EST) sequences from *P. taeda* were used to find *P. patula* putative orthologs for the above mentioned genes. Of the diagnostic defence genes that were targeted via RT-qPCR, two genes were found to be differentially regulated. *PpaAL* was up-regulated four-fold compared to the control plants sprayed with water. This gene is involved in the production of phenolic compounds, which have antifungal properties. *PpaDXS1* was significantly down-regulated. This gene is associated with the production of mono- and diterpenes, which are the building blocks for resin, and is a characteristic defence mechanism for conifers. The data suggests that chitosan treatment causes the activation of induced resistance in *P. patula,* possibly priming the plant against subsequent pathogen attack.

2.2 Introduction

Currently, the most important threat to the softwood industry is the fungal pathogen *Fusarium circinatum*. This pathogen was first discovered in 1949 in North Carolina, USA (Hepting and Roth, 1946) and has since spread to various countries including South Africa in 1994 (Viljoen *et al.*, 1994). *Pinus patula is* a commonly planted softwood in South Africa and is the most susceptible pine species to this pathogen. Manifestation of *F. circinatum* in commercial nurseries has led to large losses to the pine forestry. In pine seedlings or cuttings, infection by the fungus is associated with symptoms such as wilting, discoloration, damping of seedlings and tree death (Hammerbacher, 2005; Viljoen *et al.*, 1994). Post planting mortality constitutes a major part of the economic losses as asymptomatic contaminated seedlings remain undetected and stress factors such as establishment and summer-rainfall conditions allow the fungus to break out (Crous, 2005). The disease infection in mature trees occurs mainly due to wounds, and these can be caused by the twig, bark and cone beetles (*Ips*, *Conophthorus, Ernobius* and *Pissodes)* (Dwinell *et al.*, 1985). Symptoms of the disease in mature trees include resinous cankers found at the site of infection and discolouration of newly developed tips and shoots which eventually results in tree death (Coutinho *et al.*, 2007; Mitchell *et al.*, 2011; Storer *et al.*, 1999). These beetles have been identified as vectors carrying the pathogen, but due to the absence of the twig and bark beetles in South Africa, infestation of mature trees has been limited (Wingfield, 1999; Wingfield *et al.*, 2002).

Presently, various strategies in curbing or at least confining the disease are being pursued. Inspecting the seedlings, discarding suspected carriers of the fungus, and cleaning trays and floors with strong chlorine solution are hygiene practices implemented in the nurseries (Porter, 2010). Establishing these good hygiene practices has resulted in reduced seedling loss in the nursery to below 3% (A. Morris, Sappi Forests (Pty.) Ltd. Sappi, Howick. pers. comm.). Secondly, as there is great variation in susceptibility to this pathogen in the different pine species, usage of more tolerant pine species has also been suggested as a possibility to limit disease incidence (Mitchell *et al.*, 2011). However, the more tolerant pine species are associated with poorer wood quality and growth rate. Unique isolates of *F. circinatum* with variable degrees of virulence have been found specific to certain nurseries, thus further investigation into alternative pine species needs to be conducted (Gordon *et al.*, 2001).

An area of interest that deserves attention is exploring the avenue of manipulating induced resistance (Eyles *et al.*, 2009). Conifers have evolved both constitutive as well as inducible

defence mechanisms. Constitutive defence systems are continuously present and represent the first line of defence. Once these barriers are breached a whole signalling cascade is activated. These induced defence mechanisms involve structural and mechanical modifications where processes like lignification strengthen the cell wall. The production of chemicals such as terpenoids (Vallad and Goodman, 2004) which are characteristic for conifers and proteins with antimicrobial and antifungal properties (e.g. pathogenesis related [PR] proteins), are also responses associated with inducible defence (Eyles *et al.*, 2009). Systemic host responses are triggered via signalling molecules. In the case of systemic acquired resistance (SAR) (hemi)biotrophic pathogens may cause the increased production of salicylic acid (SA) which then leads to the accumulation of PR proteins (Durrant and Dong, 2004). The signalling molecules associated with induced systemic resistance (ISR) are jasmonic acid and ethylene, which impede the progress of necrotrophic pathogens (van Loon, 2007; Vinale *et al.*, 2008). Very similar to ISR is systemic induced resistance (SIR) which is activated by the same signalling molecules (JA and ET) as well as the activation of hypersensitive responses, but it triggers systemic proteinase inhibitors and wound response proteins (Gurr and Rushton, 2005). In contrast with ISR, SIR is activated by biotic and abiotic elicitors whereas ISR is activated by non-pathogenic rhizobacteria.

These defence responses have been effective in inducing resistance and protection against various pathogens in conifers. Pretreatment with inducers is a mechanism used for the induction of systemic resistance. The foliar application of salicylic acid (SA) primed *P. taeda* against the fungus *Diplodia pinea* and had a lasting effect for a month after the treatment (Reglinski *et al.*, 1998). Application of methyl jasmonate (MeJA) to *P. taeda* for an extended period of time caused the activation of induced resistance and reduced the performance of diprionid sawflies (Heijari *et al.*, 2008). Also, treatment with chitosan prior to inoculation with the pitch canker pathogen showed a decrease in symptom appearance in *P. radiata* (Reglinski *et al.*, 2004). Chemical analysis of the treated wood samples revealed that various *1-deoxy-D-xylulose-5-phosphate synthase (DXS)* genes responded to methyl jasmonate (Zulak *et al.*, 2009) as well as chitosan treatment in Norway spruce (Phillips *et al.*, 2007). Biocontrol agents such as *F. oxysporum* have received interest as alternative methods for disease control as it is environmentally friendly (Forestry South Africa environmental regulations). *Fusarium oxysporum* proved to be effective in improving resistance against *Fusarium* crown and rot root of tomato (Duffy and Défago, 1997). Continuous mechanical inoculation with *F. circinatum* over a period of time reduced lesion length in *P. taeda* seedlings. This implied that the induced defence of seedlings was strengthened (Bonello *et al.*, 2001; Gordon *et al.*, 2011). The phenomenon of induced resistance, due to application of

chemical inducers is therefore potentially useful in improving resistance against subsequent pathogen challenge.

In order to identify the onset of the various pathways, distinct diagnostic defence genes may be examined on the molecular level. Such diagnostic genes are for example *flavin dependent monooxygenase 1 (FMO1,* Mishina and Zeier, 2006) and *thaumatin-like* (*PR-5,* Wang *et al.*, 2010) representing the SAR type of defence. *Lipoxygenases* (LOX, Bruinsma *et al.*, 2010), *chitinase (PR-3, Liu et al., 2005)* and *plant defensin (PDF1.2, Penninckx et al., 1998)* are specific for ISR. To verify the production of terpenoids or phenolic compounds, leading to secondary metabolites and lignification, *1-deoxy-D-xylulose-5-phosphate synthase 1 (DXS1,* Phillips *et al., 2007;* Zulak and Bohlmann*, 2010)* and *phenylalanine ammonia lyase (PAL, Vogt, 2010; Yang et al., 2011)* expression levels may be interrogated. Sequence data for *P. patula* is not available, however, 410300 ESTs and 19000 unigenes are available for the closely related *P. taeda* (Neale and Kremer, 2011). These sequences provide a resource for designing expression profiling assays.

The aim of this study was to investigate whether the activation of induced resistance in *P. patula* is an effective strategy that may be exploited against *F. circinatum*. Therefore, the first objective entailed determining the usefulness of various chemical and biologically-derived treatments applied on *P. patula* in inducing resistance against *F. circinatum*. This was followed by investigating changes in expression levels of diagnostic defence genes, which could be linked to the observed activation of induced resistance. This will shed light on aspects for possible improvement in the current disease management strategies against *F. circinatum*. Furthermore, the findings provide clearer direction for future studies intended to enhance the understanding of the molecular basis of induced resistance in *P. patula* and other pine species.

2.3 Materials and Methods

2.3.1 Plant material

Pinus patula seedlings were grown from an orchard mix of seeds representing 12 open pollinated families obtained from Mondi, South Africa. The seeds were pre-germinated in a 1% (v/v) hydrogen peroxide solution at 25°C with continuous aeration using an aquarium air pump. Two individual seeds were planted out into industry standard Unigrow trays comprising 128 plastic inserts, and placed 2 cm deep and 2 cm apart into pine bark seedling soil mix (Varing Kwekery, Pretoria, South Africa). The filled trays were covered with cling wrap after watering, to conserve the moisture. Trays were placed into a growth room with the following conditions: 25°C, high humidity and a 16 hrs light and 8 hrs dark regime, for a period of ten days. Plants were watered every second day. After germination, the two seedlings in each insert were transferred into separate inserts. Once the seedlings reached one month of age, the trays were moved into a greenhouse at the Forestry and Agricultural Biotechnology Institute (FABI) disease free facility, University of Pretoria, South Africa) for the inducer application and inoculation trials.

2.3.2 Inducer preparation and concentration optimization

Ten known inducers, either chemical or biologically-derived, were selected based on literature, and their effectiveness in activating induced resistance in *P. patula* was assessed (Table 2.1). To determine the optimal concentration of the active ingredients (ai), three different dilutions of the following treatments Chitin, MeJA, SA and Bion® were tested. For the other inducers the concentrations used were proposed by the manufacturer. The fungal elicitor chitin was purchased from Sigma Aldrich (SIGMA-Aldrich, #C9752-5G, Missouri, USA) and dissolved in water to concentrations of 1 mg/ml, 10 mg/ml and 100 mg/ml (Zhang *et al.*, 2002). The bacterial crude extract flagellin was prepared from *Ralstonia solanacearum* by centrifuging the bacterial suspension at 1000 rpm for 10 min at 4°C. The pellet was re-suspended, in 10% of the original volume, boiled for 10 min at 95°C, and then centrifuged at 1000 rpm for 10min to pellet the debris. The supernatant was diluted (1:10) with water and used for spraying (Pfund *et al*., 2004; Totten and Lory, 1990). Messenger® (Insect Science, Tzaneen, South Africa) is a commercially available product with the key component harpin. This inducer was dissolved with water to obtain 165 mg of ai/L, as stipulated by the manufacturer. The non-pathogenic pathogen *F. oxysporum FO47* was cultured on $^{1}/_{2}$ potato dextrose agar (PDA) plates (Merck, Darmstadt, Germany). The mycelia was scraped off and suspended in 2 ml of 15% glycerol. The spore concentration was determined using a

haemocytometer and $5x10^4$ spores/plant were applied onto the seedlings as a soil drench (Belgrove, 2008). *Pseudomonas fluorescens* was grown for 48 hrs on LB plates from where it was transferred to 500 ml LB broth and left overnight in a shaking incubator at 27°C (200 rpm). The bacterial suspension was then diluted within 2.5 L or 5 L of water (Lemanceau *et al.*, 1992). Methyl jasmonate (MeJA, SIGMA-Aldrich #39270-7, Missouri, USA) was supplemented with 0.1% TWEEN®20 (SIGMA-Aldrich #P9416, Missouri, USA) and 0.1% EtOH to obtain concentrations of 100 µM, 250 µM and 500 µM by diluting it in water (Brownfield *et al.*, 2008). Monobasic potassium phosphate 10 g/L (1% active ingredient, SIGMA-Aldrich #P5379-500G, Missouri, USA, (Reuveni *et al.*, 2000), Bion® 0.05 g/L, 0.1 g/L and 0.3 g/L (Syngenta Crop Protection, USA, (Dietrich *et al.*, 2004) and sodium salicylate (SA) 1.2 mM, 2 mM and 5 mM (Riedel-de Haën #31493, USA, (Yao and Tian, 2005) were prepared with 0.1% TWEEN®20 and 0.1% EtOH. Kannar® (Kannar earth Sciences, South Africa) was diluted with water to a 2 ml/L concentration. Each treatment was applied onto a small set of seedlings till run-off using a small volume hand mister. The following day the trees were examined for any signs of necrotic symptoms such as chlorosis or other toxic indications to determine the highest optimal concentration that could be used for the study. Control seedlings were sprayed with a water mixture containing 0.1% TWEEN®20 and 0.1% EtOH.

Table 2.1: Chemical and biological inducers tested for their effectiveness in inducing resistance in *P. patula.* The table lists the concentrations selected for each inducer to test for toxicity.

Inducer	Concentrations tested
Chitin	1 mg/ml*, 10 mg/ml, 100 mg/ml
Flagellin	Crude extract*
Messenger®	65 mg ai/L*
Fusarium oxysporum	$5x10^5$ spores/plant*
Pseudomonas fluorescens	6 ml*, 15 ml
Potassium phosphate	1% active ingredient*
MeJA	100 µm, 250 µm, 500 µm*
Sodium salicylate	1.2 mM, 2 mM, 5 mM*
Bion®	0.05 g/L, 0.1 g/L, 0.3 g/L*
Kannar®	1 ml in 500 ml*

*Refers to the concentration selected for the trial.

2.3.3 Screening of the effectiveness of the inducers under nursery conditions in *Pinus patula* seedlings

The initial screening experiment was performed at a commercial nursery (Top Crop, Pietermaritzburg, South Africa). This nursery does not routinely produce pine seedlings and no standard hygiene practices were followed. Four month old *P. patula* seedlings (440 plants per inducer) were treated by spraying the aerial parts of the plant till run off using a fine hand held mister, with the selected concentration of the inducers Bion® (0.3 g/L), Messenger® (65 mg ai./L), Chitin (1 mg/ml), MeJA (500 µM); *F. oxysporum FO47* ($5x10^5$ spores/plant), *P. fluorescens* (6 ml), SA (2 mM), Kannar® (1 in 500 ml), flagellin and monobasic potassium phosphate (10 g/L) in a randomized block trial design. The control for the nursery trial was a mixture of water with 0.1% TWEEN®20 and 0.1% EtOH and 440 seedlings were sprayed with this mixture. The first assessment of disease symptom progression was conducted at four months by observing seedling mortality of the inducer treated plants versus the control plants.

A booster application was given to all plants two months after the first treatment, at an age of six months. Each seedling received the exact same inducer at the same concentration with a hand sprayer as with the first treatment (Bion®, 0.3 g/L), Messenger® (65 mg ai./L), Chitin (1 mg/ml), MeJA (500 µM); *F. oxysporum FO47* ($5x10^5$ spores/plant), *P. fluorescens* (6 ml), SA (2 mM), Kannar® (1 in 500 ml), flagellin and monobasic potassium phosphate (10 g/L). To verify the results under more stringent conditions a subset of 80 seedlings per inducer and control application were distributed in a randomized design and maintained at the FABI greenhouse facilities, where controlled environmental conditions were ensured. Per industry tray, five plots of 16 plants each were used. An open row between the treatments prevented any cross-contamination of the inducer treatments. A week after all the seedlings received their booster application at 6 months of age, at the same concentration in a randomized block trial design; they were challenged with *Fusarium circinatum*.

2.3.4 Evaluation of the top inducers under greenhouse conditions for induced resistance

Plants were grown and maintained under disease-free conditions in the greenhouse to ensure no other factor could trigger induced resistance except for the treatments. The nursery trial suggested that the three inducers (Messenger®, Chitin, MeJA) may be effective at inducing resistance, and were subsequently selected for assessment under greenhouse

conditions (at FABI disease free facility, University of Pretoria, South Africa). Inducers were administered at Messenger® (65 mg ai./L), Chitin (1 mg/ml), MeJA (500 µM) concentrations from the nursery trial using a portable hand spray and applied to the aerial portions of the seedlings till run off.

Greenhouse trial 1: MeJA and Messenger®

In the first greenhouse trial 96 seedlings per inducer were arranged into six plots and 48 plants for each control. For this trial only MeJA and Messenger® were tested. MeJA control plants received a water mixture, including 0.1% TWEEN®20 and 0.1% EtOH, whereas Messenger® control plants only received water. The first treatment was applied at two months of age and the booster was applied two months later. A week after the booster treatment the trees were infected with *F. circinatum*.

Greenhouse trial 2: MeJA, Messenger® and Chitosan

To confirm the results previously obtained, a follow-up trial was conducted, consisting of 116 plants per treatment, with chitosan, MeJA and Messenger®. Chitosan, the deacetylated version of chitin, was prepared according to the protocol described by Reglinski *et al* (2004). An amount of 30 g of chitosan (SIGMA-Aldrich #28191, Missouri, USA) was allowed to solubilize in 1.5 L of 1 M acetic acid for 4 hrs. The viscose solution was then freeze-dried for 24 hrs. The chitosan pellet was dissolved in 0.025 M acetic acid and the pH level adjusted to 6.0 using 1 M NaOH, resulting in a stock solution of 10 mg/ml. This was further diluted into the selected concentrations. As chitosan had not been tested previously two concentrations (1 mg/ml and 10 mg/ml) were assessed. MeJA and Messenger® were made up as described in section 2.3.2. The layout of the trial was a randomized design with 116 plants per treatment and for each group there was a separate control. Control plants for MeJA were treated with a mixture of water, 0.1% TWEEN®20 and 0.1% EtOH (100%), while the Messenger® and chitosan control plants received water. Plants were sprayed till run off at four and at six months of age a booster treatment was administered. A week after the last treatment, the plants were artificially inoculated with *F. circinatum*.

2.3.5 Artificial inoculation technique of pine seedlings with *F. circinatum*

The fungal inoculum was prepared using three isolates of *F. circinatum,* Culture Collection Numbers (FCC) FCC3577, FCC3578 and FCC3579, which were obtained from the Tree Protection Co-operative Programme (TPCP, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa) and previously identified to be highly virulent. These three isolates, reisolated from naturally infected *P. patula* seedlings in a nursery, have been previously characterized (Porter, 2010). The use of a mixture of the three fungal isolates is a well-established procedure which is routinely applied at FABI (Porter, 2010) to screen resistance in pine seedlings. This method insures proper infection of the pine seedlings. Using $^{1}/_{2}$ PDA plates (Merck) each isolate was grown individually at 25°C under fluorescent light with 12 hrs light/dark periods for ten days. The spores were then washed and collected by pouring 15% (v/v) glycerol over the surface. The spore concentration was determined using a haemocytometer to ensure an equal representation of each fungal isolate. The concentration of each isolate was then adjusted to $5x10^4$ spores/ml and poured together. Inoculations of the *P. patula* seedlings were conducted according to Porter (2010), where plants were topped, 1 cm of the apical bud removed and a volume of 10 µl of the mixed spore suspension (5000 spores/ml) was dropped onto the wound using a pipette.

2.3.6 Scoring system categorising disease severity

To visually rate the progression of disease severity in all the plant trials and determine the efficiency of the treatment to induce resistance in *P. patula* to *F. circinatum* over an eight week period, a three category scoring system was developed. Severity of disease symptoms was rated according to the appearance of the plants using a scale from 1-3. Figure 1.4 shows the phenotypes scored. A score of 1 represents healthy plants, a score of 2 are plants with symptoms of early infection, such as discolouration of the needles. The seedling on the far right in Figure 1.4 is showing die back and is scored as 3. As the symptoms of the pitch canker disease are apparent at the second week only, the symptom assessment took place from week two to week eight.

Further measurements involved the lesion length and the tree height. From those two measurements the percentage Livestem was determined. Percentage Livestem was calculated by subtracting the lesion length from the tree height and dividing that by tree height. This value was then converted to a percentage, to account for variability in tree height. Tree height was used as a covariable, which accounted for the fact that taller

seedlings may be able to escape the effect of the pathogen better. This standard measurement is routinely applied in the Central America and Mexico Coniferous Resources Co-operative (Camcore).

2.3.7 Statistical analysis of the *P. patula* lesion measurements

The software programme Analyse-it® (Analyse-it Software, Ltd., Leeds, UK) was used to perform statistical analysis on the data. The Shapiro-Wilk's test was used to establish the normality distribution of the dataset, applying the least significant difference (LSD) stringency setting. The data was not normally distributed and therefore nonparametric tests had to be used to statistically determine the effectiveness of the inducers versus controls. For this, the pairwise Kruskal-Wallis analysis was applied ($p<0.05$, Yi *et al.*, 2009).

2.3.8 Detection of putative defence gene orthologs in *P. patula*

Since different pathways are activated through induced resistance, an array of genes associated with the onset of distinct pathways, were chosen. The genes selected represented the systemic acquired resistance (SAR) pathway, such as *flavin-dependent monooxygenase 1 (FMO1,* Mishina and Zeier, 2006) and *thaumatin (PR-5, Liu et al., 2010a)*. Indicators for the induced systemic resistance (ISR) pathway were *chitinase* (*PR-3,* Shoresh *et al.*, 2010) and *lipoxygenase* (*LOX, Umate* and Tuteja*, 2011)*. Secondary metabolites play a crucial role in defence, therefore *phenylalanine ammonia lyase (PAL, Wang et al., 2008),* linked to phenolic production, and *1-deoxy-D-xylulose 5-phosphate synthase 1 (DXS1, Estévez et al., 2001)*, related to terpene defence, were selected.

The pipeline developed for this study involved initially obtaining the coding sequences (CDS) from plants such as *Arabidopsis thaliana* (from TAIR, http://arabidopsis.org), *Triticum*, *Picea* and *Nicotiana* (GenBank, www.ncbi.nlm.nih.gov/). The *Pinus taeda* expressed sequence tags (ESTs) sequences were retrieved from the ConiferGDB database (www.conifergdb.org) by performing a TBLASTX search using the CDS sequence of known plant species as the query thus allowing cross-species prediction at both the genomic and transcriptional level. ESTs with E (expect)-values < 10^{-50}, were imported into two *ab initio* prediction software algorithms namely, Genemark (http://exon.gatech.edu/eukhmm.cgi) and Genscan (http://genes.mit.edu/GENSCAN.html) to obtain the predicted protein sequences. CLC Bio Workbench 6.0 (CLC Main Workbench 6) was used to create contigs from overlapping ESTs where possible with the retrieved ESTs. Furthermore, a number of pine defence gene

orthologs were obtained from the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov). The putative orthologs obtained from NCBI for which only the CDS sequence was available were also analysed using the *ab initio* prediction software algorithms.

To validate the homology of the predicted protein a TBLASTN analysis was performed against TAIR. ClustalW (www-bimas.cit.nih.gov/clustalw/clustalw.html*)* was used to align the sequences and MEGA v5.01 (Tamura *et al.*, 2007) software was utilised to perform the phylogenetic analysis. Neighbor Joining (NJ) trees were constructed using pairwise deletion and the statistical confidence of the branch nodes was substantiated by bootstrap analysis (10000 permutations). Additionally, Maximum Likelihood trees (ML) were also constructed. For the ML analysis, sequence alignment was performed using the online version of MAFFT (http://mafft.cbrc.jp/alignment/server/) with E-INS-I setting which ensures a more accurate alignment of the sequence. The file was then saved as a Phylip format file and imported into ProTest software (Darriba *et al.*, 2011) to determine the optimal best-fit evolutionary model of amino acid substitution. These settings were used for the parameters in PhyML (Guindon and Gascuel, 2003). The clade confidence was determined using the bootstrap statistical analysis with a 1000 permutations. Both methods NJ and ML were done to add more confidence in selecting a particular ortholog.

2.3.9 Primer design and sequence validation

Putative defence gene orthologs were identified using the NJ and ML trees. The Primer Designer v4.20 computer software (Sci Ed Central, Cary, North Carolina, USA) was utilized to design primers from either the contigs or the ESTs. The criteria for the primers were: Tm (50°C - 65°C), product size (100 bp – 350 bp) and a high GC content of between 50-60%. The primers were synthesised by Inqaba (Inqaba Biotechnology, Pretoria, South Africa). Table 2.2 shows the list of putative orthologs that primers were designed for.

Pinus patula cDNA was used as the template for polymerase chain reaction (PCR) with 0.4 U Excel High Fidelity Taq (Southern Cross Biotechnologies, Cape Town, South Africa). Amplification products were resolved on a 1.5% (w/v) agarose gel. The product was gel purified from a 0.8% (w/v) agarose gel using the MinElute® Gel Extraction Kit (Qiagen Inc, Valencia, CA) and cloned into the pTOPO vector (Invitrogen, San Diego, CA). The plasmids containing the sequence were then transformed into *Escherichia coli* DH5α cells, which were made competent by using the $CaCL_2$ method (Cohen *et al.*, 1972). Selection of eight

colonies per gene was performed using the blue/white screening technique and colony PCR (1.5 mM buffer, 2.5 mM dNTPs, 10 µM of each primer, 5.0 U/µl Taq polymerase and 6 ng of template): initial denaturation step at 1 min at 95°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 53°C and 72°C for 2 min, and the extending step at 72°C for 10 min, performed with the universal M13 primer set: M13 Forward 5' CACGACGTTGTAAAACGAC 3' and M13_Reverse 5' GGAAACAGCTATGACCATG 3'. The transformed selected colonies were incubated at 37°C overnight in order to conduct plasmid isolation using the GeneJET™ Plasmid Miniprep Kit (Fermentas, Ontario, Canada). To validate the cloned fragment, sequencing was performed at the Sequencing facility at the University of Pretoria with the M13 primers. The retrieved consensus sequence was analyzed using the TBLASTX approach against the TAIR, NCBI and ConiferGDB databases.

Table 2.2: List of *P. patula* gene targets for which primers were designed for RT-qPCR expression analysis

Gene Name	Forward sequence 5'-3'	Reverse sequence 5'-3'	Amplicon size (bp)	Tm (°C)
Target genes				
PpaFMO1	TGGCCATCCTTGTACAGTG	GATAGTACGCCGTGCAGAA	153	64°C
PpaDXS1	CAGTTGCAGATGCCAGATTC	TACTGATGCCGCGATATGAG	269	64°C
PpaPAL	TCAAGAACGCAGAAGGTGAG	GACTAACTTGCGGTTCAAGC	102	64°C
PpaPR-3	ATGGAATGGTGACGGACATC	CCAGCATGTTGCAGTATCTC	111	64°C
PpaPR-5	CGGATGCGTCGCTGATCTT	GACACGTCTGAGCGGTGTT	152	64°C
PpaLOX	TGTTGGTCACGGAGACAAGAAG	GGCATGTATCCGGCATAAG	157	64°C
PpaPR-4	GCAGCAAGCGTCCAATGT	ATGCGCCAGTGTCTCTGT	223	64°C
PpaChs	GGACTGCACAGACCATTCTT	TACTTGCCTCGTCGATCTCA	269	54°C
PpaGLP7	AACGGTCTTCGTAGTGAGAG	GTAAGGAATGGCAGGAGTTG	235	51°C
PpaMHJ24	CCGATGCTGCTCTTCTAACC	GAAGCCGAGTGTCTTCGGAT	160	52°C
PpaOSM34	GTGTGAGATGTGCGACCTTC	TAGAGCGTATTCCGCCAGAG	272	55°C
PpaPR-2	GTTCGATGCGCTGGTGGATT	ATGTGCTGTCCAGGCCTCTT	201	53°C
PpaPR-1	CTTAACTCCGCTCTGGACAT	CTCCGTATTGACCACCAGAA	268	51°C
Reference genes				
Ppa26s	GGCCTGACACTCTTGATCCT	CAGTGCAGACACTCCGAATG	204	64°C
PpaARF1	GATCTCTAACAGGCGGTCAA	TCTCCATAGTGGACGGATCT	258	64°C
PpaICDH	CTCGGCATTACCGCGTACAT	TCCGGCCTCAACAGTTCCTA	173	52°C
PpaEF1	TGTCCGGCAGCTCATTGTC	GCACCAGCCTTGGTCTCTT	227	64°C
PpaCAP20	GGAGGAGCATCACCGTTA	CCACCACCATAGCGAGAA	231	60°C
PpaTUB6	AGCCGTGAATTGCTCACT	AACAGCCTCTGCCATGTT	226	61°C
PpaUBQ10	GGTGGATTCCTTCTGGATGT	TCGAGGTCGAGAGTTCTGAT	154	61°C

2.3.10 RNA extractions and cDNA synthesis

The aerial part of the treated (chitosan 10 mg/ml) pine seedlings and the controls were harvested 24 hrs after the first and booster application of the inducer. For each biological replicate 16 plants were bulked. The material (1-3 g) was then ground by using an electric grinder (IKA-Werke, Staufen, Germany) to pulverize the samples in liquid nitrogen. The method used to extract total RNA from the powder was the modified cetyl-trimethyl-ammonium-bromide (CTAB) method established by Zeng and Yang (2002). The RNA was purified with DNaseI (Qiagen Inc, Valencia, CA) followed by treatment with the RNeasy MinElute® Cleanup kit (Qiagen Inc, Valencia, CA) as stipulated in the manufacturer's protocol, to ensure no genomic contamination. The RNA, which was heated to 65°C for 10 min and then cooled for 2 min, was electrophoresed in a standard 1% (w/v) formaldehyde agarose gel. For concentration determination the samples were both analyzed using the Nanodrop® ND-100 Spectrophotometer (Nanodrop technologies, ND1000, DE, USA) and quality and quantity investigated on a Experion capillary electrophoresis device (Experion, Bio-Rad Laboratories, South Africa).

The first strand cDNA was synthesised using 1 µg of purified total RNA. Added to the RNA were 0.5 µl of rRNasin® RNase Inhibitor (Promega, Wisconsin, USA) and 0.5 µg/µl of oligo-dTs and incubated for 10 min at 70°C. Next, 1 µl Improm-II Reverse Transcriptase (Promega, Madison, Wis.) were included in the reaction together with 4 µl 5X Buffer, 2.4 µl $MgCl_2$ and 0.2 nM dNTP's. Thermal cycler conditions were followed as stipulated in the manufacturer's manual i.e. 25°C for 10 min, 42°C for 60 min and 70°C for 15 min. To ensure that no genomic contamination was present in the cDNA, a polymerase chain reaction was conducted with *PpPR-4* primers. The *PpPR-4* gene was amplified both from genomic DNA (650bp) and cDNA (2200bp) spanning an intron of 430 bp. The conditions for the reactions were as follows: 10 ng of 1:10 cDNA as template and cycle conditions: 1min at 94^{o}C followed by 30 cycles of 30 sec at 94^{o}C, 30 sec at 60^{o}C, 1 min at 72^{o}C and a final hold at 4^{o}C. The products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel. The negative control for this reaction was a no template control.

2.3.11 Reverse transcription – quantitative PCR (RT-qPCR) analysis of induced plant material

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were adhered to for reverse transcriptase quantitative PCR (RT-qPCR, (Bustin *et al.*, 2009). Each 11 µl RT-qPCR reaction consisted of 0.2 nM of LightCycler® 480 SYBR Green I Master mix (Roche, Mannheim, Germany), 10 nM of each primers and 2.5 ng/µl of *P. patula* cDNA template. The reaction was performed on the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, GmbH, Basel, Switzerland) with an initial incubation step at 95°C for 5 min and amplification of 45 cycles of 95°C for 10 sec, 64°C for 10 sec and 72°C for 15 sec. Two holds of 95°C for 5 sec, 65°C for 1 min and 95°C continuously with 10 acquisitions per 1°C and a cooling step of 40°C for ten sec were performed, to obtain a melting curve. Technical repeatability was ensured, by amplifying each biological replicate in triplicate. Various housekeeping genes *tubulin 6 (PpaTUB6), ubiquitin (PpaUBQ), actin (PpaACT), elongation factor-1 alpha (PpaEF1)* and *18s rRNA (Ppa18s)* were tested for stable expression. The selected reference genes were *ADP-ribosylation factor-1* (*PpaARF1)* and *proteosome subunit* (*Ppa*26S) with stable expression. Table 2.3 shows the primers used for RT-qPCR analysis. Standard curves were constructed from selected dilutions of 1:10, 1:20, 1:50, 1:100, 1:500 and 1:1000 using a pool of cDNA from the harvested material, as template to assess the amplification efficiency for each primer pair. According to Hellemans *et al.* (2007), a sample maximization method was employed whereby the maximum amount of samples were analyzed per run and therefore different genes were assessed in different runs. This method accounts for technical variation and therefore no inter-run calibrators were required. The crossing points were calculated by determining the absolute quantification /2nd derivate maximum and that data was imported into qBASE*plus* v1.0 (www.qbaseplus.com Biogazelle, Ghent, Belgium) where the relative quantification and normalization (Hellemans *et al*., 2007) based on reference genes was performed.

The regression line calculated determined the efficiency of the primer pair. The threshold of the r^2- value (correlation coefficient) was set to >0.980 and used to determine how well the data fit the regression line. The slope was evaluated by the efficiency value (E) which was around 2. The last value used to evaluate the standard curves was the standard error. Once all the parameters were set, the qBASE*plus* v1.0 software calculated the CNQR value and the standard error of the CNQR value for each biological replicate. The normalized relative quantities, expressed as relative expression ratio in arbitrary units (AU, Hellemans *et al.*, 2007) and the standard error for each biological replicate were then exported to Microsoft®

Excel 2007 (Microsoft, Redmond, WA). The biological standard deviations and errors were determined using all three biological replicates for the chitosan induced and water control samples. Finally the Student's *t*-test was applied to investigate significance between the chitosan induced and the water control. Statistical significance was then computed by a one-tailed Student's *t*-test ($p<0.05$) between induced and control seedlings for all the selected marker genes and graphs were generated in Microsoft® Excel 2007.

To validate the presence of a single product and ensure the correct product size, samples were analyzed on a 1.5% (w/v) agarose gel. In addition, a random sample for each gene was selected as a representative and sequenced.

Table 2.3: List of selected *P. patula* gene targets with the primers designed for RT-qPCR expression analysis

Gene Name	Forward sequence 5'-3'	Reverse sequence 5'-3'	Amplicon size (bp)	Tm (°C)
Target genes				
PpaFMO1	TGGCCATCCTTGTACAGTG	GATAGTACGCCGTGCAGAA	153	64°C
PpaDXS1	CAGTTGCAGATGCCAGATTC	TACTGATGCCGCGATATGAG	269	64°C
PpaPAL	TCAAGAACGCAGAAGGTGAG	GACTAACTTGCGGTTCAAGC	102	64°C
PpaPR-3	ATGGAATGGTGACGGACATC	CCAGCATGTTGCAGTATCTC	111	64°C
PpaPR-5	CGGATGCGTCGCTGATCTT	GACACGTCTGAGCGGTGTT	152	64°C
PpaLOX	TGTTGGTCACGGAGACAAGAAG	GGCATGTATCCGGCATAAG	157	64°C
PpaPR-4	GCAGCAAGCGTCCAATGT	ATGCGCCAGTGTCTCTGT	223	64°C
Reference genes				
Ppa26s	GGCCTGACACTCTTGATCCT	CAGTGCAGACACTCCGAATG	204	64°C
PpaARF1	GATCTCTAACAGGCGGTCAA	TCTCCATAGTGGACGGATCT	258	64°C

2.4 Results

2.4.1 Effectiveness of inducers in triggering induced resistance in *P. patula*

Evaluation of the ten selected treatments, which have previously shown promise in priming the plant against subsequent pathogen challenges, was conducted under both nursery and greenhouse environment. The nursery environment (reflecting naturally high levels of *F. circinatum)* and controlled conditions in the greenhouse (representing a disease free environment) were chosen to determine the efficiency of the inducers. The optimal concentration of Bion®, Chitin, MeJA; *P. fluorescens* and SA were chosen based on the lack of visible toxic symptoms or chlorosis. Therefore the highest concentration was selected for subsequent trials (results not shown). For the other inducers the same concentration as shown in table 2.1 was applied for all trials.

The majority of the seedlings grown in a nursery (TopCrop, located in Kwazulu-Natal, South Africa) were naturally infested with *F. circinatum,* as tip die-back, reddish brown needle discolouration was evident (Mitchell *et al.*, 2011). After inducer application at four months, a first evaluation of the effectiveness was determined by observing the survival rate of plants sprayed with the various inducers. Four of the inducers (Bion®, Messenger®, MeJA, Chitin) appeared to have improved plant survival, compared to control plants which were treated with water. As Bion® is the commercially available analogue to SA both treatments were found to perform similarly well. However, the difference between treated and control plants for all treatments was not statistically significant ($p>0.05$, Kruskal-Wallis test, Figure 2.1).

A subset of 80 plants per inducer was then screened under artificial conditions. They were artificially inoculated with the pathogen *F. circinatum,* a week after a booster application at six months of age was given to them. Booster applications have been shown to increase the success of the inducers in activating induced resistance (Heijari *et al*., 2008). The efficiency of each inducer was calculated at six weeks post inoculation. Chitin treated seedlings at a concentration of 1 mg/ml compared to the water sprayed control plants had significantly reduced lesion lengths (**$p<0.01$, Kruskal-Wallis test). These seedlings, sprayed with chitin, had 80% of the stem still healthy (Figure 2.2) whereas there was only 68% healthy tissue in the control plants ($p<0.004$). The lesion size was reduced to an average of 21 mm compared to 35 mm in the control plants suggesting that chitin treatment reduced the severity of the disease associated with *F. circinatum*. Treatment with three other inducers: Bion® ($p<0.03$, Kruskal-Wallis), MeJA ($p<0.05$, Kruskal-Wallis), and Messenger® ($p<0.05$, Kruskal-Wallis), also showed a significant reduction in the lesion length in the pine seedlings after *F.*

circinatum inoculation compared to control (uninduced) seedlings (*p<0.05, Kruskal-Wallis test, Figure 2.2).

Interestingly, the opposite effect was seen with Kannar® and *Pseudomonas fluorescens* treatments as they caused an increase in the severity of the disease compared to the control plants (Figure 2.2). The lesion length was on average 8 mm longer in the Kannar® (p<0.005, Kruskal-Wallis) and *Pseudomonas fluorescens* (p<0.03, Kruskal-Wallis) induced seedlings compared to the control (uninduced) seedlings at six weeks post inoculation. Additionally, the disease symptoms were more severe in the seedlings and more prominent phenotypic characteristics of *F. circinatum* infection were displayed, such as browning of the needles.

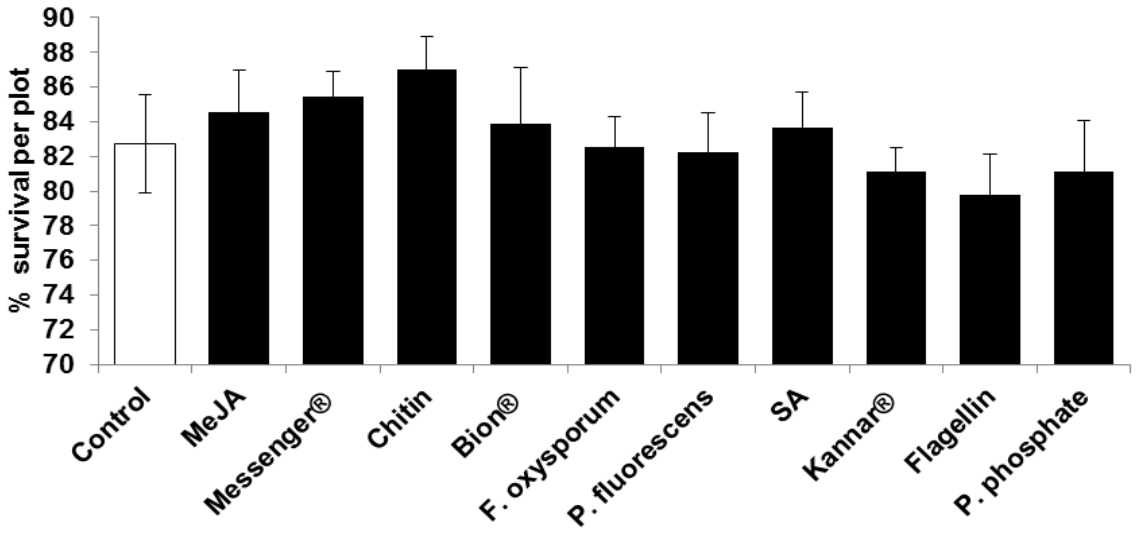

Figure 2.1: **Effectiveness of chemical and biological inducers in enhancing survival of *P. patula* seedlings compared to untreated plants under nursery conditions after the first treatment**. The number of dead plants was counted in each of the 11 replicates and the average percentage of plant survival per replicate was calculated for 440 plants per treatment. Error bars represent standard error.

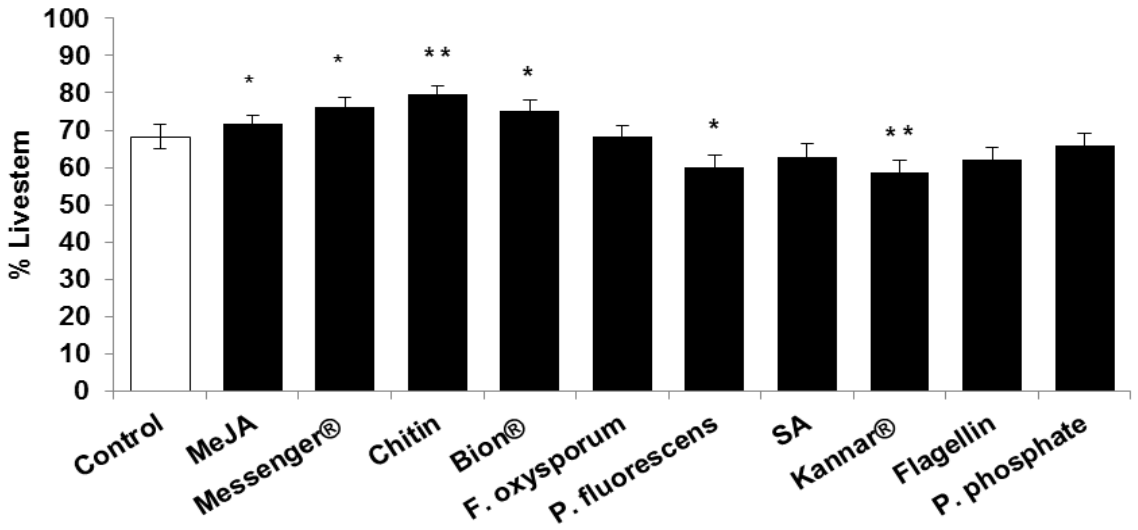

Figure 2.2: **Disease symptoms in *P. patula* seedlings, 6 weeks post inoculation with *F. circinatum* under artificial conditions**. Eighty plants per treatment were taken to determine the % Livestem. Error bars represent standard error. A ** indicates a significance of $p<0.01$ (Student's *t*-test), while a * indicates significance at $p<0.05$ between treated and control (uninduced) plants.

To validate the results from the nursery trial, additional trials were performed in the disease - free environment of a greenhouse. Plants were grown in growth rooms, then sprayed and challenged with *F. circinatum*. The top inducers identified from the nursery trial were assessed: chitosan, Messenger® and MeJA. In the first greenhouse trial MeJA and Messenger® were applied at two months and at four months in order to observe any change in early age spraying. The measurements in Figure 2.3 point out that the two inducers were effective at different times of the trial. MeJA significantly curbed *F. circinatum* lesions at two weeks post inoculation, with approximately 85% Livestem ($p<0.05$, Kruskal-Wallis). However, in the following weeks the induced plants did not show a difference compared to the controls, and on average they had larger lesions than the control seedlings. Messenger® treated plants showed significant reduced symptom development compared to the control plants only later in the trial at week five ($p<0.05$, Kruskal-Wallis), suggesting a delayed response of the commercial treatment. However, both the inducers did not have a long lasting effect.

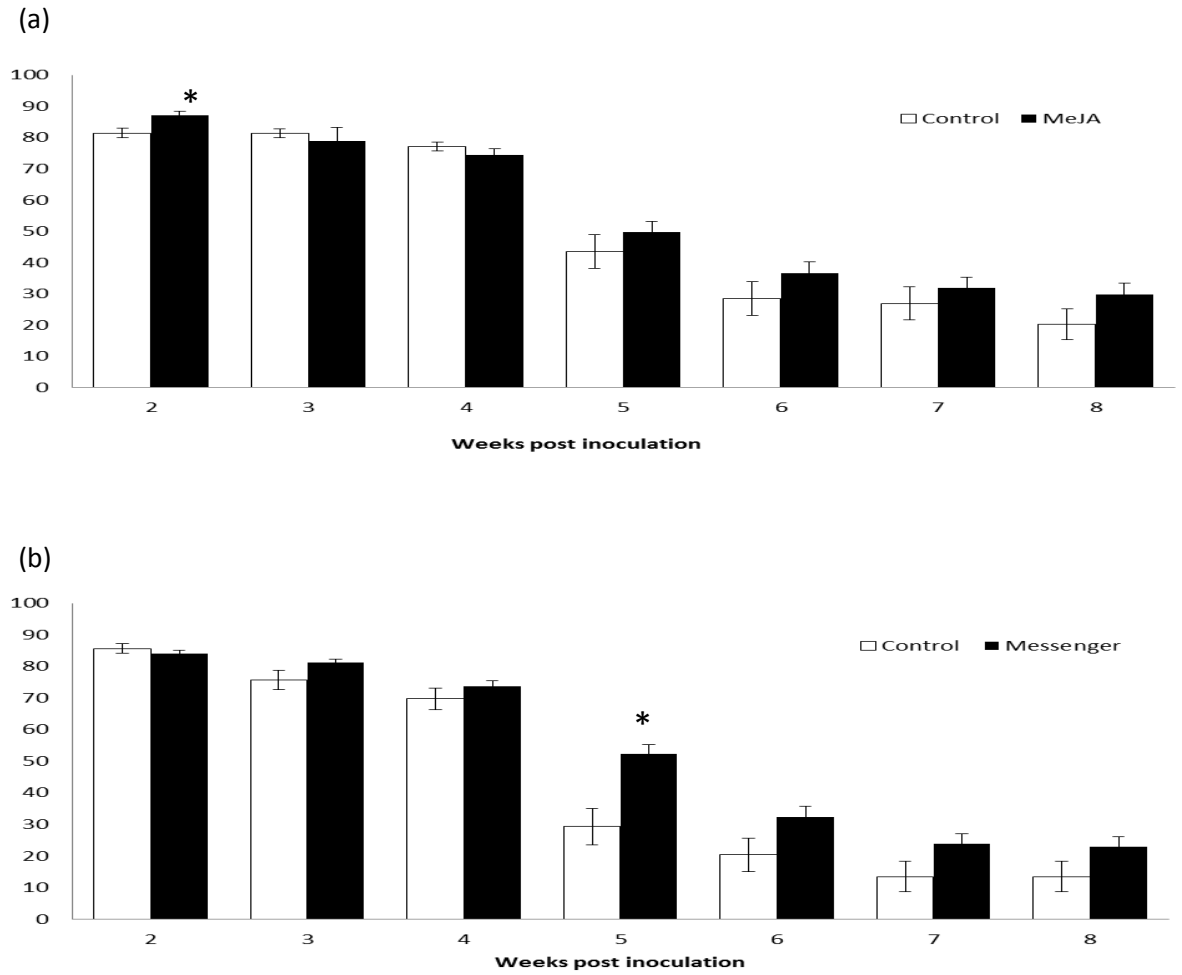

Figure 2.3: **The effect of MeJA and Messenger® on disease progression in *P. patula* seedlings during an eight week period post inoculation with *F. circinatum*.** The x-axis represents the weeks post inoculation. The y-axis represents the %Livestem. 80 plants per treatment were used to determine % Livestem after two treatments with (a) MeJA and (b) Messenger® followed by inoculation with *F. circinatum*. The plants were treated with both inducers at two months and four months of age, and were challenged with the pathogen a week after the booster treatment. The controls of MeJA were sprayed with a mixture of water, ethanol and TWEEN®20, whereas the control plants for Messenger® only received water. The * indicates a significant difference between the induced and control plants where $p<0.05$ (Kruskal-Wallis test).

In the second greenhouse trial, chitosan, the de-acetylated form of chitin, was included, with 112 seedlings per inducer. The treatments administered to the pine trees at four and six months of age, were Messenger®, MeJA and chitosan, the latter in concentrations of 1 mg/ml and 10 mg/ml. Again MeJA showed a significant ($p<0.05$, Kruskal-Wallis) response in reducing lesion length caused by *F. circinatum* in the second week, in respect to the control

seedlings, which is attributed to rapid priming of the plants. However, this protection does not last long and only delays the symptoms (Figure 2.4a). Messenger® showed no significant response which may be due to the larger seedling number compared to the previous trial, or the age difference in the plants (Figure 2.4b). The most effective biochemical inducer in this trial was chitosan, at a concentration of 10 mg/ml. The inducer was successful in significantly reducing disease severity in comparison to the control plants ($p<0.05$, Kruskal-Wallis) over a window period of week 2 to 6 (Figure 2.4c). This indicates a rapid as well as a long lasting effect of the treatment. However, this significance in reduced disease severity was not sustained for more than 6 weeks.

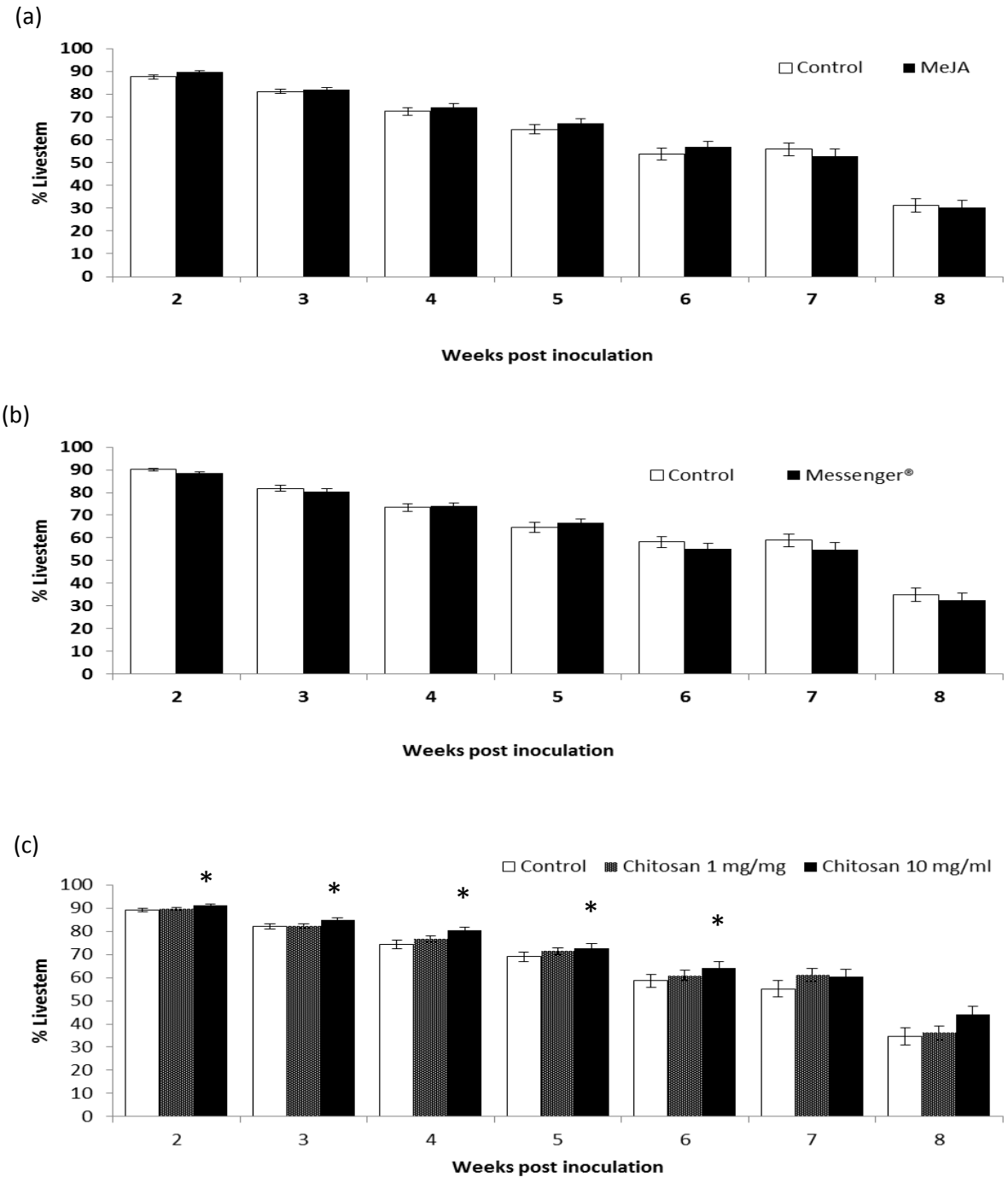

Figure 2.4: **The effect of MeJA, Messenger® and Chitosan treatment on disease progression in *P. patula* seedlings during an eight week period post inoculation with *F. circinatum*.** 112 plants per treatment were used to determine the % Livestem. The inducers MeJA (a), Messenger® (b) and Chitosan (c) were sprayed till run-off at four months and six months of age and a week later they were challenged with the pathogen. The controls for both MeJA and chitosan resembled a mixture of water ethanol and TWEEN®20, components added to the active ingredient of the inducers. Messenger® control was just water. The x-axis represents the weeks post inoculation with the fungus and the y-axis represents the % Livestem. The * indicates $p<0.05$ (Kruskal-Wallis test) a significant difference between the induced and control plants.

2.4.2 Identification of putative defence marker orthologs in *P. patula* using phylogenetics

The complete genome sequence for a pine species is not yet available, thus for the purpose of this study, the 410 000 ESTs available for *P. taeda* were used to identify *P. patula* orthologs. The ESTs range in length from 150 bp to around 800 bp and were derived from various conditions, referred to in supplementary table S1. The genes *flavin-dependent monooxygenase 1 (FMO1,* Mishina and Zeier, 2006), *thaumatin (PR-5, Liu et al., 2010a)*, *chitinase* (*PR-3,* Shoresh *et al.*, 2010), *lipoxygenase* (*LOX, Umate* and Tuteja*, 2011), phenylalanine ammonia lyase (PAL,* Wang *et al.*, 2008) and *1-deoxy-D-xylulose 5-phosphate synthase 1 (DXS1,* Estévez *et al.,* 2001) were selected. As all the trees drawn are amino acid based, two prediction software programs, Genemark (http://exon.gatech.edu/eukhmm.cgi) and Genscan (http://genes.mit.edu/GENSCAN.html) were used. The predictions obtained from both softwares for all plant species are included into the phylogenetic trees and referred to as GM (Genemark) and GS (Genscan). Both sequences for contigs and ESTs were added to all the phylogenetic analysis except for the *FMO1* gene family.

Flavin-dependent monooxygenase 1 (*FMO1*) is a well-known defence marker gene for the SAR pathway associated with salicylic acid (Mishina and Zeier, 2006). Its ortholog was selected based on a Maximum Likelihood phylogenetic tree (ML, Figure 2.5), using the JTT + G evolutionary model (G = 1.452, ProTest) and a Neighbour Joining tree (NJ, Supplementary Figure S7). Though TBLASTX analysis of the *Arabidopsis FMO1* gene in ConiferGDB detected two ESTs, there were no significant matches in the NCBI database. To ensure better identification of the ortholog, all *Arabidopsis* and *Picea* (spruce) *FMO1* gene family members were added to the list of protein sequences. The protein sequence of the RTAL1 4 EST corresponds to the *Arabidopsis* gene with a bootstrap value of 45% in the ML tree and in the NJ tree it corresponds to RTFE1 with a 96% bootstrap support. The expected (E) values obtained for the two protein sequences (RTAL1 E-value = 0.01 and RTFE1 E-value = $2e^{-21}$), when TBLASTN analysis was performed in TAIR, provided support for RTFE1 as the possible ortholog.

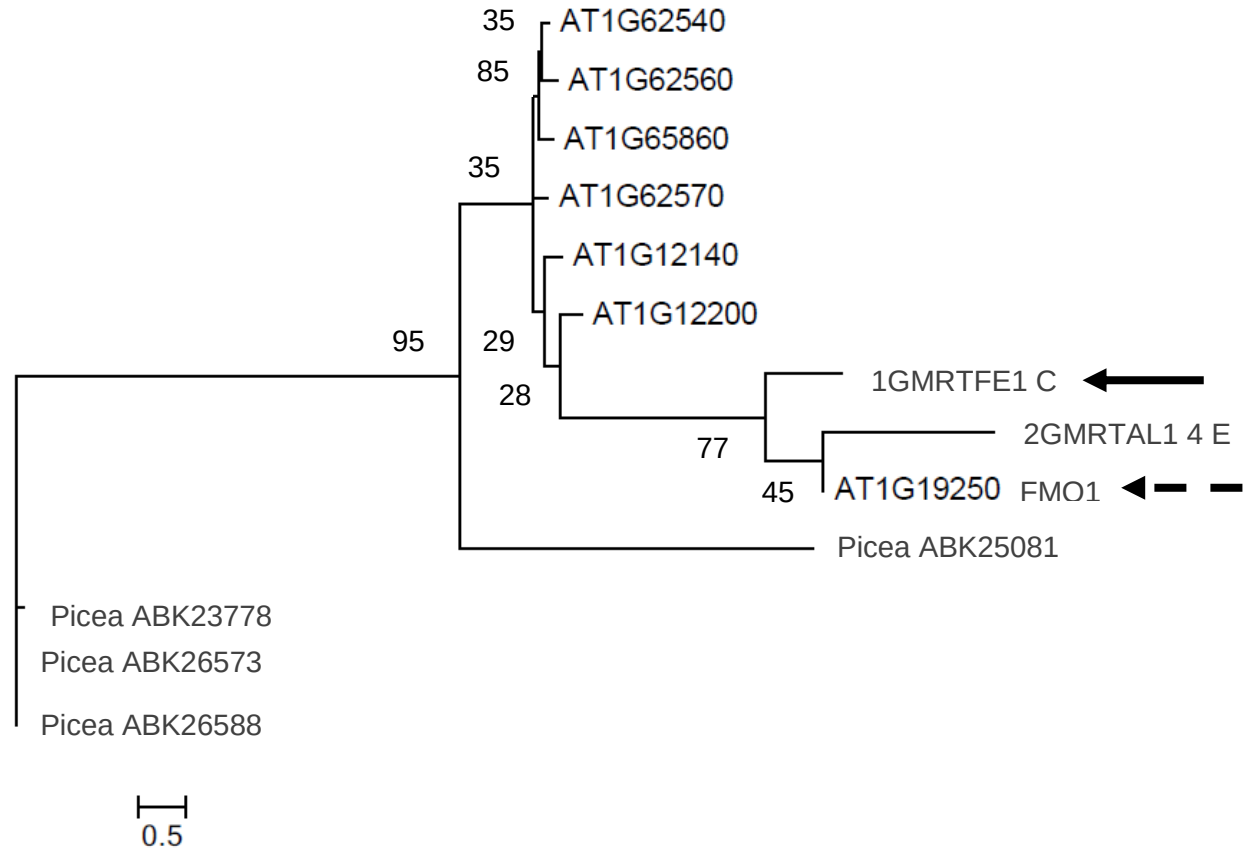

Figure 2.5: Maximum likelihood phylogenetic tree showing the relationship between the *FMO* (*flavin-dependent monooxygenase*) gene sequences of various plant species and the putative *PpaFMO1* ortholog. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea* genes as found in NCBI. The dotted arrow represents the *Arabidopsis* gene and the solid arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

The *DXS (1-deoxy-D-xylulose 5-phosphate synthase)* gene was selected as it was shown that its up-regulation influenced the defence response of *Picea.* Three genes in spruce encode for *DXS* and are involved in the terpenoid – based defence, a key defence mechanism in conifers. Sequences of *DXS* are available from NCBI for both *Pinus taeda* (*Pt*) and *Pinus densiflora (Pden)*, both found in the same subgenus of *Pinus*. These sequences (unpublished) were added to the analysis. The *DXS* type 1 was selected, specifically contig3 (Supplementary Figure S1) which is *P. taeda* sequence, as well a close relationship to *Arabidopsis* (Figure 2.6). The sequence of this contig grouped closely to the *Picea* sequence and *P. densiflora.* The relationship to *Picea* was well supported in both the ML (evolutionary model JTT + G, G=0.686) and the NJ (Supplementary Figure S8) trees. Hence contig3 was

the representative chosen for this gene and primers were designed to amplify a 268 bp fragment. The contig was constructed as an additional verification of the *PtDXS* sequences available on GenBank.

Figure 2.6: Maximum likelihood phylogenetic tree showing the relationship between the *DXS (1-deoxy-D-xylulose 5-phosphate synthase)* gene sequences of various model organisms and the putative *PpaDXS1* ortholog. In this tree sequences from *Piuns densiflora* (Pdens), *P. taeda* (Pt), *Nicotiana*, *Populus* and *Arabidopsis* were included**.** EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea, Nicotiana, Populus* and the *Pdens and Pt* genes as found in NCBI. The dotted arrow represents the *Picea* gene and the solid arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

For the *PAL* gene, a known marker for the phenylpropanoid pathway which is associated with secondary metabolite production, six EST sequences were retrieved from ConiferGDB. *Pinus pinaster* and *P. taeda* sequences from NCBI, and sequences for other plant species (*Arabidopsis, Triticum* and *Nicotiana)* from the same gene family were added to the trees. Two contigs were created from some of the ESTs obtained from ConiferGDB. The model that best fit the given data for creating the maximum likelihood (ML) tree was LG+I+G (G = 2.008) based on the ProTest software (Figure 2.7). From the neighbour joining phylogenetic (NJ, Supplementary Figure S9) tree and the ML tree that were constructed for *PpaPAL*, primers were designed to amplify a 1023 bp product based on the sequence from contig1. The general *P. taeda PtPAL* and a *P. pinaster PpinPAL2* sequence derived from NCBI (unpublished) make up this contig, providing added confidence in the selected sequence. Although all four *Arabidopsis* sequences grouped closely together, there are different expression patterns between *PAL1, PAL2* and *PAL3, PAL4* (Huang *et al.*, 2010; Olsen *et al.*, 2008; Rohde *et al.*, 2004; Wanner *et al.*, 1995), hence *PpinPAL1* and *PpinPALl2* are found together. With the TBLASTN results from TAIR, contig1 (Supplementary Figure S2) was used as the representative sequence for *PpaPAL*. The identification of the putative ortholog is based on nucleotide sequence available for pine, and further analysis still needs to be conducted once the genome of *P. taeda* becomes available.

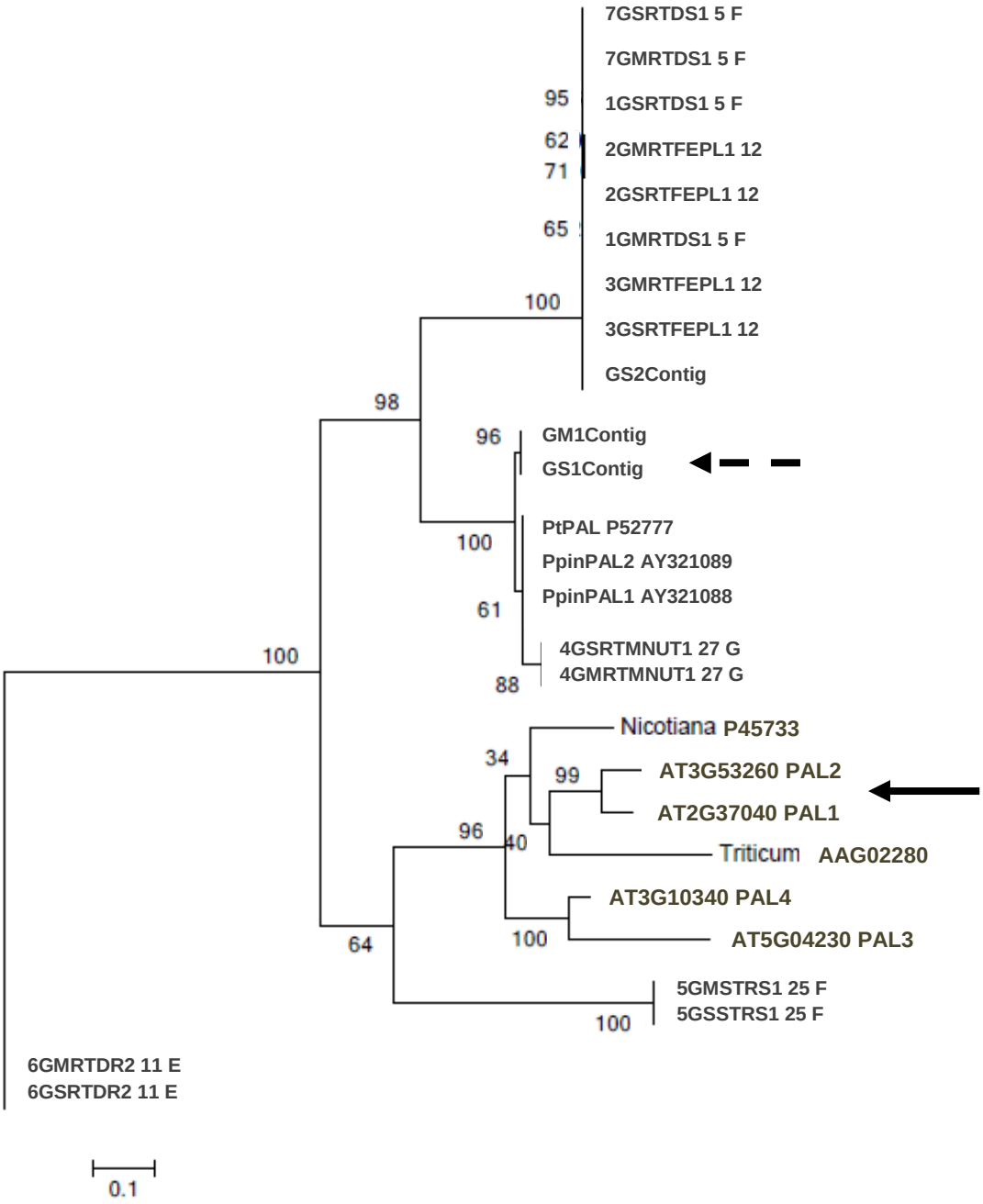

Figure 2.7: Maximum likelihood phylogenetic tree showing the relationship between the *PAL (phenylalanine ammonia lyase)* gene sequences of various model organisms and the putative *PpaPAL* ortholog. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea, Nicotiana, Triticum, Pinus pinaster (Ppin)* and *P. taeda (Pt)* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

Another diagnostic defence gene that was chosen was *PR-3.* This gene is known to be involved in induced systemic resistance pathway (ISR). The *Arabidopsis* sequence of *PR-3* (*chitinase*) grouped closely with the ESTs derived from ConiferGDB in the ML tree (Figure 2.8). The resulting phylogenetic tree was constructed using the evolutionary model based on a WAG matrix (G = 2.239). In the NJ tree (Supplementary Figure S10) a clearer identification was possible as the *Arabidopsis PR-3* grouped in the same clade as the selected contig and its ESTs, yet the bootstrap support was lower in the NJ tree than in the ML tree. The primers designed from contig1 (Supplementary Figure S3) amplify a 202 bp fragment. Additionally confidence in the *PpaPR-3* ortholog was substantiated by using TBLASTN to TAIR to determine similarity and obtaining high E-values ($4.4e^{-86}$).

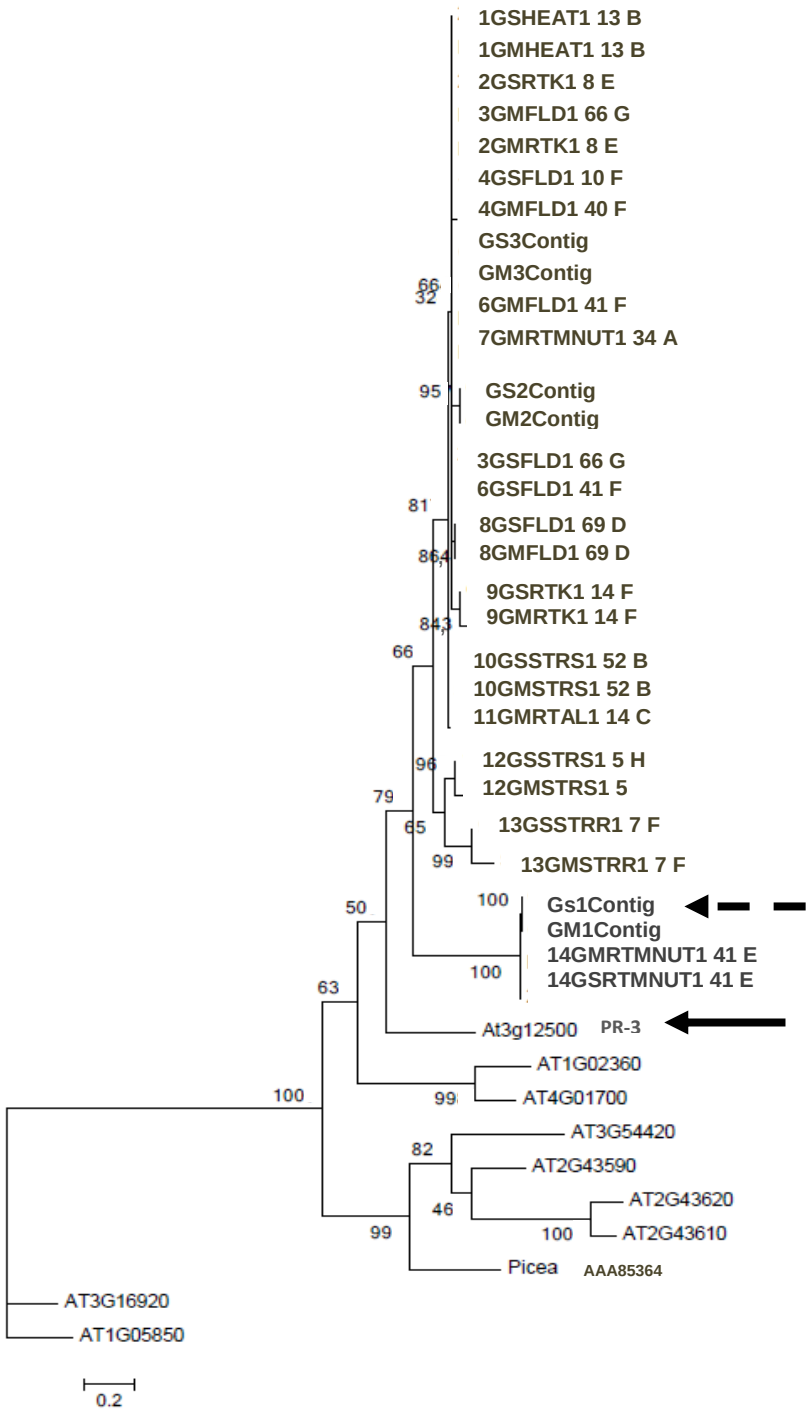

Figure 2.8: Maximum likelihood phylogenetic tree showing the relationship between the *PR-3 (chitinase)* gene sequences of various model organisms and the putative *PpaPR-3* ortholog. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

In addition to *FMO1, PR-5 (thaumatin-like* gene*)* was selected as a marker for the SAR pathway. The ML tree (Figure 2.9) and NJ tree (Supplementary Figure S11) for *PR-5* indicate a close relationship between all three contigs, the ESTs and the *Arabidopsis PR-5*. Sequences for *P. monticola* were available and ML phylogenetic analysis was conducted using these sequences, but no clear tree could be inferred. *P. monticola* belongs to a different subgenus (*Strobus*), and in order to improve the primer design to be *P. patula* specific, these sequences were removed from the phylogenetic analysis. The ML tree was drawn using the WAG+I+G (G = 2.087) evolutionary model obtained from ProTest. There was high bootstrap support for the main clade (1000). All three contigs were used to perform a TBLASTN analysis in TAIR. The highest e-value obtained was for contig1 ($3.8e^{-80}$) and primers were designed to specifically amplify a 152 bp region of contig1 (Supplementary Figure S4).

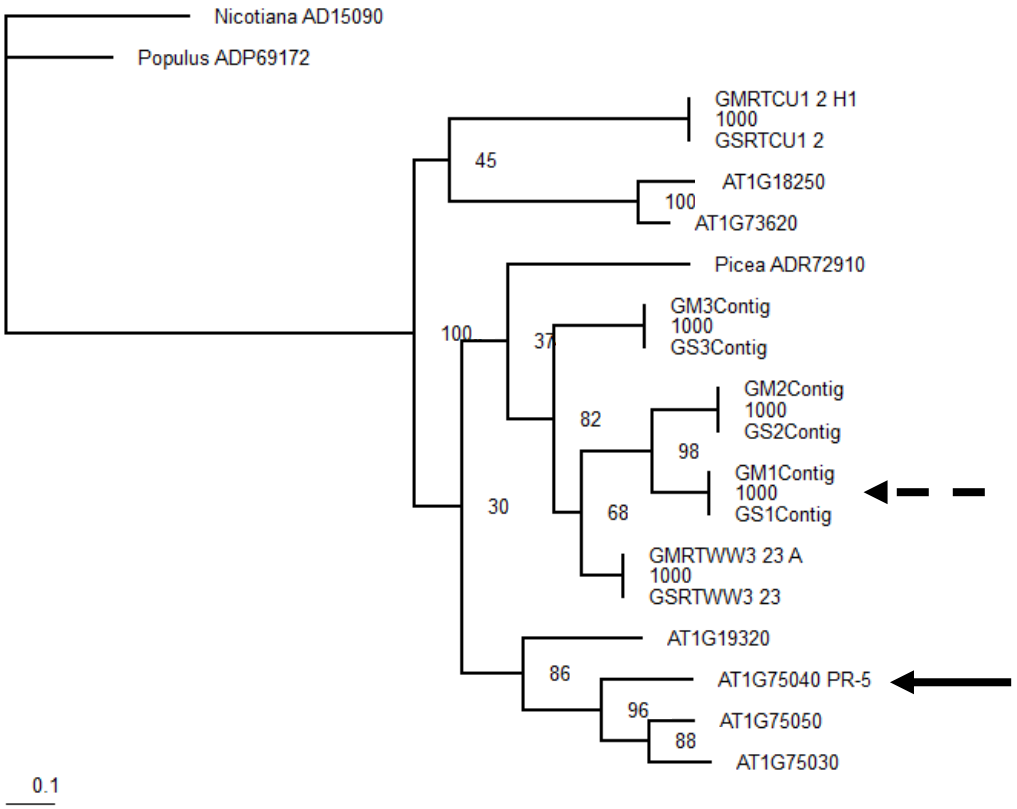

Figure 2.9: Maximum likelihood phylogenetic tree showing the relationship between the *PR-5* (*thaumatin-like*) gene sequences of various model organisms and the putative *PpaPR-5* ortholog. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea, Nicotiana* and *Populus* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

In order to find the ortholog for *LOX1 (lipoxygenase)*, a representative of the ISR pathway, a ML tree with the LG+G+I+F (G = 1.758) evolutionary model (Figure 2.10) and a NJ tree (Supplementary Figure S12) was used. As the *LOX* gene family encodes six proteins in *Arabidopsis* (Umate and Tuteja, 2011) all of them were added, as well as two sequences from *Populus*. Due to similar oxygenase activity *LOX1 and LOX5* have positional specificity and are classified as *9S-lipoxygenase*, whereas *LOX2, LOX3, LOX4* and *LOX6* are known as *13S-lipoxygenase* (Bannenberg *et al.*, 2009). As limited sequence was available there was difficulty in resolving this gene family Therefore, contig 2 (Supplementary Figure S5) is a representative of that group for this study.

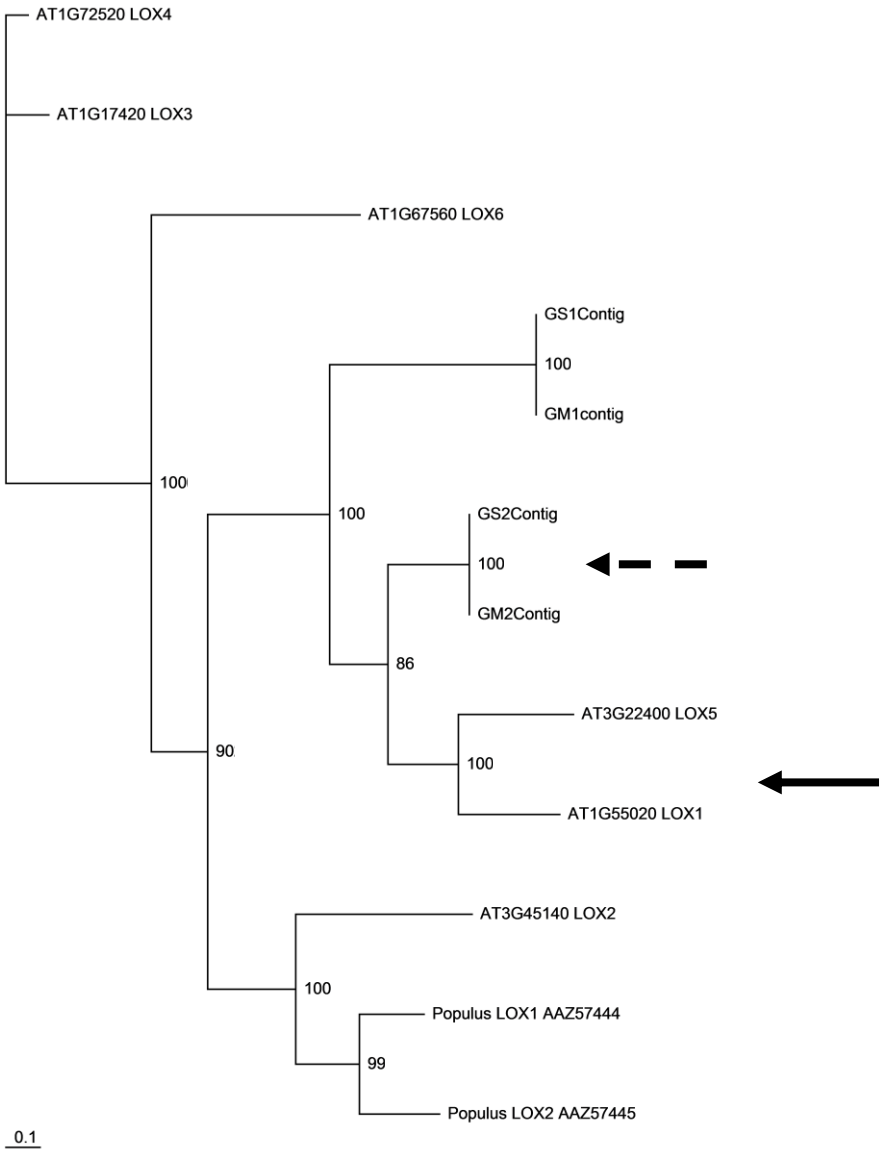

Figure 2.10: Maximum likelihood phylogenetic tree showing the relationship between the *LOX (lipoxygenase)* gene sequences of various model organisms and the putative *PpaLOX* ortholog. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Populus* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

The hevein-like protein also referred to as PR–4, is a defence gene associated with the ISR defence pathway. This gene in *Arabidopsis* has no other associated proteins so just one *Arabidopsis*, *Nicotiana* and *Populus* sequence were used to draw up the trees. The contig (Supplementary Figure S6) was assembled from all the ESTs identified and translated into a protein sequence. In order to construct a more robust tree the translated ESTs making up the contig were included. The ML tree drawn (evolutionary model WAG+I+G, G = 0.724, Figure 2.11) supported a close relationship to the tobacco *PR-4* with bootstrap statistics of 0.76, whereas the NJ tree (Supplementary Figure S13) did not clearly associate them. At present, contig1 is the chosen representative and primers based on this sequence were designed to amplify a 200 bp fragment.

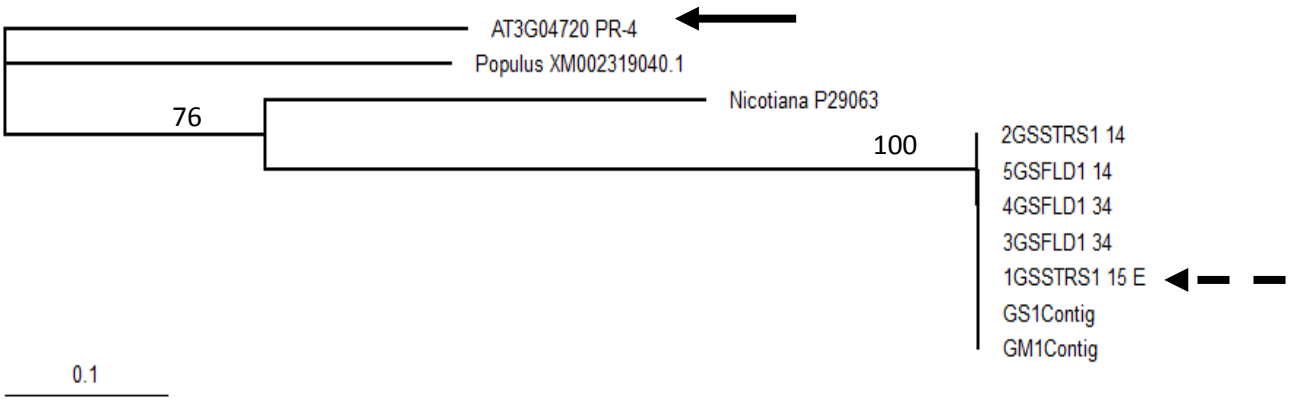

Figure 2.11: Maximum likelihood phylogenetic tree showing the relationship between the *PR-4 (hevein-like*) gene sequences of various model organisms and the putative *PpaPR-4* ortholog. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Populus* and *Nicotiana* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

2.4.3 Detecting reference gene orthologs in *P. patula* using phylogenetics

In order to conduct accurate gene expression analysis, reference genes (also known as house keeping genes) are required. The same methodology to find orthologs was applied as with the defence genes. The first reference gene analyzed was the *26s* proteasome gene, which is a proteosome subunit. This gene has been used in several *Arabidopsis* expression analysis studies (Czechowski *et al*., 2005). The ML tree (Figure 2.12) shows several ESTs and contig1 in the same clade with 99% boots trap support. The NJ (Supplementary Figure S16) supports this. Contig1 (Supplementary Figure S14) was chosen as the representative ortholog for this gene, and confidence in contig1 was substantiated by the TBLASTN results back to TAIR of contig1 and the two ESTs. The primer pair was designed to amplify a 204 bp fragment of the contig.

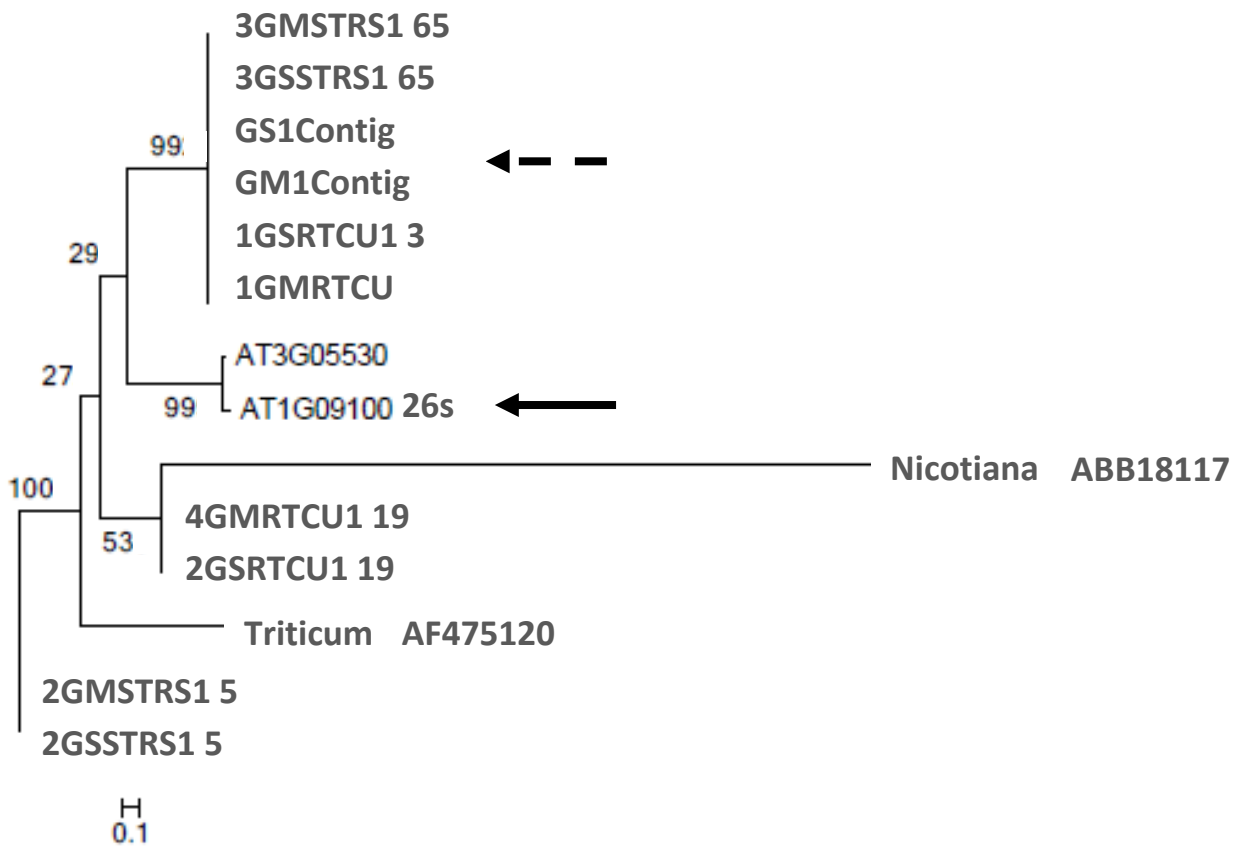

Figure 2.12: Maximum likelihood phylogenetic tree showing the relationship between the *26s (proteosome subunit)* gene sequences of various model organisms and the putative *Ppa26s* ortholog. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Nicotiana* and *Triticum* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

The second putative ortholog for a reference gene identified using a ML tree (evolutionary model JTT+G, G = 2.457) (Figure 2.13) and a NJ tree (Supplementary Figure S17) was *ARF1 (ADP-ribosylation factor-1)*. With high bootstrap support in both trees and the *Arabidopsis* gene found in the same clade as contig1, this contig (Supplementary Figure S15) was selected as a representative of the *ARF* gene family. The primers amplify a 258 bp fragment.

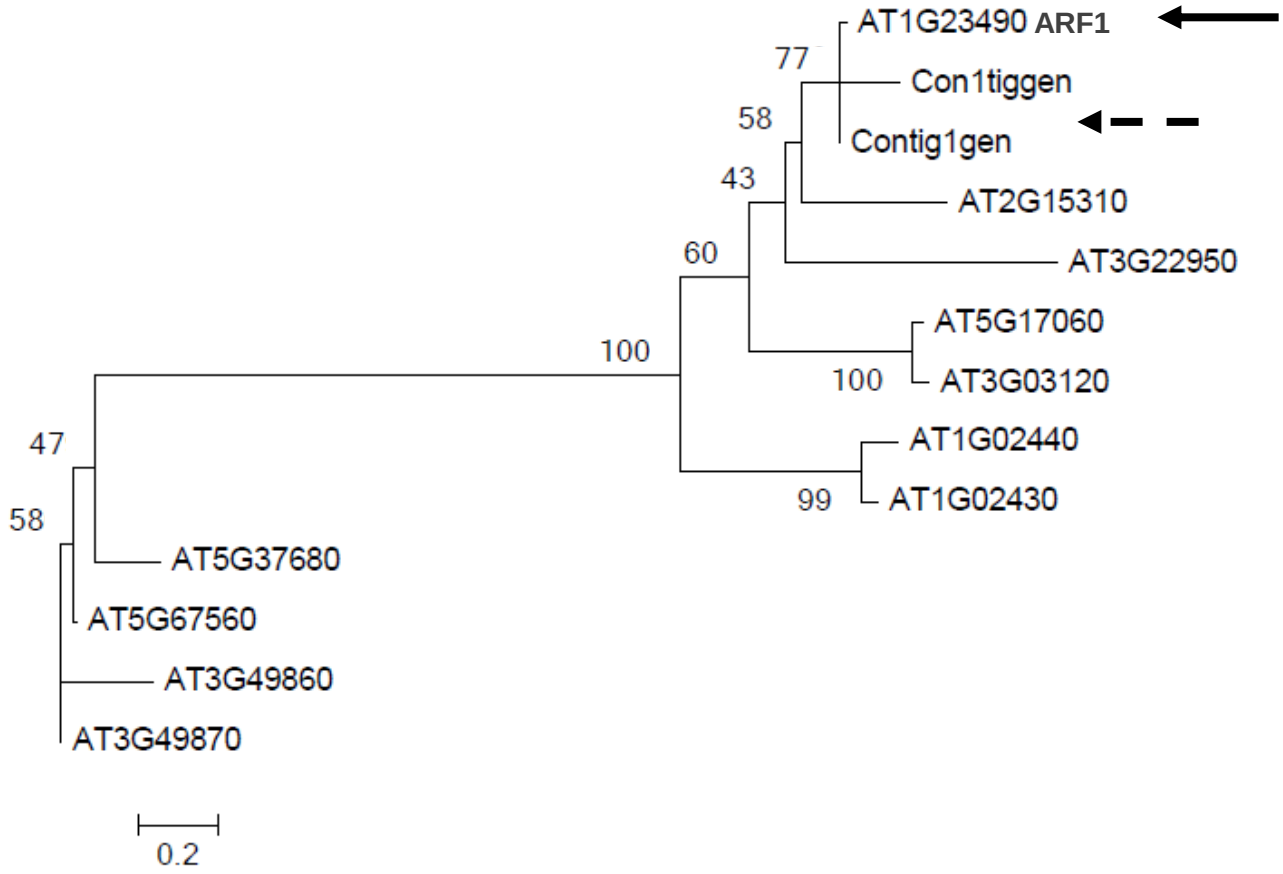

Figure 2.13: Maximum likelihood phylogenetic tree showing the relationship between the *ARF (ADP-ribosylation factor-1)* gene sequences of various model organisms and the putative *PpaARF* ortholog. EST names are provided as found in ConiferGDB and *Arabidopsis* ATG numbers in TAIR. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The ML bootstrap values were obtained from 1000 replicates. The branch length is proportional to the amino acid substitutions per aligned site.

2.4.4 Expression of known defence related marker genes in response to chitosan application

To relate the findings of the plant trials to the activation of systemic resistance, putative orthologs for known diagnostic defence genes were investigated for transcriptional changes that could be attributed to the application of chitosan. For this purpose, expression analysis of defence related marker genes was performed on *P. patula* seedlings treated with chitosan 10 mg/ml and control harvested 24 hrs after the first and booster application of the second greenhouse trial using RT-qPCR.

Total RNA from the samples harvested 24 hrs after the first and booster treatment was extracted and analyzed on a formaldehyde gel to verify the quality and integrity of the RNA (Figures 2.14 and 2.15). The RNA bands are clearly visible, which is an indication of good quality RNA that was not degraded, and cDNA synthesis was followed. An intron spanning primer pair *(PpaPR-4),* amplifies a 650 bp size fragment which is indicative of gDNA and a 250 bp fragment for the cDNA. This test was used for quality control. All six biological replicates together with a genomic DNA positive control and a cDNA positive control were examined (Figures 2.17 and 2.18). The purpose of this step was to ensure that no gDNA was present in the cDNA samples and this can be clearly seen in the figures below. A faint amplicon is visible in the no template control. This indicated some degree of contamination in the reaction. Repeated rounds of optimization using *PpaPR-4* were unsuccessful. Thus expression analysis of *PpaPR-4* was discontinued in this study.

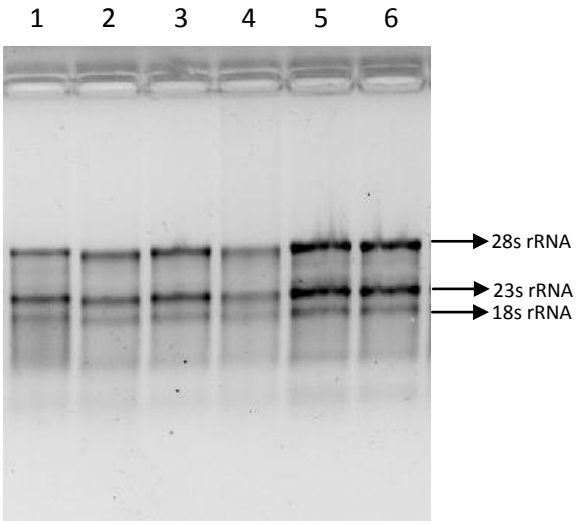

Figure 2.14: Total RNA extraction from *P. patula* samples harvested 24 hrs after the first chitosan treatment on a formaldehyde agarose 1% (w/v) RNA gel: Samples represent total RNA (500 ng) extracted from tissue treated with chitosan (10 mg/ml) and water control harvested 24 hrs after the first application. Lanes 1 to 3: control biological replicates. Lanes 4 to 6: treated biological replicates.

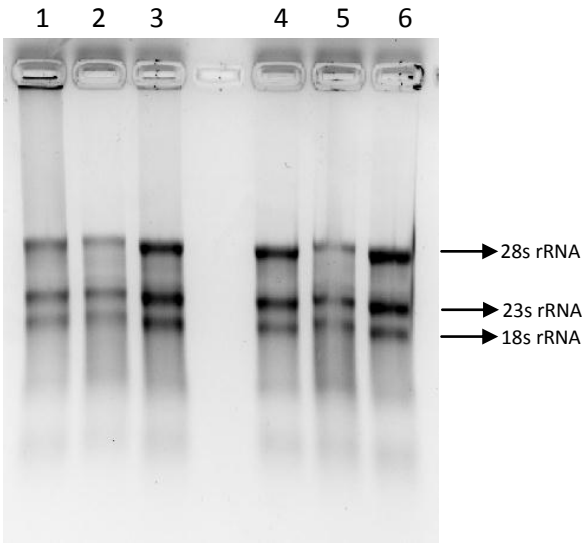

Figure 2.15: Total RNA extraction from *P. patula* samples harvested 24 hrs after the booster chitosan treatment on a formaldehyde agarose 1% (w/v) RNA gel: Samples represent total RNA (500 ng) extracted from tissue treated with chitosan (10 mg/ml) and water control harvested 24 hrs after the booster application. Lanes 1 to 3: control biological replicates. Lanes 4 to 6: treated biological replicates.

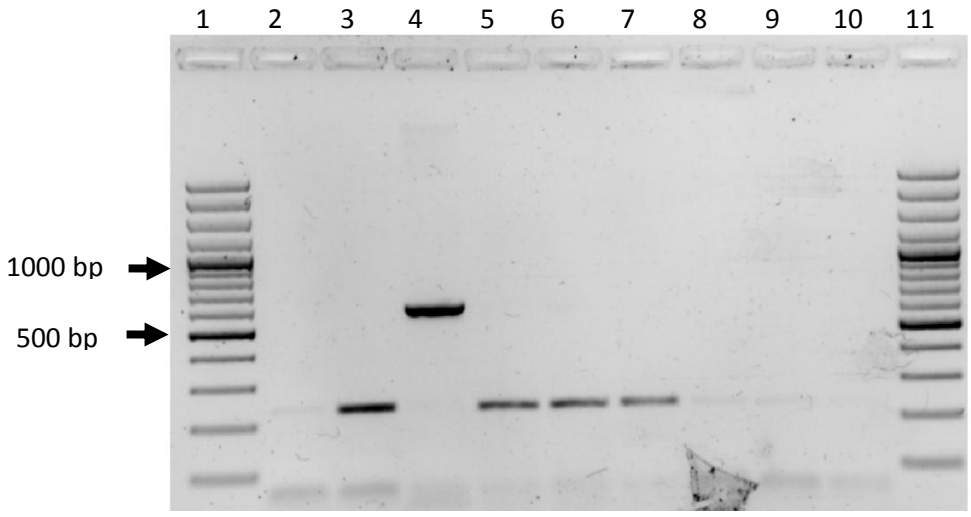

Figure 2.16: Gel electrophoresis on 1.5% (w/v) agarose gel using the *PpaPR-4* intron spanning primer pair to identify genomic DNA contamination: Samples represent cDNA synthesized from all three biological replicates of first application of chitosan (10 mg/ml) treated tissue. Lane 1 and 11: 100 bp DNA ladder plus (Fermentas); Lane 2: Negative control; Lane 3: Positive control of *P. patula* cDNA; Lane 4: Positive control of *P. patula* genomic DNA; Lane 5 to 7: three control samples; Lane 8 to 10: three chitosan treated samples.

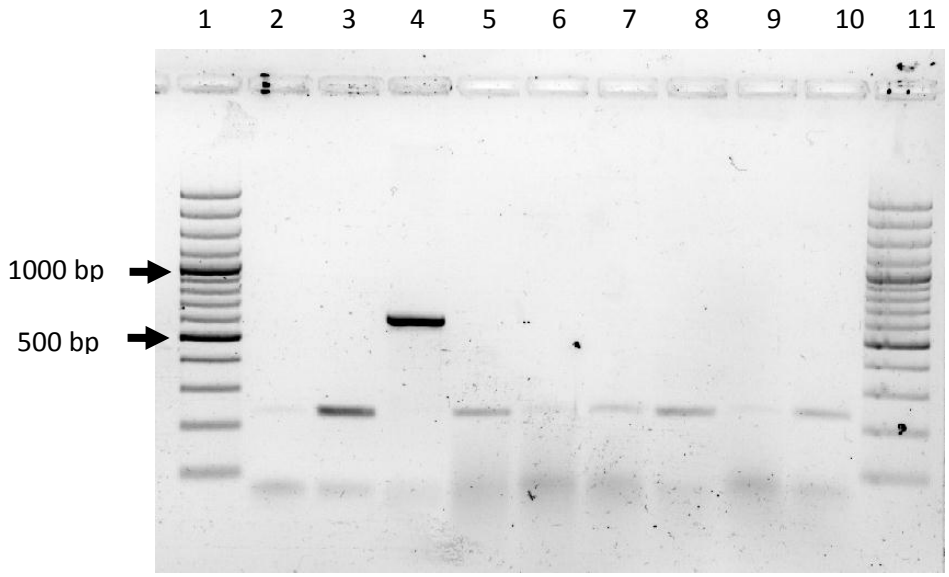

Figure 2.17: Gel electrophoresis on 1.5% (w/v) agarose gel using the *PpaPR-4* intron spanning primer pair to identify genomic DNA contamination: Samples represent cDNA synthesized from all three biological replicates of the booster application of chitosan (10 mg/ml) treated tissue. Lane 1and 11: 100bp DNA ladder plus (Fermentas); Lane 2: Negative control; Lane 3: Positive control of *P. patula* cDNA; Lane 4: Positive control of *P. patula* genomic DNA; Lane 5 to 7: three control samples; Lane 8 to 10: three chitosan treated samples.

2.4.4.1 Primers designed for the marker genes

Known defence marker gene sequences from *Arabidopsis*, various conifers and other species were compiled. Phylogenetic analysis was performed and the closest associated sequences were chosen for primer design. Not all primers were used, as they could not be optimized for use on the LightCycler® 480.

2.4.4.2 Marker genes expression analysis using RT-qPCR

To correlate the reduction of lesion length in chitosan treated seedling to the activation of induced resistance, the expression of known defence-related genes *PpaFMO1, PpaDXs1, PpaPAL, PpaPR-3, PpaPR-5* and *PpaLOX* were assessed. The plant trials revealed that chitosan had the potential to reduce disease symptoms for an extended period of up to six weeks after a very severe inoculation technique with *F. circinatum* spores. Therefore, samples harvested for that inducer 24 hrs subsequent to both applications were analyzed. As the RNA yield was fairly low, ranging from 200 ng/µl to 1000 ng/µl, 16 plants were bulked

for each biological replicate. After cDNA synthesis and clean-up, the concentration of the cDNA was determined to be approximately 2 ng/µl.

Following multiple attempts to obtain stable reference genes, the two reference genes that complied with the standards of the qBASE*plus* v1.0 software, were *Ppa26s* and *PpaARF1*. The use of two reference genes ensure that the following analysis was reliable and complies with MIQE regulations (Bustin *et al.*, 2009).

Expression level analysis of the diagnostic defence genes were done to examine whether the effect of the inducer in priming the plant for subsequent pathogen attacks could be linked to differential expression of the marker genes. This would reveal the activation of induced resistance caused by the application of chitosan. The melting curve profiles of the target genes showed single peaks for each gene thereby indicating that a single product was amplified (Supplementary Figures S22 and S24). The correct size amplicon was confirmed by performing agarose gel electrophoresis (Supplementary Figure S18). Although optimization was conducted (temperature gradients were tested and new primers were designed), primer dimers were evident in some reactions (Supplementary Figure S18). These primer dimers were not detected in the no template control reactions based on the melting curve analysis (Supplementary Figures S22 and S24) and thus did not affect quantification of target genes. To ensure that the amplicon was the target of interest, samples were sequenced and a BLAST analysis was performed against TAIR (Supplementary Table S6).

To perform analysis on the qBASE*plus* v1.0 software, crossing point values (Cq) were generated by the LightCycler® 480 absolute quantification software and transferred to qBASE*plus* v1.0. The software calculated the relative quantity of the expressed target genes by taking the reference genes into account and normalizing the target genes expression against that. For each reference gene, two stability factors were calculated. The first factor is the stability parameter (M) which determines the stability of the reference gene across other reference genes, having a threshold value not higher than 0.5. The higher this value is, the less stable the expression of the gene (Vandesompele *et al.*, 2009). The second factor looks at the expression levels across the sample set of the individual gene and is known as the coefficient of variation (CV). This factor needs to be more stringent and has a threshold value of 0.2 (Vandesompele *et al.*, 2009). The lower the M and CV values, the higher the stability of the reference gene pair (Hellemans *et al.*, 2007). For the first application of chitosan sample set, it was found that *PpaARF1* (M = 0.468, CV = 0.158) and *Ppa26s* (M =

0.468, CV = 0.168) was the most stable set of reference genes (Supplementary Table S2, Supplementary Figure S19). For the booster treatment samples the same reference genes were used and resulted in the M and CV values of *PpaARF1* (M = 0.468, CV = 0.168) and *Ppa26s* (M = 0.468, CV = 0.165) (Supplementary Table S4, Supplementary Figure S20). Another evaluation of the experiment is done as the software determines the percentage reproducibility, based on the technical replicates. This value needs to be at a minimum of 85%. Any one of the technical replicates that was an outlier due to the Cq value, with a difference greater than 0.5, was excluded in the further analysis. The reproducibility for the first sample set was identified as 89%, whereas the second experimental reproducibility was 90%.

The relative quantities for each gene pair were determined from the Cq values and the standard curves (Supplementary Figure S21 and Figure S23). For both time points the guidelines for the r^2- value, E value and standard error were followed, ensuring robust results (Supplementary Table S3 and S5, Taylor *et al.*, 2010). From there the determined CNQR values were examined and biological replicates with an abnormally high standard error were rejected, due to great variation. The biological standard error was used to determine the significance using the Student's *t*-test to identify significant differential regulation.

While the r^2- values according to the MIQE regulations should ideally be 0.98, values between 0.8 and 0.95 were obtained for the standard curves generated for each target and reference gene (Supplementary Table S3). Although not optimal, these standard curves were utilised for further analysis. It is acknowledged that this would affect the accuracy of the quantification results to a certain degree.

The analysis of the expression data was performed using the chitosan 10 mg/ml samples harvested 24 hrs after the first application to elucidate the possible activation after one treatment. The expression profiles revealed no significant differential regulation of *PpaLOX, PpaPR-5* and *PpaPR-3* compared to the control samples in their expression profiles (Figure 2.18). *PpaLOX* and *PpaPR-3* represented markers for the activation of the ISR pathway, whereas *PpaPR-5* was used to investigate the SAR pathway. The three expression profiles of the diagnostic defence genes reflect large biological variation between the replicates, making any inferences difficult. As none of the profiled genes were significantly up- or down-regulated (Student's *t*-test), the next step was to profile samples harvested from the booster application.

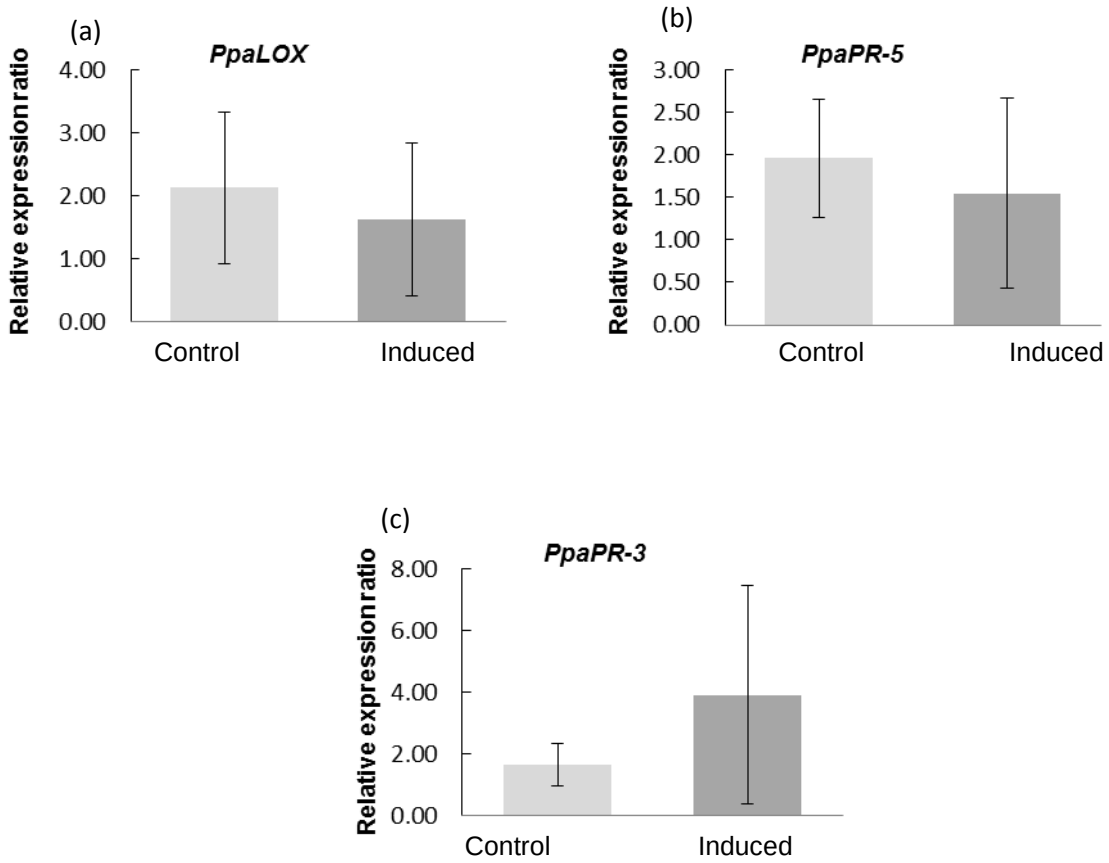

Figure 2.18: **Relative expression profiles of the target putative defence gene orthologs in *P. patula* from the samples taken 24 hrs after the first chitosan application**. The y-axis represents the relative expression ratios and the x-axis the two samples: control (light grey) versus induced material (dark grey). In (a) the *PpaLOX*, (b) *PpaPR-5* and *PpaPR-3* gene is represented. The error bars drawn represent the biological standard error (n=3). The * indicates the significant differential expression between the control and induced material with $p<0.05$ (Student's *t*-test).

At 24 hrs after chitosan application, no significant difference was observed for the defence marker genes tested, thus it was decided to determine whether the booster application affected the expression profile of another subset of defence marker genes. Due to the depletion of the harvested tissue the expression of *PpaPAL*, *PpaDXS1* and *PpaFMO1* was not profiled 24 hrs after the first chitosan application. Transcript expression levels of the known defence genes *PpaFMO1*, *PpaPR-3*, *PpaPAL* and *PpaDXS1*, using the *P. patula* material harvested at 24 hrs after booster application of chitosan 10 mg/ml, were assessed to associate the lesion length reduction to induced resistance activation. It was observed that

the defence gene (*PpaPAL*), linked to the jasmonate pathway (Wang et al., 2008), had an increased transcript expression that was statistically significantly ($p<0.05$, Student's *t*-test) higher than the control (Figure 2.19A). The target gene was four times more up-regulated than the respective control. The (*PpaDXS1*) had a significantly ($p<0.05$, Student's *t*-test) lower expression in the induced plant material compared to the control (Figure 2.19B). Expression profiles of the putative orthologs of (*PpaPR3*), which is associated with the ISR pathway (Liu *et al.*, 2005), and (*PpaFMO1*), a known SAR marker (Mishina and Zeier, 2006), indicated no significant differential expression (Figure 2.19C and D).

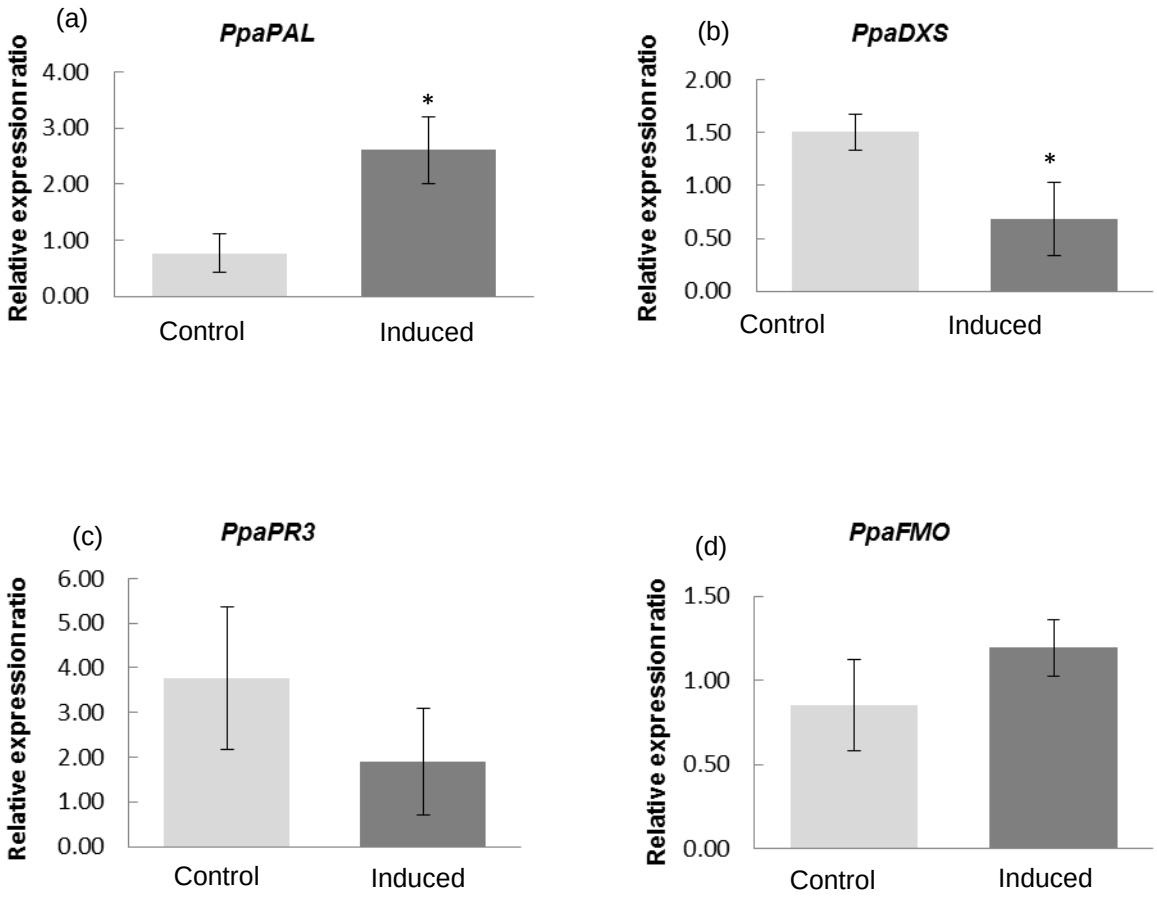

Figure 2.19: **Relative expression profiles of the target defence gene orthologs in *P. patula* from the samples taken 24 hrs after the booster chitosan application**. The y-axis represents the relative expression ratios and the x-axis the two samples control (light grey) versus induced material (dark grey). In (a) the *PpaPAL*, (b) *PpaDXS1*, (c) *PpaPR-3* and (d) *PpaFMO1* gene is represented. Error bars drawn represent the biological standard error (n=3). The * indicates the significant differential expression between the control and induced material with $p<0.05$ (Student's *t*-test).

2.5. Discussion

Pine is one of the most prevalent types of trees planted by forestry companies for specifically solid wood, pulp and paper. A current threat to the industry, causing great losses is the damage caused by the fungus *F. circinatum.* The fungus causes the death of young seedlings during the post planting phase, which increases the costs for re-establishment of pine trees. The approach of induced resistance could form part of an integrated management strategy together with good nursery hygiene practice.

Systemic inducible defence pathways can be activated through the exogenous application of chemicals (Schreiber and Desveaux, 2008; Verhage *et al.*, 2010). This study examined the effectiveness of several chemical and biologically-derived inducers in reducing disease symptoms caused by the fungus *F. circinatum* on *P. patula*. Furthermore, the activation of induced resistance in *P. patula* was determined at a molecular level by looking at the expression levels of known defence genes. The data presented in the study shows that exogenous application of chitosan on *P. patula* seedlings was able to reduce disease severity caused by *F. circinatum* infection. Following chitosan treatment, the diagnostic defence gene *PpaPAL*, an enzyme involved in the phenylpropanoid pathway, showed an increase in transcript abundance in *P. patula*, suggesting stimulation of plant defence responses. A decrease in transcript abundance of *PpaDXS1*, which encodes an enzyme involved in the first steps methylerythritol pathway, in the chitosan treated seedlings compared to the control was observed. Investigating the expression of these diagnostic defence genes may provide clues as to how resistance against *F. circinatum* in *P. patula* may be enhanced.

2.5.1 Identification of chitosan as a promising resistance inducer in *Pinus patula*

A panel of ten different inducers (MeJA, Messenger®, Chitin, Bion®, *F. oxysporum*, *P. fluorescens*, SA, Kannar®, flagellin, potassium phosphate and chitosan) selected from literature were tested for their efficacy in improving tolerance against *F. circinatum* on *P. patula*. Tests were performed both in the nursery and the greenhouse. Bion®, Messenger®, Chitin, MeJA and SA application reduced lesion length after manual inoculation with the pathogen at six weeks post application in nursery (Figure 2.1 & Figure 2.2). Subsequent greenhouse studies conducted on the top three inducers, MeJA, Chitosan (derivative of Chitin) and Messenger®, revealed that Chitosan at 10 mg/ml showed a significant reduction

in lesion length compared to the control plants for a period of six weeks (Figure 2.4). The percentage difference in lesion length observed between the control and treated seedlings is small as high inoculum dosage and a severe inoculation technique was applied, possibly masking the actual potential of the chitosan.

Chitosan is a derivative of chitin, which is made by de-acetylation of chitin. Chitin is a polysaccharide and represents the main structural component of fungal cell walls, as well as the exoskeleton of arthropods and insects. The polysaccharide is able to cause a response that mimics the activation of local and systemic defence signalling cascades (Kombrink *et al.*, 2011; Zhang and Zhou, 2010). Chitosan's natural occurrence in the environment makes chitosan an alternative to hazardous pesticides and promotes the agricultural application of this inducer (El Hadrami *et al.*, 2010; Ramírez *et al.*, 2010). Variation in effectiveness of the polysaccharide has been determined to be influenced by the molecular weight, degree of N-acetylation and pH of the solution (Raafat and Sahl, 2009). Many studies identified a variety of defence responses elicited in reaction to chitosan. Chitosan was shown to activate reactive oxygen species, one of the earliest observable plant defence strategies. In addition to this, chitosan enables the production of phytoalexins and PR proteins, which are part of the direct chemical defences (Hadwiger and Beckman, 1980; Povero *et al.*, 2011; Ramonell *et al.*, 2002). Physical defence barrier activation such as formation of callose and lignification of cell walls are other key functions activated, making the polymer chitosan attractive as a foliar treatment agent (Conrath *et al.*, 1989; El Hadrami *et al.*, 2010; Pearce and Ride, 1982). Furthermore, this polymer has been proven to exercise inhibitory effects on hyphal growth and prevent spore germination of fungi (Amborabé *et al.*, 2008; Benhamou and Thériault, 1992; El Ghaouth *et al.*, 1994). Application of chitosan on grapevine stimulated defence reactions and prevented *Botrytis cinerea* growth (Aranaz *et al.*, 2009; Trotel-Aziz *et al.*, 2006).

Chitosan at a concentration of 10 mg/ml was required to activate induced resistance over a period of six weeks (Figure 2.4). This was in accordance with what was observed in *P. radiata* where treatment with chitosan (10 mg/ml) resulted in a reduction of disease incidence of 60% and had a lasting effect over a six week period (Reglinski *et al.*, 2004). In that study (Reglinski *et al.*, 2004) the authors attributed the lower disease severity to the activation of induced resistance, which explains what was observed in the current work. Both percentage Livestem and disease incidence are used to express induced resistance. Disease incidence is an absence or presence of disease symptoms, whereas percentage Livestem indicates any delayed effect on symptom development in diseased plants. In this

study all plants were symptomatic and therefore percentage Livestem was used. Induced resistance was not only observed in pine through the application of chitosan, but also in spruce, grape, cucumber and *A. thaliana,* emphasising the potential chitosan in enhancing induced defences (Ferri *et al.*, 2009; Lippert *et al.*, 2009; Ma *et al.*, 2011; Povero *et al.*, 2011).

Screening of pine infected with *F. circinatum* in a controlled environment and measuring lesion length has been correlated to survival rate of pine in the natural setting (Smith, 2011). The longer the lesion, the lower the chance of survival of that pine seedling in the field. This correlation allows the use of greenhouse screening techniques to estimate performance in the natural environment. Although chitosan provides a measure of protection in *P. patula* against *F. circinatum* in this study, it seems small. However, the larger impact and the accumulative effect of the treatment have to be considered. Spraying pine seedlings at an early stage will prime the seedlings and will also increase post planting survival. Having 5% more seedlings surviving at the nursery stage results in increased plant establishment in the field. This will have an impact on the amount of wood and the other end products for the forestry sector. Most importantly, the method used to investigate the effectiveness of the inducers was severe, as high concentrations of the fungus were applied to *P. patula* and the trees were wounded for infection, which does not reflect natural infection in the nursery. Therefore, seeing a small significant effect under such severe inoculation conditions emphasises the potential of chitosan to activate induced resistance.

2.5.2 Other inducers that influenced disease severity

The other inducers such as Bion®, Messenger®, MeJA and SA showed promising results in priming the *P. patula* seedlings against *F. circinatum,* but only for a short period (Figure 2.2). The commercially available product Messenger® has been used effectively against the soil borne pathogen *Fusarium* spp. in tomato, cucumber and wheat (Wei and Betz, 2007). Bion® has been successfully applied against *Phytophthora cinnamomi* in *P. radiata (Ali et al., 2000)*. Further investigations into dosage as well as application frequency and timing need to be done to improve the potential of these inducers for activation of induced resistance in *P. patula*.

Interestingly, of the ten inducers that were tested, two inducers (Kannar® and *Pseudomonas fluorescens*) had an opposite effect to the rest, and actually enhanced symptom severity of the disease (Figure 2.2). Plants have specific defence pathways that are effective against

necrotrophic pathogens, such as the JA and ET pathways, whereas the SA pathway is associated with biotrophs (Glazebrook, 2005). The balance required between JA and SA is dependent on the nature of the pathogen, its lifestyle and can be the determining factor as to whether a plant will succumb to infection (Bari and Jones, 2009; Beckers and Spoel, 2006; Grant and Jones, 2009; Kunkel and Brooks, 2002; Mur *et al.*, 2006; Schenk *et al.*, 2000). Activation of the wrong defence pathway would merely provide ideal conditions for the pathogen to thrive and therefore one would observe an increase in disease severity. Specifically for the bacterial biocontrol agent *P. fluorescens,* most of the responsive transcripts would be associated with bacterial defence responses which are different to fungal responses. These biocontrol agents may be competing for nutrients or space with the pathogen, which results in an antagonistic response to the pathogen, or by producing antimicrobial compounds and activating a host induced defence response (Compant *et al.*, 2005). Kannar® is a product just released into the market recently with little information available. It is known to enhance plant health, but it also seemed to have activated the wrong defence pathway for *F. circinatum*. From this study it is not recommended to use these commercial products as means to boost pine seedlings against *F. circinatum*.

2.5.3 Comparison of field versus greenhouse conditions

The field trial in this study reflects the natural conditions in which the seedlings are grown. As the fungus *F. circinatum* is present and established in South Africa since the 1990s, the *P. patula* seedlings are challenged with natural concentrations of the pathogen in the nursery (Mitchell *et al.*, 2011; Viljoen *et al.*, 1994). The actual potential of the inducers may have been masked due to early induction of systemic resistance caused by the presence of the pathogen. In the first trial, no clear difference between the different treatments was observed which could be explained by the fact that the treated and control plants may have had activated induced resistance prior to the trial. This phenomenon of early induction of resistance was observed when *P. radiata* was repeatedly mechanically inoculated with *F. circinatum* and a progressive reduction in lesion size was visible (Bonello *et al.*, 2001). A correlation has been made between lesion length in greenhouse studies and observed mortality in the field (Smith, 2011) and this allows for effective evaluation of resistance in various pine species by means of artificial inoculation. In the greenhouse, seedlings grew in a disease free environment whereby any response could directly be related to inducer treatment effectiveness. Comparing nursery trials to greenhouse studies therefore allows one to differentiate between the masked effect and the actual inducer potential. For industry the ability to infer direct relationships between greenhouse studies and field trials is useful as

any possible improvement to the management strategies against *F. circinatum* should first be tested under controlled conditions.

2.5.4 The purpose of the booster application

Studies have demonstrated that numerous applications of inducing agents actually increased the level of systemic induced resistance. Research on the long term effect of MeJA on *P. sylvestris* was conducted and it was shown that application of MeJA three times a year was more effective than once a year (Heijari *et al.*, 2008). This was confirmed by measuring terpenoid levels. One application of MeJA only had a lasting effect for a short period of time (<20 days), whereas multiple applications enhanced the total defence level. Cumulative evidence shows that increased induction of peroxidase adds to induced resistance which is activated by inducers. Hammerschmidt *et al*. (1982) found that there was a parallel relationship between the number of lesions and the increase in peroxidase activity, but also that the peroxidase levels were higher in cucumber plants which were given a booster application. Treating cucumber with various inducers showed that a second treatment was more effective against powdery mildew and increased peroxidase levels (Alkahtani *et al.*, 2011). This was also true for *F. circinatum* whereby repeated mechanical inoculation of *P. taeda* with the pathogen caused a progressive reduction in lesion length as the plant was primed for subsequent challenges with that pathogen. Lastly, pine trees are long lived trees and therefore booster applications would be necessary to extend the period of induced resistance.

In this study it was observed that the booster application affected the expression of *PpaPAL* by up-regulating it, and also caused a down-regulation of *PpaDXS1*. *PpaPR-5* and *PpaLOX* were examined only after the first application, which indicated no differential regulation and thus were not profiled in material treated with a booster application. The gene *PpaPR-3* was profiled both 24 hrs after the first treatment with chitosan as well as after the booster application. No differential regulation was observed at both these time points. This indicates that the booster application was more effective in priming the pine seedlings based on the responses observed for *PpaPAL* and *PpaDXS1*. However, as only *PpaPR-3* could be compared between the two applications more genes need to be profiled to make more inferences on the effectiveness of a booster application.

2.5.5 The inoculation technique employed currently

Important criteria for an inoculation technique, such as robustness, repeatability and ease of applying the pathogen come into consideration when conducting plant trials. Various types of inoculation techniques have been tested to screen for *F. circinatum* tolerance (Barrows-Broaddus and Dwindell, 1984; Dvorak *et al.*, 2009; Kuhlman, 1987; Viljoen *et al.*, 1994). The method used in this study is employed at FABI disease clinic. The technique, established by Porter (2010) employs a topping approach, whereby the top of the seedling is cut off and fungal inoculum is pipetted onto the wound, ensuring that each tree obtains the same amount of spores. The certainty of each plant receiving the same dosage of pathogen is not present when making use of a spraying or soil drenching method. Topping is a harsh practice and may lead to breaching of the defence systems, thereby masking induced resistance activated by biochemical treatments. More severe symptoms are to be expected due to the extreme inoculation technique employed during the greenhouse trials compared to the nursery situation. This gives the impression that the inducer is less effective than it could potentially be, because the seedlings have been overwhelmed with fungal spores. It is hypothesised that inducer treated seedlings experiencing undisrupted inoculation, would perform better. The margin of disease severity decreased due to inducer application would be larger compared to control seedlings. On the other hand, this technique gave clear indications on the success of the inducers tested in providing protection to the plant under these harsh conditions, which are not commonly found in nurseries. Screening for resistance against *F. circinatum* in pine using the inoculation method applied in this study has been used by the forestry sector, in order to estimate survival rate of seedlings in the field.

2.5.6 Phylogenetic approach taken to identify putative defence marker genes

Various algorithms can be employed to perform phylogenetic analysis. For the purpose of this study, neighbour joining (NJ) and maximum likelihood (ML) were the algorithms used. The Neighbour joining algorithm constructs trees from evolutionary distance data. These trees are based on the least total branch length and require a shorter amount of computational time compared to most other algorithms. Yet, as a result of this, the algorithm could yield a suboptimal tree (Saitou and Nei, 1987). To ensure the optimal selection of orthologs, maximum likelihood (ML) was used as a second approach to ensure more statistical power. ML phylogenetics exhaustively tries to choose the best tree under certain criterion from a large number of trees (Saitou and Imanishi, 1989). For this reason the tree created is much more reliable, but this method requires far more computational time.

Elucidation of true orthologs is dependent on the amount of data available for the species of interest. Having too little information may lead to missing the actual functional ortholog as many copies may be present in the genome. As pine is a gymnosperm with a large genome size of 28,000 Mbp, many repeated DNA sequence is present in the genome. The increase in genome may be due to duplication, and the duplicate genes may not even be functional orthologs, or there are multiple copies of the functional genes present. Having this in mind, phylogenetics is the foundational step to identifying possible putative defence marker genes. To follow on, functional studies will be needed to clarify which of the orthologs is the true ortholog.

For the purpose of this study all possible ESTs were included and the closest sequence to the model organism was chosen. There is limited sequence data available and only from the pine species *P. taeda*, a close relative to *P. patula*. This made ortholog predictions difficult. Despite this, the phylogenetic approach provided a starting point in identifying the defence responses associated with chitosan treatment in *P. patula*. Currently the genome of *P. taeda* is being sequenced and this will in future allow for more detailed studies to be conducted (Neale and Kremer, 2011).

2.5.7 Defence responses elicited by chitosan application in *P. patula*

Pinus defence responses are highly integrated and coordinated, and may include inducible chemical or protein based defence mechanisms and or anatomical defences (Eyles *et al.*, 2009). This resistance can be enhanced through inducer application and verified by expression profiling of diagnostic defence genes. Chitosan treatment appeared to reduce *F. circinatum* associated disease symptoms in *P. patula* seedlings and therefore differential regulation of the identified diagnostic defence genes were examined. The four-fold increase in expression of *phenylalanine ammonia lyase* (*PpaPAL*) is attributed to the application of chitosan (Figure 2.19). This can be correlated to previous studies conducted in carrots and grapevine which also showed increased transcript levels of *PAL* after chitosan treatment and increased tolerance when challenged with a necrotrophic fungus (Ferri *et al.*, 2009; Jayaraj *et al.*, 2009).

PAL mediates the carbon flux that is required for the phenylpropanoid pathway (Hahlbrock *et al.*, 1989). Its biosynthesis is triggered by various stimuli such as pathogen attack, tissue wounding and numerous environmental factors. But most importantly the phenylpropanoid pathway results in the production of flavenoids, isoflavenoids, lignin and phenolics (Osakabe

et al., 2009). These metabolites lead to the activation of chemical defences and anatomical defences such as lignification of the cell wall. Accumulation of phenolic compounds is associated with both passive and systemic defence and the magnitude of defence is linked to the increase of precursors of phenolic acids (Singh *et al*., 2010).

PAL is related to induced resistance, yet it cannot be assigned to any specific defence pathway as it triggers a co-operative mechanism between the salicylic acid and jasmonate pathways (Yang *et al.*, 2011). Studies conducted in grapevine and carrots demonstrated that the application of chitosan resulted in the induction of *PAL* and an increased tolerance to infection with a necrotrophic fungus *Botrytis cinerea* was observed (Trotel-Aziz *et al.;* 2006, Ferri *et al.;* 2009, Jayaraj *et al.,* 2009). A microarray experiment with chitosan treated *A. thaliana* revealed that defence genes such as camalexin and CoA-ligase, associated with the last steps of the phenylpropanoid pathway were up-regulated (Povero *et al.*, 2011). In light of available knowledge and the result obtained from the expression profile of *PpaPAL*, it is hypothesised that the phenylpropanoid pathway may provide a clue as to what defence mechanisms are employed by *P. patula* in order to combat *F. circinatum* infection. In order to substantiate this hypothesis further, expression analysis using various marker genes from the phenylpropanoid pathway need to be performed.

Expression analysis also indicated differential regulation of 1-deoxy-D-xylulose 5-phosphate synthase 1 (*PpaDXS1*). A three-fold down-regulation was observed at 24 hrs after the booster application of chitosan in *P. patula* (Figure 2.19). This gene, *PpaDXS1* is known to play a role in catalysing the first steps of the methylerythritol phosphate pathway, which takes place in the plastid. Furthermore, it is essential for the synthesis of products such as pyridoxal, thiamine and terpenes. Additionally, *DXS* promotes the onset of the isopentenyl diphosphate and dimethylallyl diphosphate pathways (Kim *et al*., 2009). Zulak and Bohlmann, (2010), have identified two types of the *DXS* enzyme. Type I has one isoform (*DXS1*) and is important for the production of isoprenoids of general metabolites such as carotenoids and phytol and occurs in mostly photosynthetic tissue. Type II has its role in production of specialized metabolites and involves two isoforms (*DXS2a, DXS2b*). In *Arabidopsis,* the type II *DXS*s are absent, explaining the low production of monoterpenes and diterpenes. *DXS* is involved in the production of resin, a terpene commonly associated with coniferous trees. This type of resistance barrier is known to be present in older trees, but is not the line of defence in place for young seedlings. It was hypothesised that chitosan may increase the expression of the resin as a means of defence, however, the profiling of *PpaDXS1* revealed that the pathway was suppressed. The down-regulation may be ascribed

to the carbon partitioning that leads to suppression of some defence pathways such as the methylerythritol phosphate pathway, in order to accommodate the increased carbon need for the phenylpropanoid pathway. During the first year of growth, carbon is mostly assigned to the growth of the plant and not to resin production (Hudgins *et al.*, 2006; Wainhouse *et al.*, 2009). After the growth spurt of the first year, a drastic growth was observed in duct size and therefore in the amount of resin (Hudgins *et al.*, 2006; Wainhouse *et al.*, 2009). A positive correlation of resin duct size and the quantity of resin has been observed in several studies. In this study only *PpaDXS1* in *P. patula* was able to be profiled, so further research is required to examine the *PpaDXS2* transcript levels. Various time-points need to then be included for the different growth stages of *P. patula*. This will provide an idea as to which *PpaDXS* gene is the diagnostic marker to use for terpenoid defence.

The other four genes that were profiled in this experiment were *thaumatin-like* (*PpaPR-5*), *flavin-dependent monooxygenase* (*PpaFMO1*), *lipoxgenase* (*PpaLOX*), and *chitinase* (*PpaPR-3*). The *thaumatin-like* gene (*PpaPR-5*) was used as the diagnostic defence gene for the systemic acquired resistance pathway (SAR) in analysing the sample harvested 24 hrs after the first chitosan spray (Figure 2.18). Thaumatin-like proteins (PR-5) are involved in the reaction of plants towards stresses such as pathogen attack, drought and cold temperatures. The biological function of this gene includes antifungal activity, glucanase activity and the activation of the SAR pathway (Liu *et al.*, 2010a). A recent example of increased expression of *PR-5,* is found in a tolerant European plum cultivar (*Prunus domestica*), which was infected with *Monilinia fructicola.* When compared with the profile of a susceptible cultivar, the increased expression of PR-5 could explain the possible tolerance to the pathogen (El-Kereamy *et al.*, 2011). A similar observation was made in wheat in response to stripe rust (Wang *et al.*, 2010). In western white pine (*P. monticola*) thaumatin-like proteins (PmTLP) were identified using a proteomic approach. The authors investigated mRNA expression of the *PR-5* genes in response to the pine blister rust pathogen *Cronartium ribicola* and the *PmTLP* family was found to be involved in resistance against this pathogen (Liu *et al.*, 2010b). For this study it was hypothesised that chitosan treatment on *P. patula* seedlings may have caused an increase in transcript abundance of *PpaPR-5.* But the analysis indicated no differential regulation after the first treatment. This is not in line with what Jayaraj *et al*. (2009) observed, when carrots were treated with chitosan. They observed an elevated response of *PR-5* in their data compared to the control 10-70 hrs after treatment. As only limited sequence for *P. taeda* is available perhaps a more conclusive identification of *PR-5* orthologs will be achieved once the genome sequence is made available and differential regulation of the correct gene ortholog would be observed. Further

profiling at various time-points should be done in order to obtain more conclusive results with respect to the function of *PpaPR-5* in defence in *P. patula*.

Flavin-dependent monooxygenase 1 has numerous functions in pathogen resistance. The function of *FMO1* is said to involve the oxidation of nucleophilic substrates and is possibly involved in cellular redox changes caused by reactive oxygen species (Lenk and Thordal-Christensen, 2009; Sehlmeyer *et al.*, 2010). The gene is a marker for the SAR pathway in *A. thaliana,* where its local and systemic expression was identified during inoculation with *Pseudomonas syringae* pv. *tomata* DC3000 (Mishina and Zeier, 2006). It's crucial role in SAR was confirmed in studies done by Jing *et al*. (2011; Mishina and Zeier, 2006). It was observed that SAR was no longer able to function in a knock–out mutant of *FMO1*. Besides the priming of the SA-dependent defence pathway, *FMO1* also causes the priming of callose deposition and seems to regulate cell death (Ent *et al.*, 2009). Furthermore, there is a requirement for *FMO1* upregulation for the presence of basal defence, as well as for the triggering of resistance gene-mediated defence (Schlaich, 2007). Seeing that chitosan is a fungal derivative and that ISR is associated with fungal pathogen attack, we do not expect to see SAR related genes playing a role. This could explain why there is no differential regulation observed at 24 hrs after the booster chitosan application when *PpaFMO1* was profiled (Figure 2.19).

In order to encompass a wider range of defence pathways, the ISR pathway was examined by profiling the expression levels of the marker defence gene *chitinase* (*PR-3*). The chitinase enzymes play a crucial role in hydrolysing chitin which is a major fungal cell wall component. It was thus expected that the application of a deacetylated version of chitin would have an effect on the regulation of *PR-3*. During fungal attack, chitinase is commonly one of the prominent plant defence genes that respond in combating the pathogen. Increased levels of chitinase in carrots and grapevine as a result of chitosan treatment was associated with the enhanced resistance present against *Alternaria radicina* (Jayaraj et al., 2009). In the current study, *PpaPR-3* showed no significant differential regulation after both the first and booster application of chitosan in *P. patula*. The fact that no differential expression between treated and control seedlings was observed 24 hrs after the first treatment could be due to the fact that the first application did not have a noticeable effect (Figure 2.18). Furthermore large biological variation was observed and hence little can be inferred from the results. The expression profile for *PpaPR-3* 24 hrs after the booster treatment also showed no significance, therefore, chitosan seems to have no effect on the differential regulation of chitinase (Figure 2.19). The deficiency of *PR-3* induction is in line with the work conducted

by Venter (2004) who demonstrated that *P. patula* seedlings challenged with *F. circinatum* showed no detectably induced levels of *chitinase*. This deficiency was explained to be a potential reason as to why *P. patula* is highly susceptible to the fungus (Venter, 2004). Perhaps the seedlings revert to alternative pathways, such as the phenylpropanoid pathway, to help defend themselves.

Another representative of the ISR pathway that was profiled was *lipoxygenase* (*PpaLOX*). The *LOX* enzymes are important for the octadecanoid pathway, leading to the biosynthesis of jasmonic acid (Bell *et al.*, 1995). The *PpaLOX* gene was selected, since in the study performed by Trotel-Aziz *et al*. (2006), investigating defence responses in grapevine, *LOX* was stimulated by chitosan application. Peak levels were reached at 6 hrs post treatment. In the current study *lipoxygenase* showed no significant increase in activity, compared to the control at 24 hrs post first chitosan application (Figure 2.18). Trotel-Aziz *et al.* (2006) observed an increase in *LOX* expression with increasing chitosan treatment, therefore profiling after the second/booster application may show an increase in *LOX* activity.

2.6 Conclusion and Future prospects

The study revealed that out of a panel of ten chosen inducers, chitosan application had a significant effect in reducing lesion length, suggesting the onset of induced resistance. This observation is congruent with previous studies in the field. Treatment with chitosan was performed on seedlings at the nursery stage. Application of the treatment at this time would increase the survival rate after planting. Therefore, chitosan seems to offer potential for protecting pine seedlings against *F. circinatum*. As only two concentrations were examined, there is room for improvement in that a dose response study can be performed in future. Chitosan is very difficult to dilute due to its coarse structure, making application on a larger scale impractical. As work on chitosan has just started, possible development as a commercially available product will make usage friendlier. In this study, a very destructive inoculation technique was applied as the top part of the seedlings were cut off. This makes the effectiveness of the inducer more convincing, since it was effective under severe stress imposed on the plant. Yet, a comparison should be done using non-destructive inoculation techniques to determine the response to chitosan. Such non-destructive methods could be root or soil drenching, ensuring no physical barriers of the plant are being breached thus not activating defence prior to infection.

The knowledge of induced resistance (IR) is at its early stages. Making use of IR as a protective measurement for plants, is currently at its infancy; hence it is important to fill gaps in knowledge surrounding the mechanisms of IR (Eyles *et al.*, 2009). Future research provides opportunities to identify IR defence mechanisms that are unique defences to long lived trees such as pine trees. Strong interest is being expressed in using IR agriculturally as a means of crop protection, as it is an eco-friendly alternative strategy that can be employed to combat pests (Eyles *et al.*, 2009). Applying IR in the field needs risk assessment and investigation into cost-benefit. In the field, various environmental factors influence the impact and efficiency of IR responses in the host, therefore this strategy needs to be deployed in a smart and sensible way. Clearly critical questions still need to be tackled to exploit IR. However, the results concerning chitosan as an inducer of induced resistance in *P. patula* look promising. We therefore suggest further testing of chitosan treatment as part of an integrated management strategy in conjunction with good hygiene practices to improve resistance in *P. patula* seedlings against *F. circinatum*.

Currently whole genome sequencing of *P. taeda* is underway which will provide a platform for further studies on defence mechanisms in *Pinus* (Neale and Kremer, 2011). In the meantime, putative orthologs were investigated using 410 000 ESTs available to identify the response to chitosan. The expression profiles give a glimpse of the possible line of defence required to protect against *F. circinatum*, seeing that *PpaPAL* the defence marker gene for the phenylpropanoid pathway was increased four-fold. This pathway is known to cause the production of secondary metabolites which have antifungal properties explaining the reduction in the lesion size. Furthermore, the upregulation of *PpaPAL* may be an indication of cell wall lignification as secondary metabolites function in anatomical defences as well. The markers used in this study are the first ones used in *P. patula* and provide a starting point in developing our knowledge of pathogen defence mechanisms in this conifer. More genes do need to be explored and a whole microarray profile will aid in expanding the knowledge on defence mechanisms present in *P. patula.* This knowledge can be used for breeding and biotechnology strategies to enhance resistance in *P. patula* against pathogens.

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3. Concluding Remarks

Induced resistance is a defence mechanism by which a plant is able to improve its resistance against subsequent challenges by a pathogen. Associated with induced resistance is the activation of SAR, ISR and SIR but also anatomical modifications, as well as the production of antimicrobial proteins and chemicals. Further downstream each of those responses lead to the activation of certain defence genes which could then be used to investigate the onset of induced resistance. The principle of induced resistance has been used in agriculture, where inducers were sprayed onto crops have been shown to elicit a defence response and prime the crop.

The main aim of this study was to elucidate defence responses associated with inducer application in *Pinus patula*. Firstly, various inducers were tested for their effectiveness in reducing disease severity caused by *F. circinatum.* Phylogenetic analysis was done to detect possible putative orthologs of key defence marker genes. Subsequently molecular analysis was performed to identify the activation of induced resistance. Expression profiles of the putative orthologs were elucidated by means of RT-qPCR and revealed differentially expressed genes.

This MSc study has identified chitosan as biochemical treatment which reduced disease symptoms over a period of time. Furthermore, we were able to identify six putative defence gene orthologs. The expression profiles of those genes revealed that induced resistance seemed to have been triggered by chitosan and was effective in priming *P. patula*. Though the amount of sequence data available for pine and the number of defence genes profiled were limitations for this study, it provides insight as to what resistance pathway may be effective to reduce *F. circinatum* disease severity in the pine seedlings.

Future avenues from this study would be to conduct large scale profiling of the entire transcriptome which will provide a broader view of the defence mechanisms in pine. Investigation of more time points, as well as profiling response of the other inducers used in this study that showed significance in reducing lesion length is of great interest. What expression profiles would be observed compared to chitosan? A big leap forward in understanding the complexity of pine defence is to be expected once the genome of *P. taeda* is made available. Additionally, gene functionality studies as well as chemical analysis will allow for a clearer understanding of pine – pathogen interactions. Following from this,

breeding strategies can be explored to improve resistance which may not just be conifer resistance against *F. circinatum* in pine.

Supplementary Data

Supplementary Data A

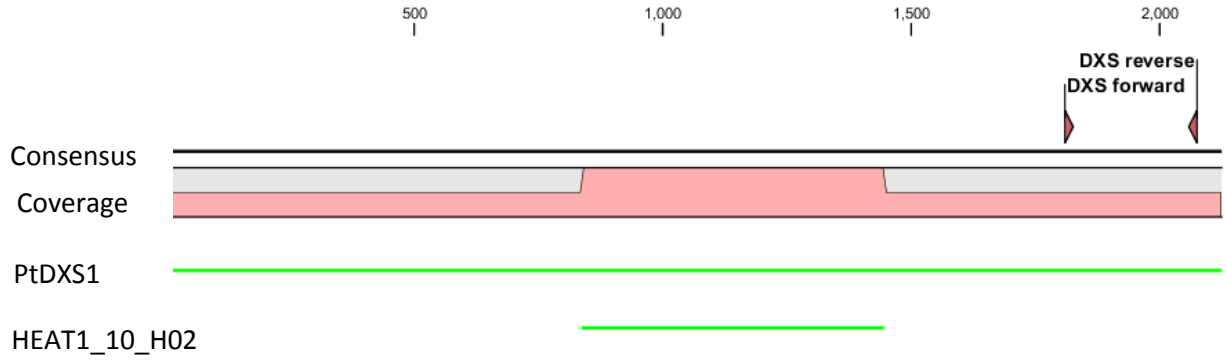

Figure S1: *PpaDXS1* (*1-deoxy-D-xylulose 5-phosphate synthase)* contig map. The two ESTs, retrieved both from NCBI and ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

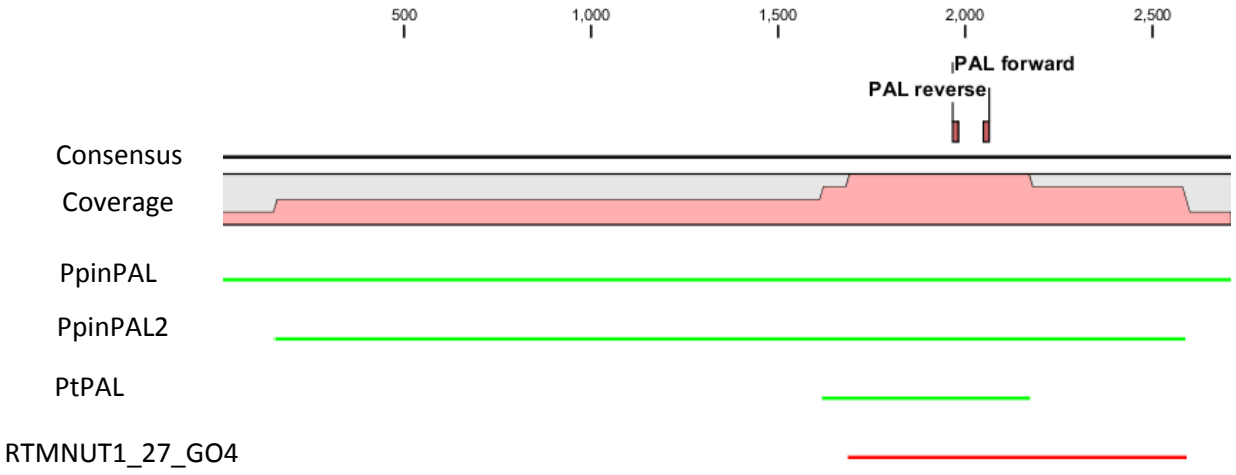

Figure S2: *PpaPAL* (*phenylalanine ammonia lyase)* contig map. The four ESTs, retrieved both from NCBI and ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

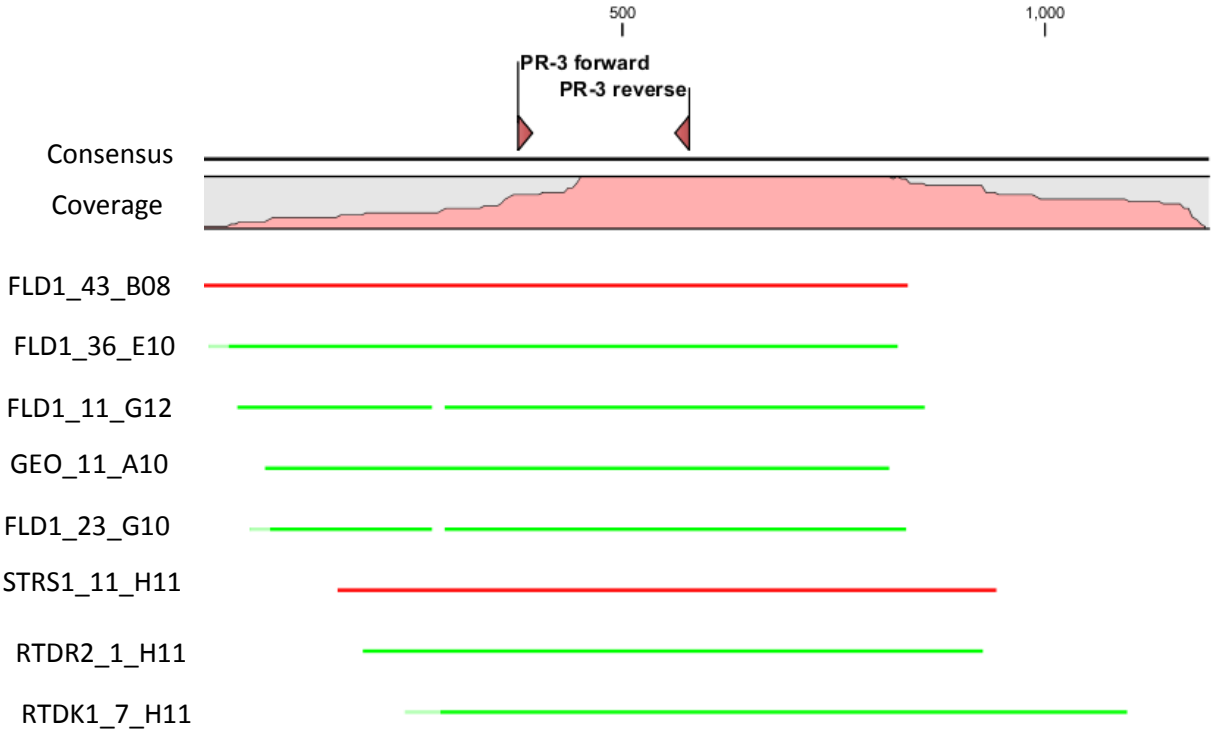

Figure S3: *PpaPR-3 (chitinase)* contig map. The eight ESTs, retrieved from ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

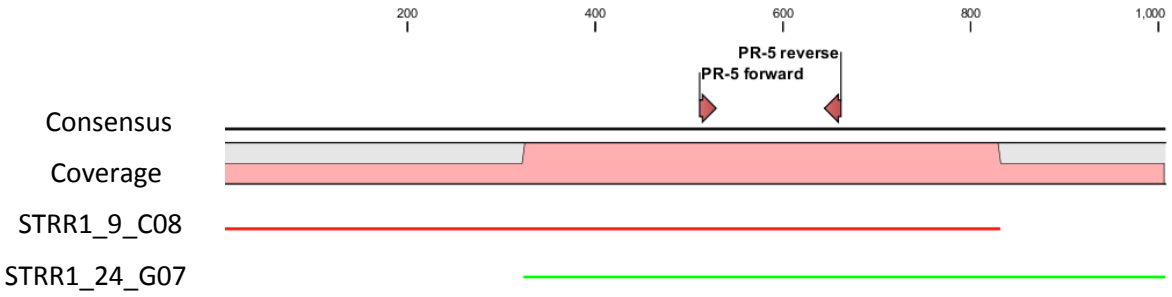

Figure S4: *PpaPR-5 (thaumatin-like)* contig map. The two ESTs, retrieved from ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

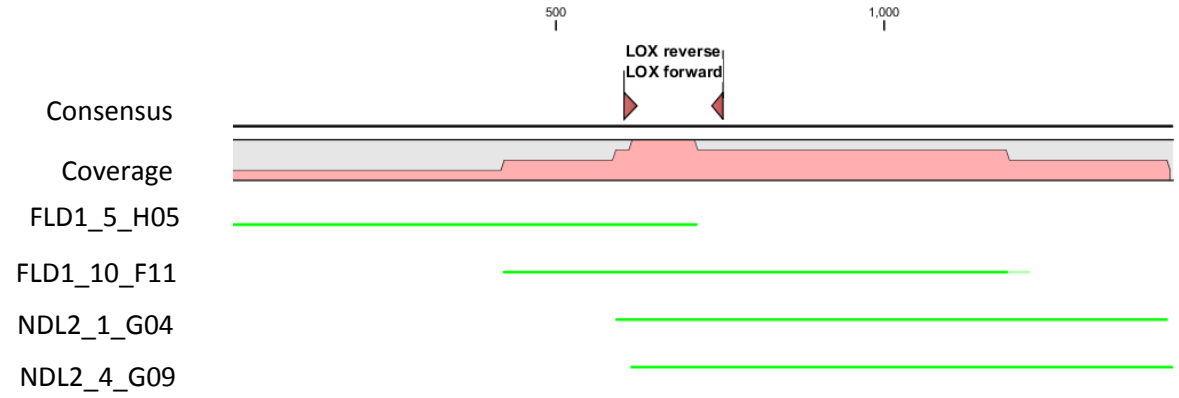

Figure S5: *PpaLOX* (*lipoxygenase*) contig map. The four ESTs, retrieved from ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

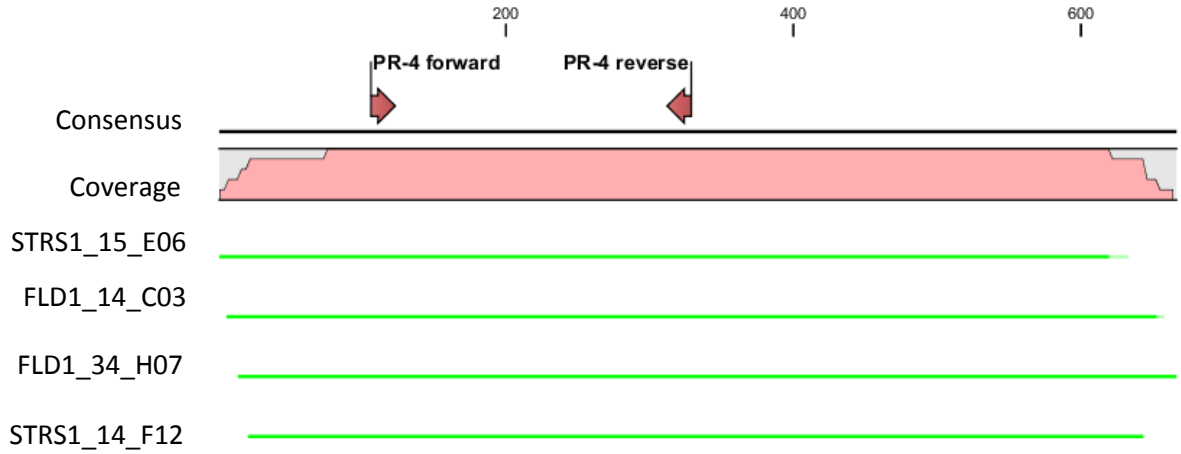

Figure S6: *PpaPR-4 (hevein – like protein)* contig map. The four ESTs, retrieved from ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

Supplementary Data B

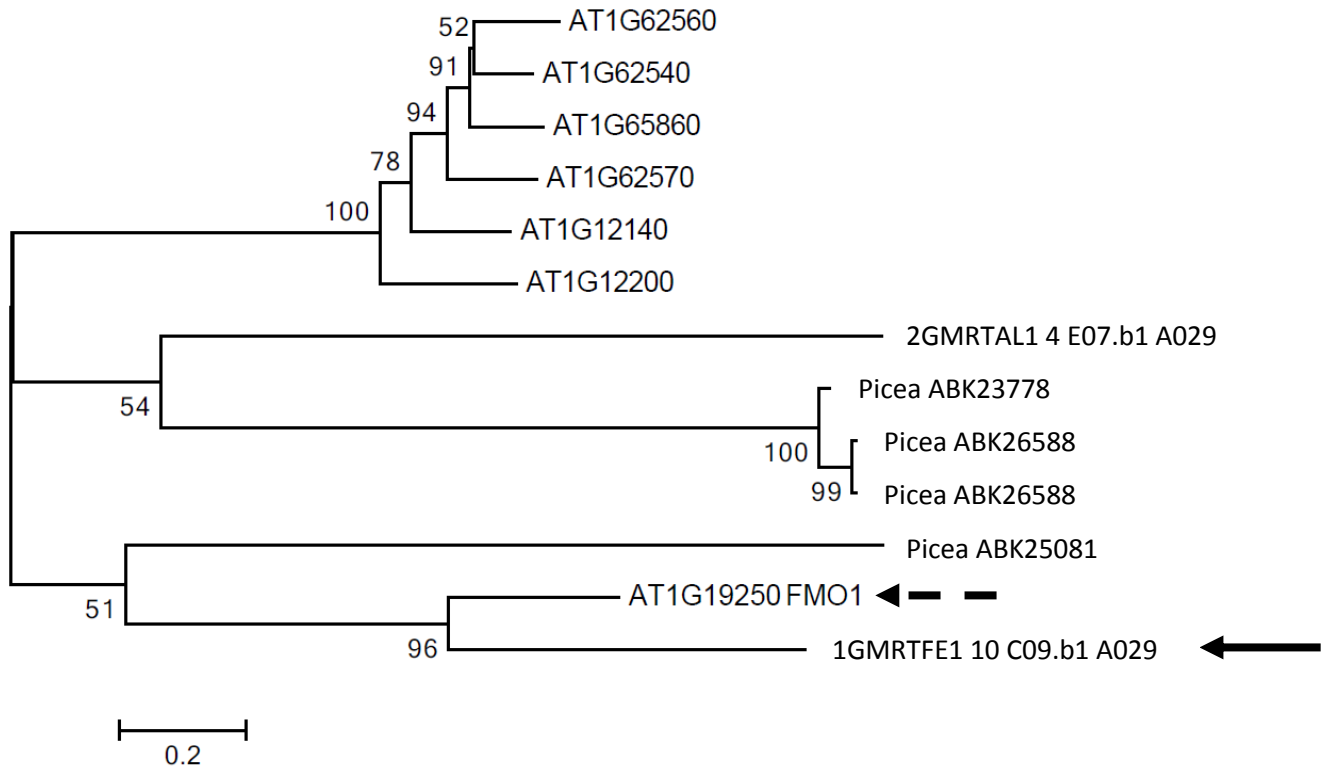

Figure S7: Neighbour joining tree for the plant *PpaFMO1 (flavin dependent monooxygenase)* resistance gene based on amino acid sequences. . EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea* genes as found in NCBI. The dotted arrow represents the *Arabidopsis* gene and the solid arrow highlights the sequence chosen as template for primer design The length of each branch is proportional to the substitutions per site given by the scale. The numbers found at the branches are the percentage of bootstrap replications (10000) supporting the branch.

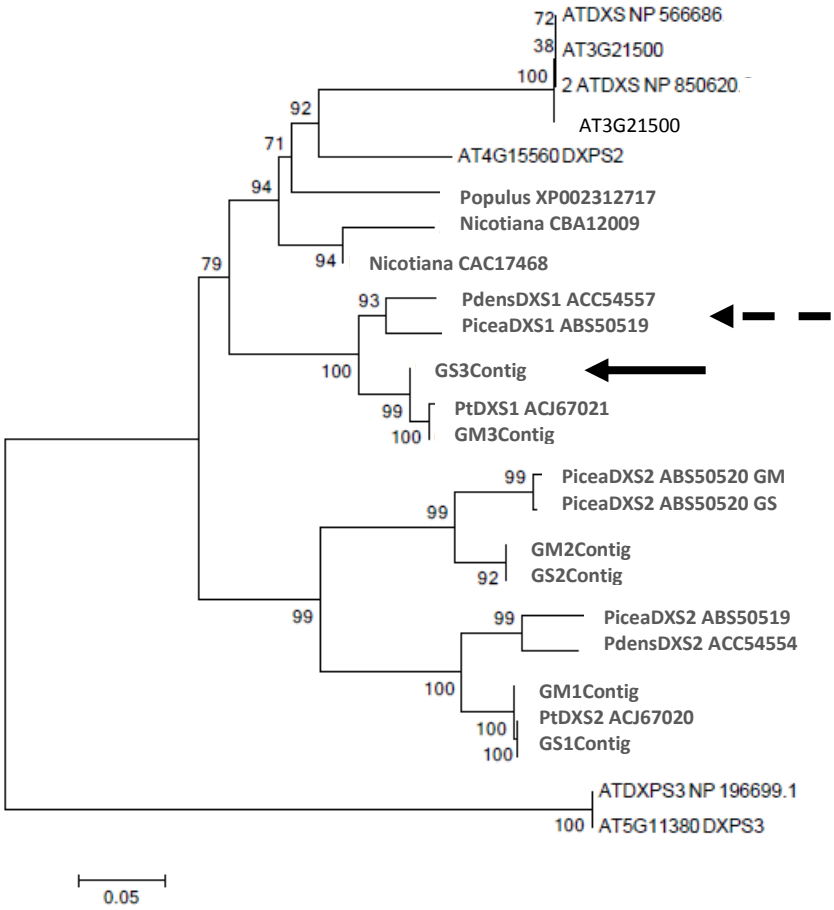

Figure S8: Neighbour joining tree for the plant *PpaDXS1* (*1-deoxy-D-xylulose 5-phosphate synthase)* resistance gene based on amino acid sequences. In this tree sequences from *Piuns densiflora* (Pdens), *P. taeda* (Pt), *Nicotiana*, *Populus* and *Arabidopsis* were included. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea, Nicotiana, Populus* and the *P. densiflora (Pdens)* and *P. taeda (Pt)* genes as found in NCBI. The dotted arrow represents the *Picea* gene and the solid arrow highlights the sequence chosen as template for primer design. The length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch.

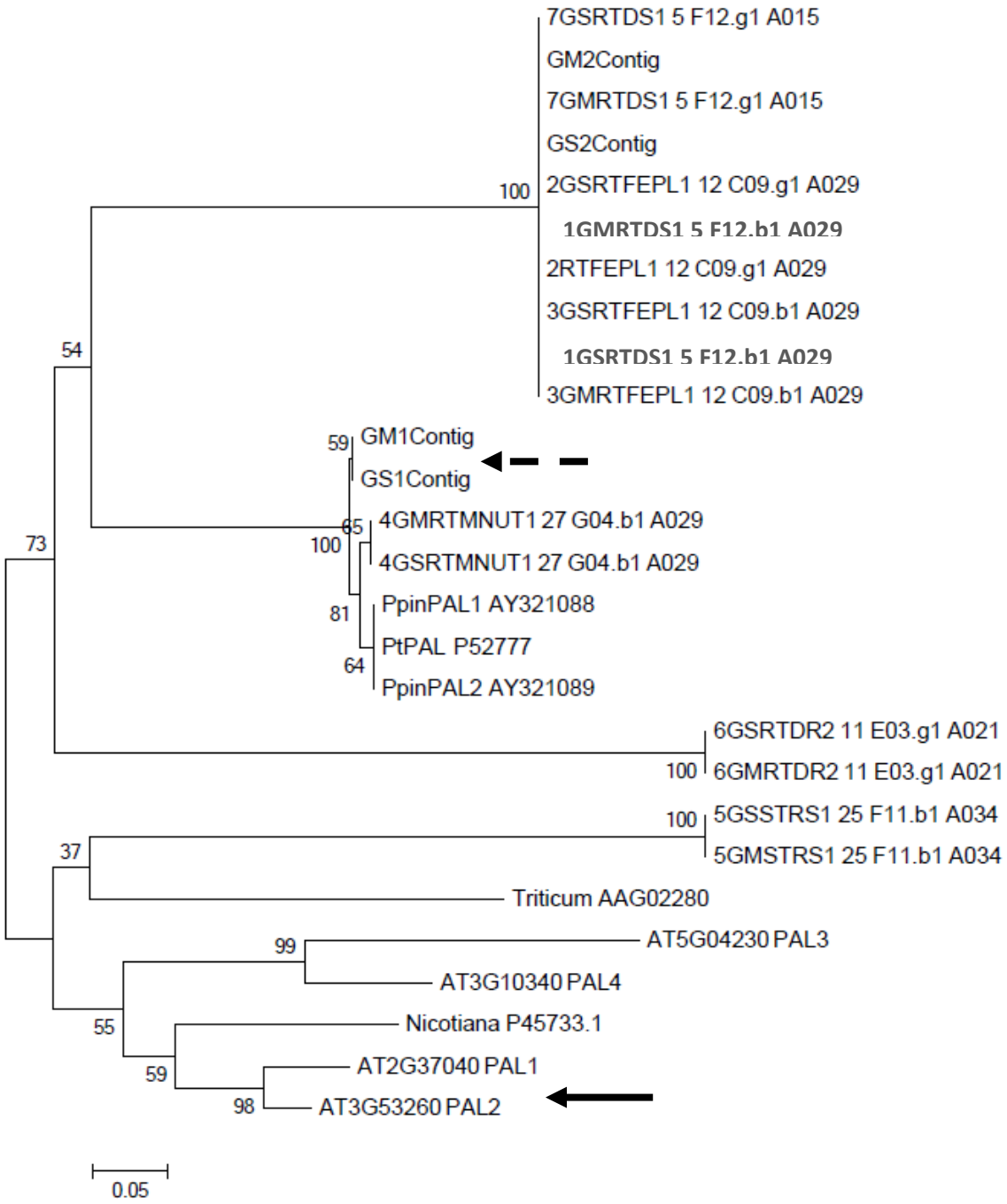

Figure S9: Neighbour joining tree for the plant *PpaPAL* (*phenylalanine ammonia lyase)* resistance gene based on amino acid sequences. . EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea, Nicotiana, Triticum, Pinus pinaster (Ppin)* and *P. taeda (Pt)* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch. The sequences of tobacco, wheat and spruce were included.

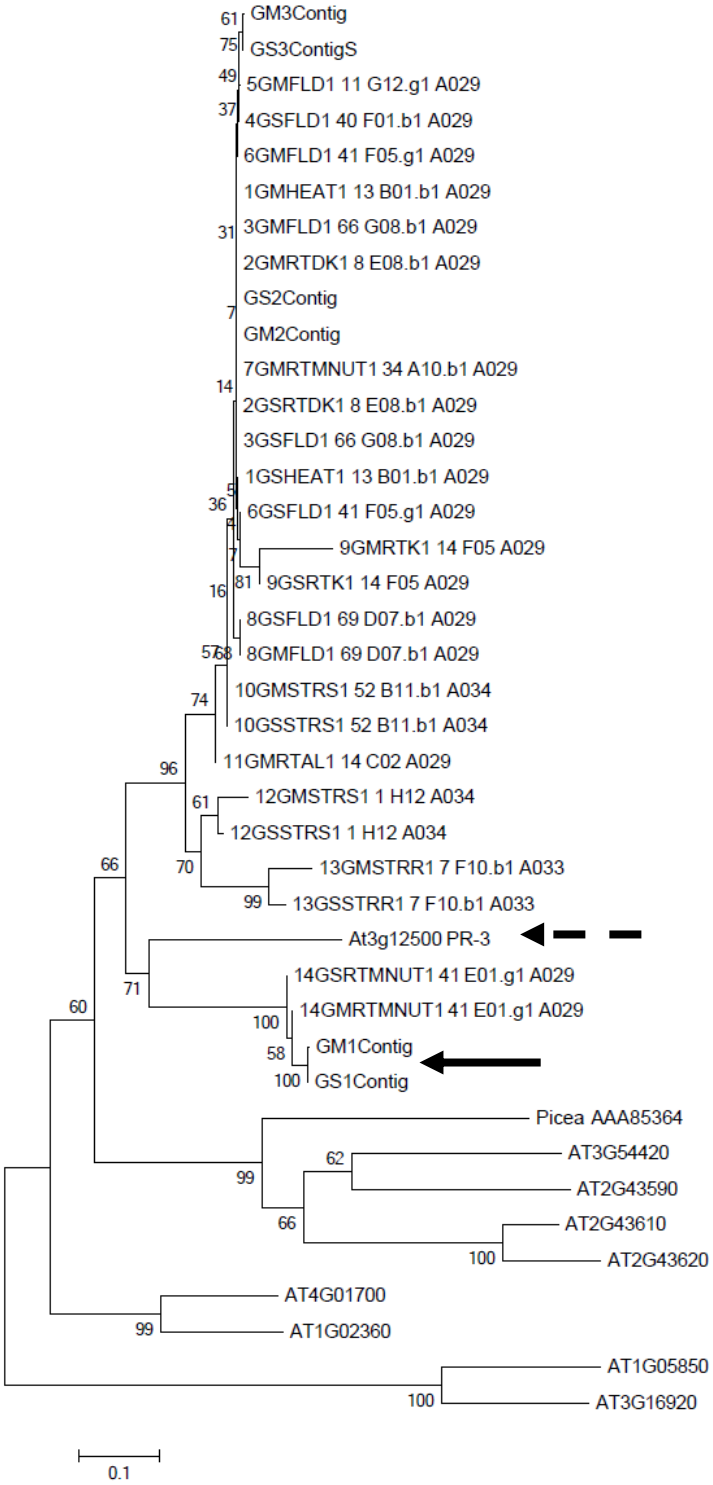

Figure S10: Neighbour joining tree for the plant *PpaPR-3 (chitinase)* resistance gene based on amino acid sequences. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch.

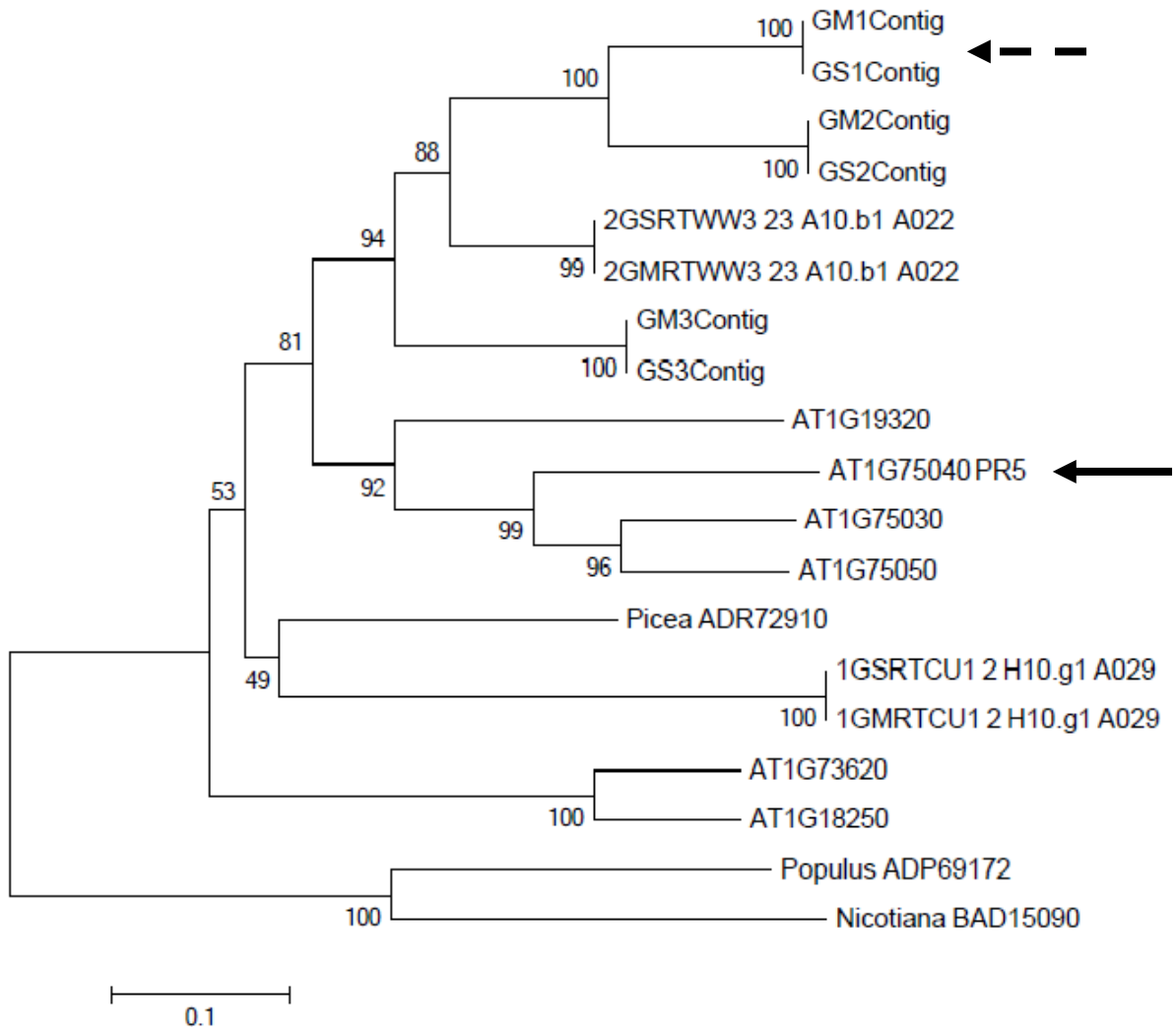

Figure S11: Neighbour joining tree for the plant *PpaPR-5 (thaumatin-like)* resistance gene based on amino acid sequences. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Picea, Nicotiana* and *Populus* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch.

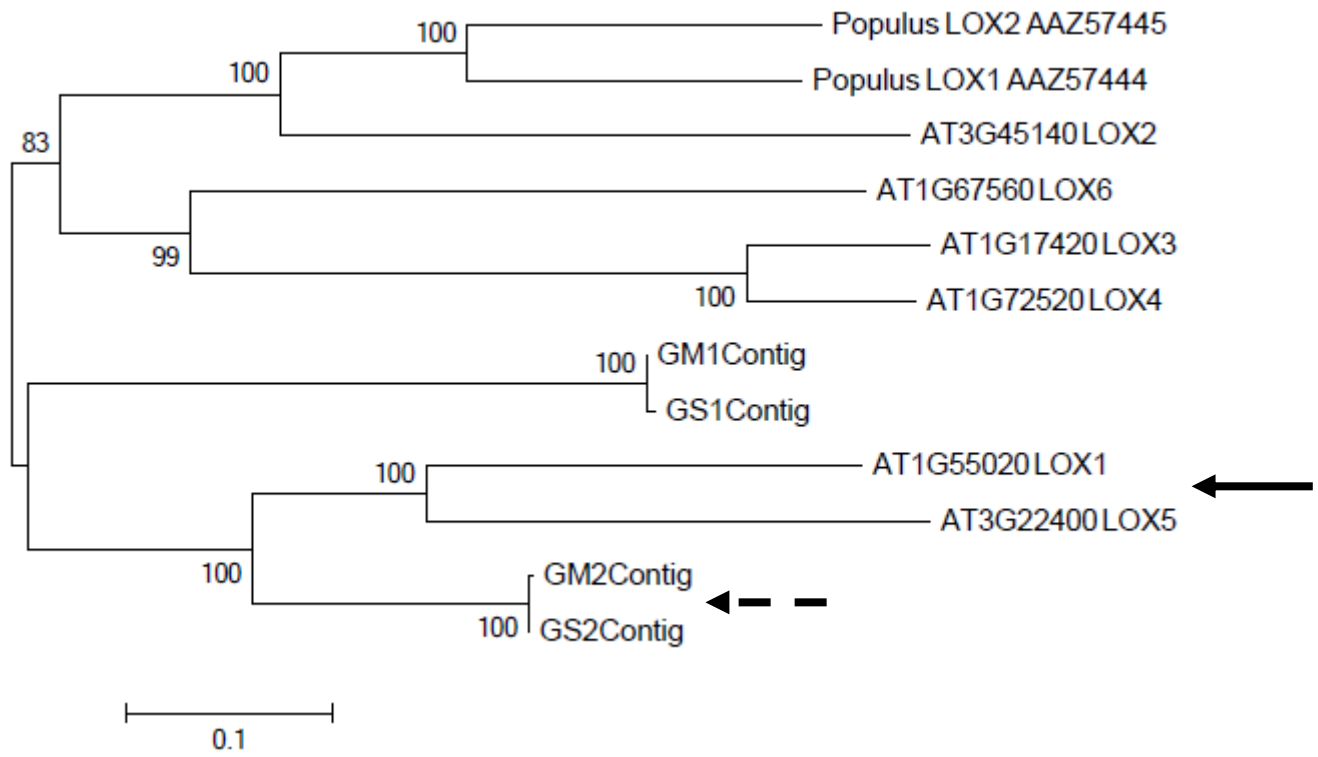

Figure S12: Neighbour joining tree for the plant *PpaLOX* (*lipoxygenase*) resistance gene based on amino acid sequences. . EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Populus* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer designThe length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch.

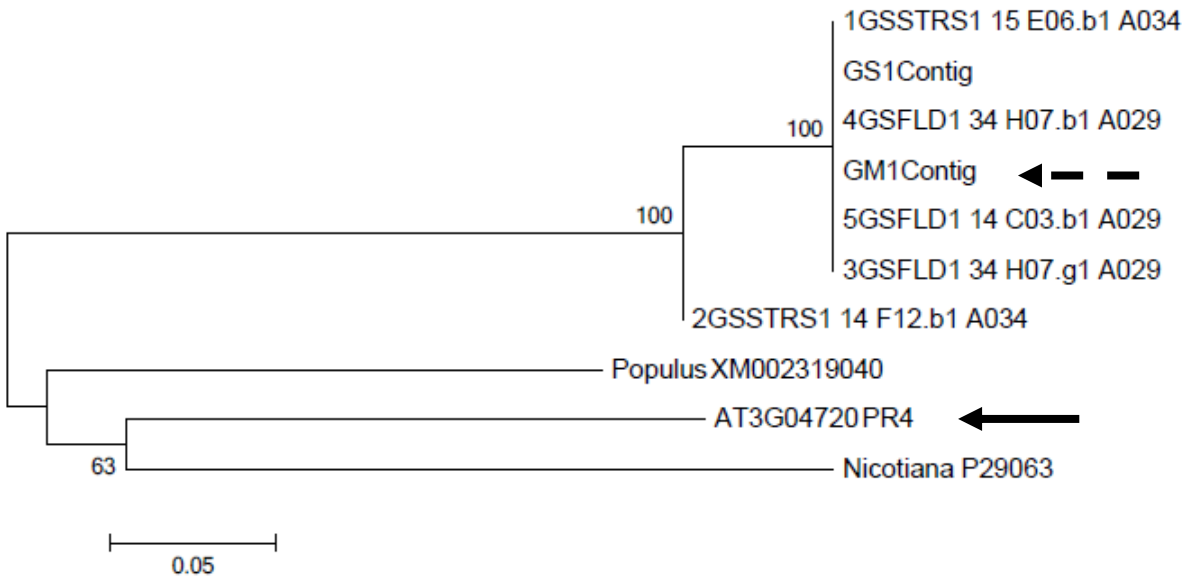

Figure S13: Neighbour joining tree for the plant *PpaPR-4 (hevein – like protein)* resistance gene based on amino acid sequences. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Populus* and *Nicotiana* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch.

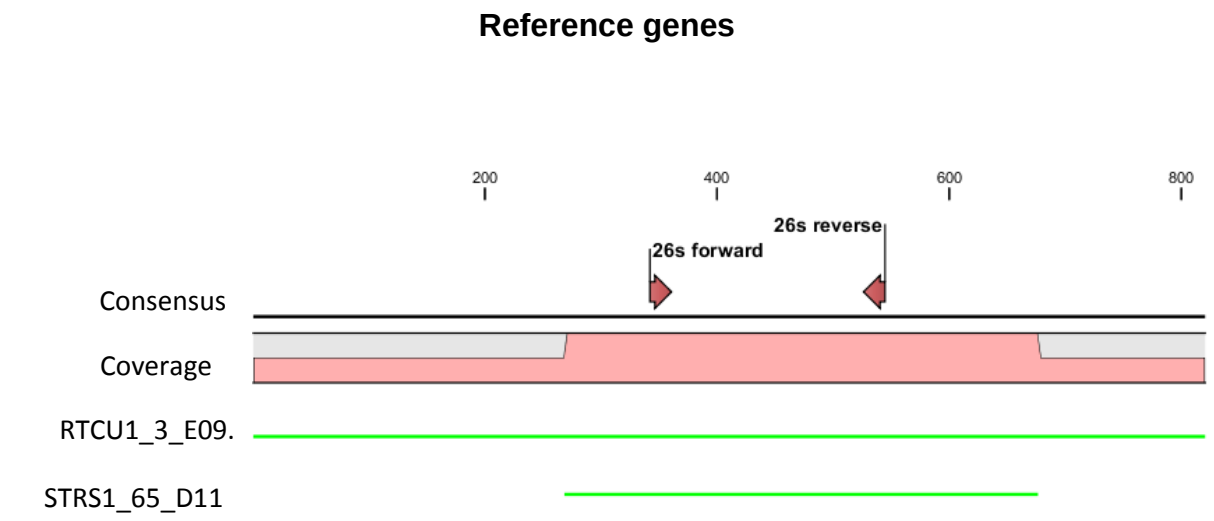

Figure S14: *Ppa26s (proteosome subunit)* contig map. The two ESTs, retrieved from ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

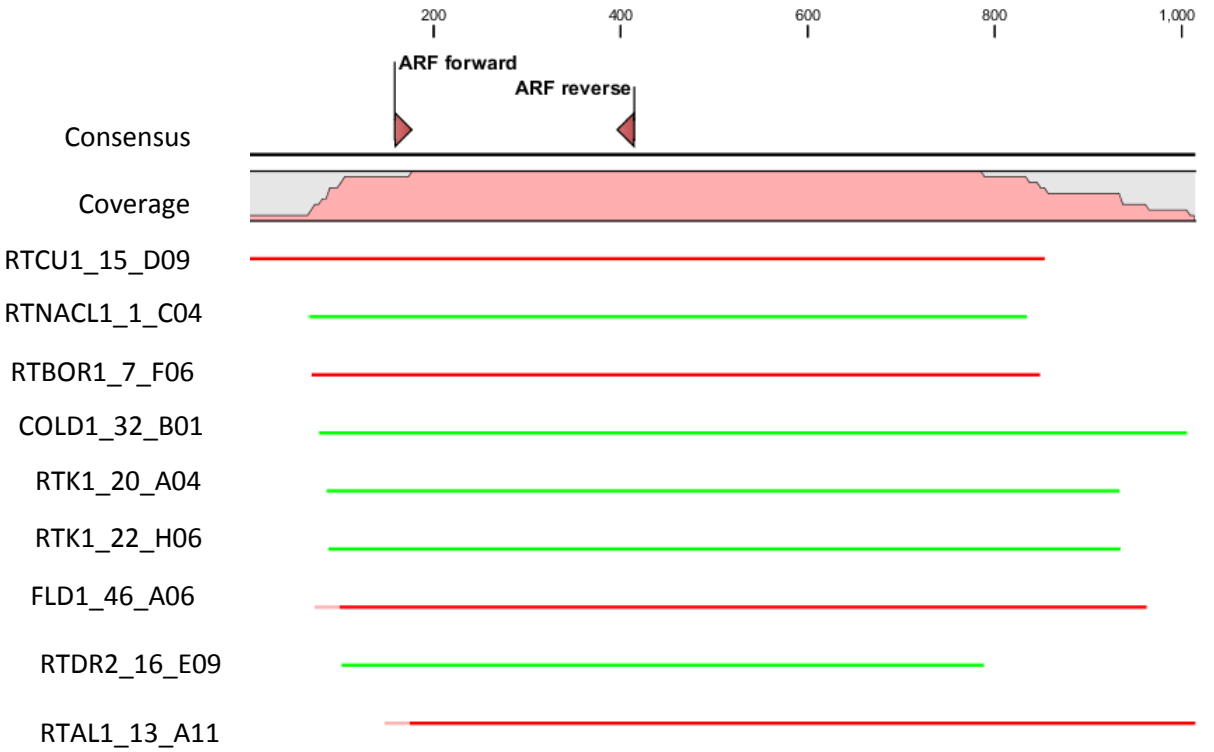

Figure S15: *PpaARF1* (*ADP-ribosylation factor-1*) contig map. The nine ESTs, retrieved from ConiferGDB, making up the consensus sequence, are shown and furthermore the coverage is indicated. The position of the designed primer pair is indicated by the red arrows on the consensus sequence.

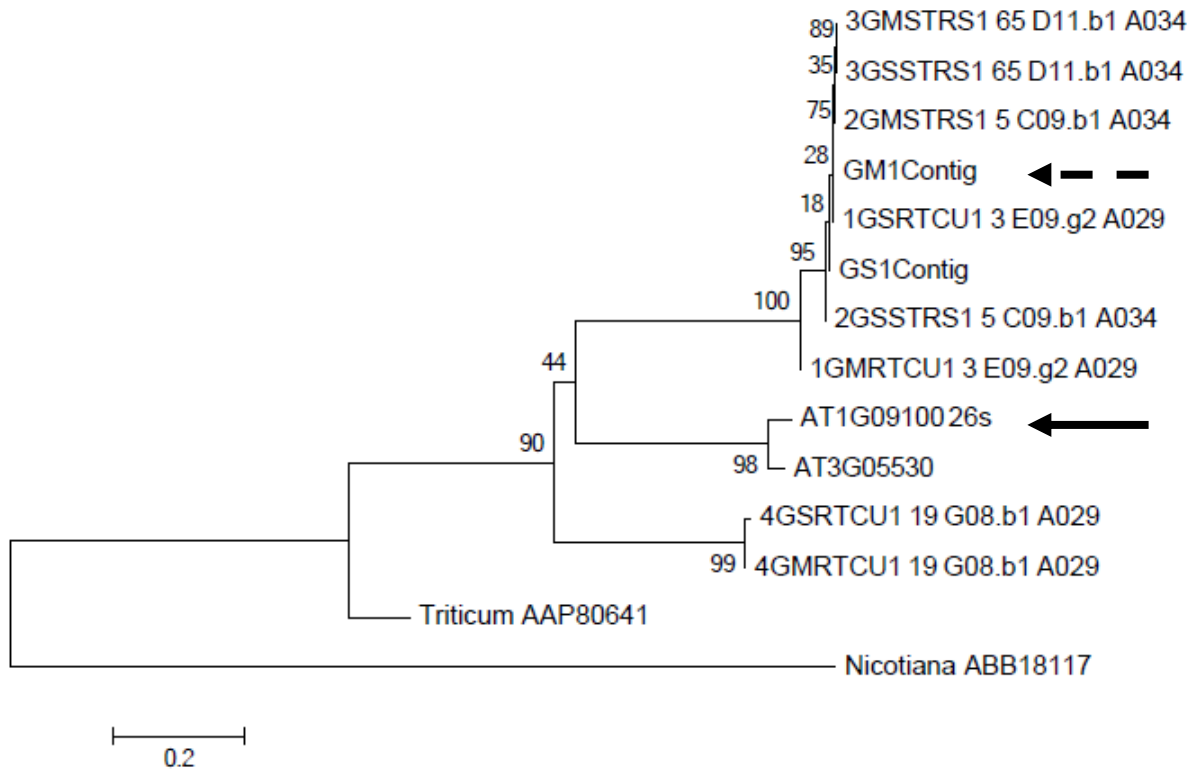

Figure S16 Neighbour joining tree for the plant *Ppa26s (proteosome subunit)* resistance gene based on amino acid sequences. EST names are provided as found in ConiferGDB, *Arabidopsis* ATG numbers in TAIR and *Nicotiana* and *Triticum* genes as found in NCBI. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design.The length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch.

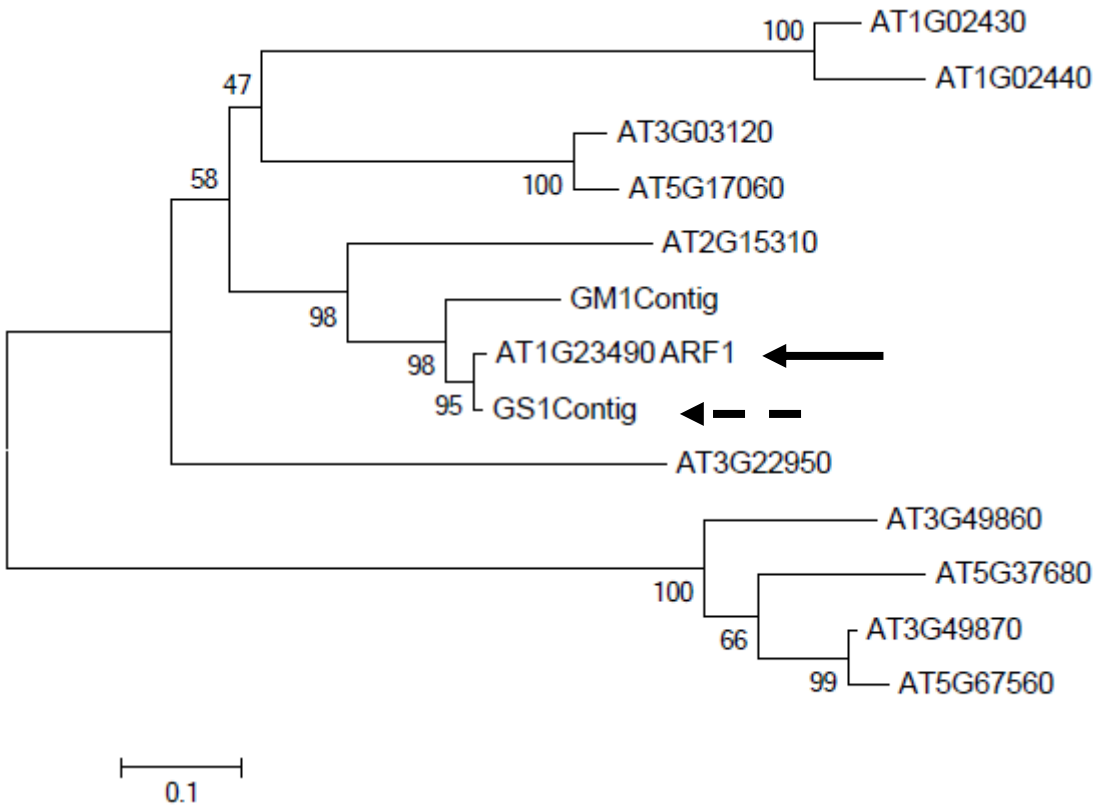

Figure S17: Neighbour joining tree for the plant *PpaARF1* (*ADP-ribosylation factor-1*) resistance gene based on amino acid sequences. . EST names are provided as found in ConiferGDB and *Arabidopsis* ATG numbers in TAIR. The solid arrow represents the *Arabidopsis* gene and the dotted arrow highlights the sequence chosen as template for primer design. The length of each branch is proportional to the substitutions per site given by the scale. The numbers found on the branches are the percentage of bootstrap replications (10000) supporting the branch.

Supplementary Data C

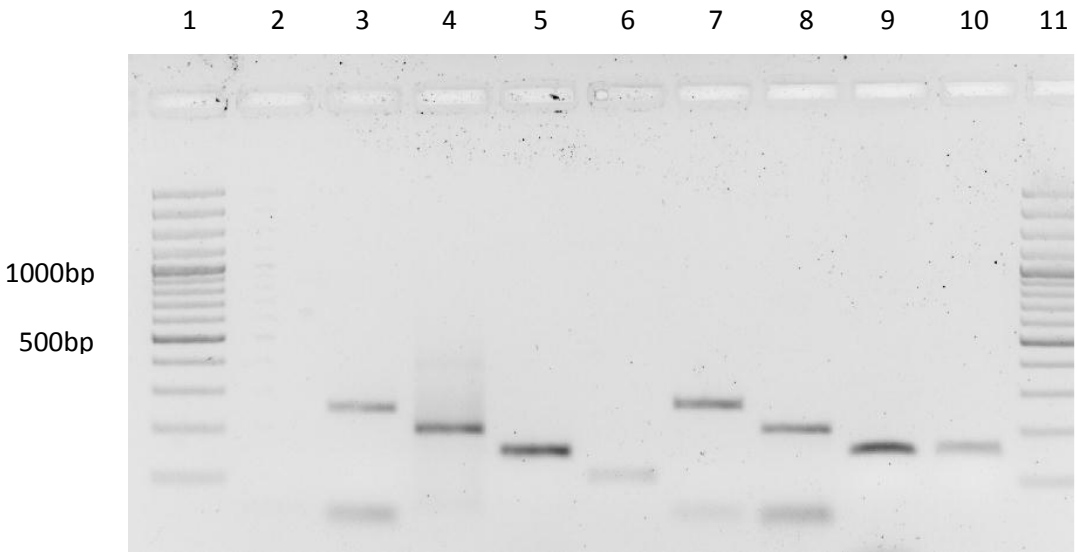

Figure S18: RT-qPCR products of chitosan trial: Products of reference and target specific primers were analyzed on a 1.5% (w/v) agarose gel. Each sample is a pool of three technical replicates. Lane 1 and 11: 100bp DNA ladder plus (Fermentas); Lane 2: negative control; Lane 3: *PpaARF1*; Lane 4: *Ppa26s*; Lane 5: *PpaLOX*; Lane 6: *PpaPAL*; Lane 7: *PpaDXS1*; Lane 8: *PpaPR3*; Lane 9: *PpaFMO1* and Lane 10: *PpaPR5*.

Supplementary Data D

Table S1: Summary of the abbreviations used in ConiferGDB for the various classes of ESTs:

ConiferGDB abbreviations	Description of EST origin
COLD	root cold
FLD	root flooded
GEO	root gravitropism
HEAT	root at 37°C
NDL	needles
NXNV	xylem normal wood vertical
RTAL	root plus added aluminium
RTBOR	root plus added boron
RTCNT	roots minus micronutrients
RTCU	roots plus copper
RTDK	roots under dark conditions
RTDR	roots recovering from drought
RTDS	drought stress roots
RTFEPL	roots plus iron
RTK	roots minus potassium
RTMNUT	roots minus micronutrients
RTNACL	roots plus added NaCL
RTWW	well watered roots
STRS1	shoot tip pitch canker

Table S2: Determining the stability of the reference genes for the samples taken 24hrs after the first chitosan application.

Reference Target	M	CV
Ppa26s	0.468	0.165
PpaARF1	0.468	0.158
Average	0.468	0.162

Table S3: The amplification efficiency of the target and reference genes for 24hrs after the first chitosan treatment

Target gene	Efficiency (E)	SE	R2 - value
PpaPR-3	2.124	0.071	0.859
PpaPR-5	1.822	0.051	0.925
PpaLOX	2.095	0.094	0.845
Ppa26s	1.993	0.065	0.843
PpaARF1	1.865	0.032	0.952

Table S4: Determining the stability of the reference genes for the samples taken 24 hrs after booster chitosan application.

Reference Target	M	CV
Ppa26s	0.468	0.165
PpaARF1	0.468	0.168
Average	0.468	0.162

Table S5: The amplification efficiency of the target and reference genes for 24 hrs after the booster chitosan treatment

Target gene	Efficiency (E)	SE	R2 - value
PpaFMO1	2.493	0.134	0.919
PpaDXS1	2.209	0.113	0.904
PpaPAL	2.589	0.116	0.925
PpaPR-3	2.248	0.115	0.871
Ppa26s	1.985	0.041	0.917
PpaARF1	2.195	0.076	0.808

Supplementary Data E

Table S6: TBLASTX results of the RT-qPCR products against the TAIR and NCBI database: Each target gene was sequenced from the RT-qPCR product and a BLASTN conducted against ConiferGDP to verify that the right EST was being picked up, in the case of a contig that it blasted against that. Additionally, a TBLASTX analysis was conducted against the TAIR v10 and the NCBI database and the results of which are shown in the table below.

	ATG number	TAIR results	NCBI Hit	NCBI accession number	NCBI E-values
Target genes					
PpaFMO1	AT1G19250	$4e^{-8}$	*Pinus radiata*	JO261748	$1e^{-23}$
PpaDXS1	AT4G15560	$2e^{-43}$	*Pinus taeda*	EU862298	$1e^{-51}$
PpaPAL	AT3G53260	$9e^{-3}$	*Pinus taeda*	AC241300	$3e^{-14}$
PpaPR-3	AT3G12500	$6.63e^{-89}$	*Pinus contorta*	HM219849	$1e^{-39}$
PpaPR-5	AT1G75040	$1e^{-8}$	*Pinus glauca*	BT114867	$1e^{-17}$
PpaLOX	AT1G55020	$8e^{-17}$	*Pinus taeda*	FJ096002	$3e^{-27}$
PpaPR-4	AT3G04720	$3e^{-32}$	*Picea sitchensis*	BT071506	$4e^{-47}$
Reference genes					
Ppa26s	AT1G09100	$4.00e^{-16}$	*Picea glauca*	BT115913	$4e^{-38}$
PpaARF1	AT1G23490	$3e^{-46}$	*Picea sitchensis*	BT071679	$4e^{-48}$

Table S7: Sequences of each of the contig regions of the defence marker genes.:

	Sequence 5' to 3'
Target genes	
PpaFMO1	GATAGTACGCCGTGCAGAAAACCCTGAGCTGGCCTTTCTAGTAAGAACTGTGCAAAACGTGTATTATAAAAGAGTTGTATAGGC AATCCGTAGAGCTCGAAAGAAATAAGCGCCCAGTGCGCTCTTCTGAAGACCACTGT
PpaDXS1	CAGTTGCAGATGCCAGATTCTGCAAGCCACTTGATGATGATCTAATTCGATCTCTTGCAAGGGAGCACGAGGTATTAATAACAG TCGAGGAGGGAACAATAGGAGGTTTGGGAACACATGTTGCTCATTTTCTGGCTTTAGATGGTTTCTTGGACGGGAAATTAAAGT GGAGACCAATGGTTCTTCCTGACCACTACATCGAACACGGGGCTCCGAGTGATCAAATGATTGAAGCCGGTTTGACAGCGTCT CATATCGCGGCATCAGTG
PpaPAL	ACGCAGAAGGTGAGAAGGATCCCAACACTTCCATTTTCAATAAGATTCCTGTGTTTGAAGCCGAGCTGAAGGCACAGCTTGAAC CGCAAGTTAGTCTGGC
PpaPR-3	GGATTGCCTTGCTCCTCTTTGAAACAATAACCCCACGCGTACGCGCCGTCTGGGGCTGTCGCCCATCCTCCTGTGGTTTCGTG GGAGGTTTGGCCAAAGAAAGCGGCGGGCTCTCTTTTCCGAGTAGCGACATCGCCGGTGGTGCCGAAGGAGGGAAAAGCATTG GCAGCCGCAATGAAGGCAGAATAGGTGTAGAATCCGCTGG
PpaPR-5	CGGATGCGTCGCTGATCTTCTGACCAACTGCCCGGCCTCACTAATTGTCGCCGACAAAGAGGGGCGAGTGATTGCCTGCAGG AGCGCGTGCGATGCGTTCAAGCTTCCCGAGTACTGCTGCACAGGCGACCACAACACCGCTCAGACGTGTC

PpaLOX	TGTTGGTCACGGAGACAAGAAGGACGAGACATGGTGGTACAGCCTGGAGAGCGTCGAAGAAGTTGAAAAAGTAATAACAACAA TCATATGGGTAGCCTCGGCTCTTCACGCTGCGGTTAATTATGGGCAATACTCTTATGCCGGATACATGCC
PpaPR-4	GCAGCAAGCGTCCAATGTGAGGGCAACTTATAATTACTATAACCCACAAAGCATTGGCTGGGATTTGGGTAAGGCCTCTGCATA CTGTGCTACCTGGGATGCCAGCAAGCCCCTGGAGTGGAGGAAGAAATATGGGTGGACTGCCTTCTGTGGACCTGTTGGACCC CATGGCCAGGCATCCTGTGGAAAATGCTTGAAGGTTACTAACAGAGACACTGGCGCAT
Reference genes	
Ppa26s	TCAGGCAGACACTCCGAATGTCAGCACCTGTAGAATTGGGGCATAACCGGGAAAGCAGCTCAAAACGAATGTCTCGTTCACAA TTCATGGTACGTGTATGAATCTTAAAAATCTGAGTCCTGCTTTCCAAATCTGGTAAGCCAAATTCAACCTTTCTGTCCAACCGAC CAGGCCGTAATAATGCAGGATCAAGAGTGTCAGGCC
PpaARF1	GATCTCTAACAGGCGGTCAAGATGGGGCTCACTTTTACCAAGCTTTTTAGTCGGCTCTTCGCAAAGAAGGAAATGCGCATTCTG ATGGTTGGTCTCGATGCCGCTGGTAAGACGACCATCTTGTATAAGCTGAAGCTGGGAGAAAACGTTACGACCATCCCTACCATC GGATTCAATGTGGAAACCGTCGAGTACAAGAACATCAGCTTCACTGTGTGGGATGTTGGGGGTCAGGACAAGATCCGTCCACT ATGGAGA

Supplementary Data F

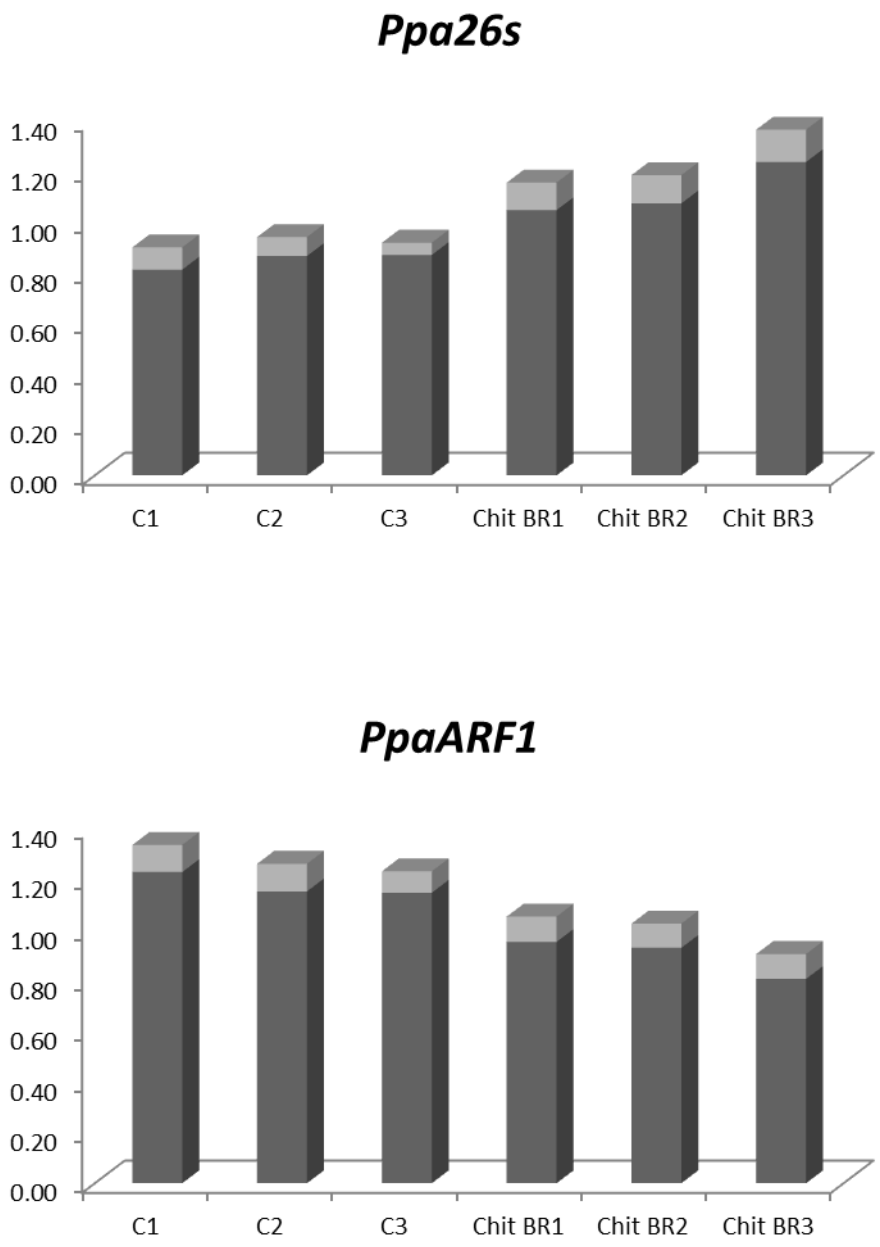

Figure S19: Expression Profiles of reference genes for the 24 hrs after first chitosan treatment experiment. The y-axis represents the relative expression ratios in arbitrary units. The x-axis represents the samples that were analyzed. The light grey segments of the graphs represent the standard error amongst the technical replicates for each sample. Samples abbreviated as e.g. C refers to control samples and Chit BR1 = chitosan biological sample 1. Stability criteria: *PpaARF* (M = 0.46; CV = 16.5%) and *Ppa26s* (M = 0.46; CV = 16.5%).

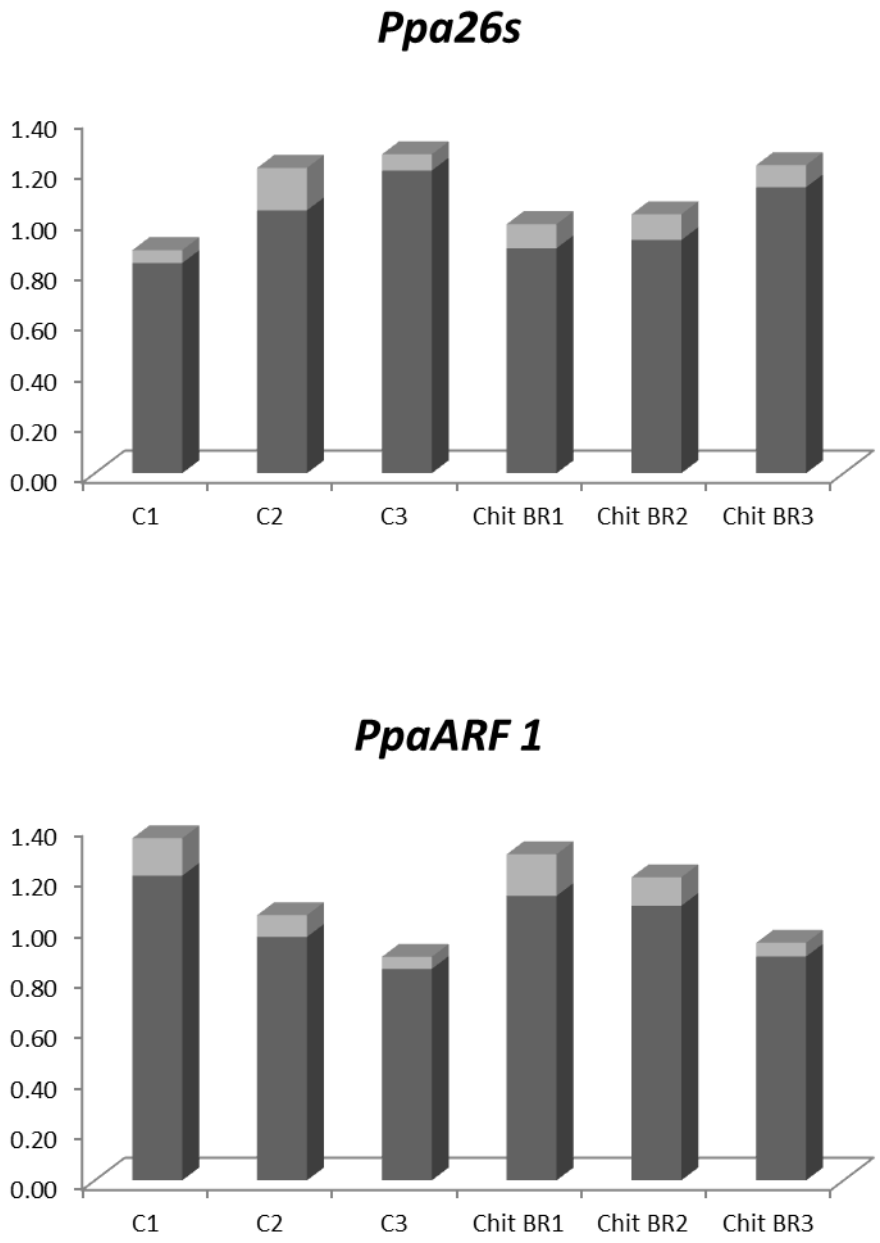

Figure S20: Expression Profiles of reference genes for the 24 hrs booster chitosan treatment experiment. The y-axis represents the relative expression ratios in arbitrary units. The x-axis represents the samples that were analyzed. The light grey segments of the graphs represent the standard error amongst the technical replicates (n=3) for each sample. Samples abbreviated as e.g. C refers to control samples and Chit BR1 = chitosan biological sample 1. Stability criteria: *PpaARF* (M = 0.46; CV = 16.5%) and *Ppa26s* (M = 0.46; CV = 16.5%)

Supplementary Data G

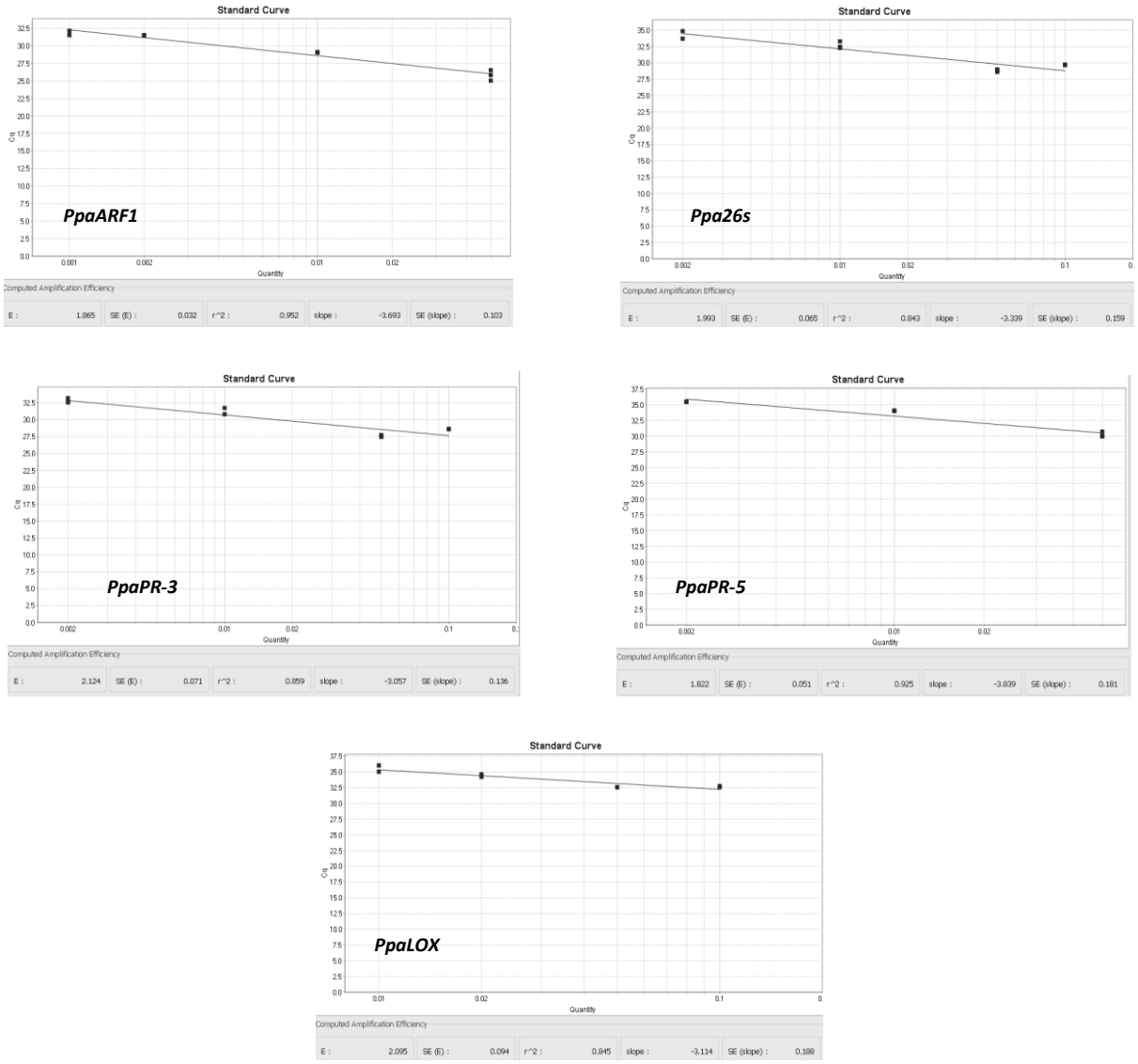

Figure S21: Standard Curves of all the targets and reference genes: These graphs were generated from the qBASE*plus* v1.0 software using the samples harvest 24 hrs after the first chitosan application.

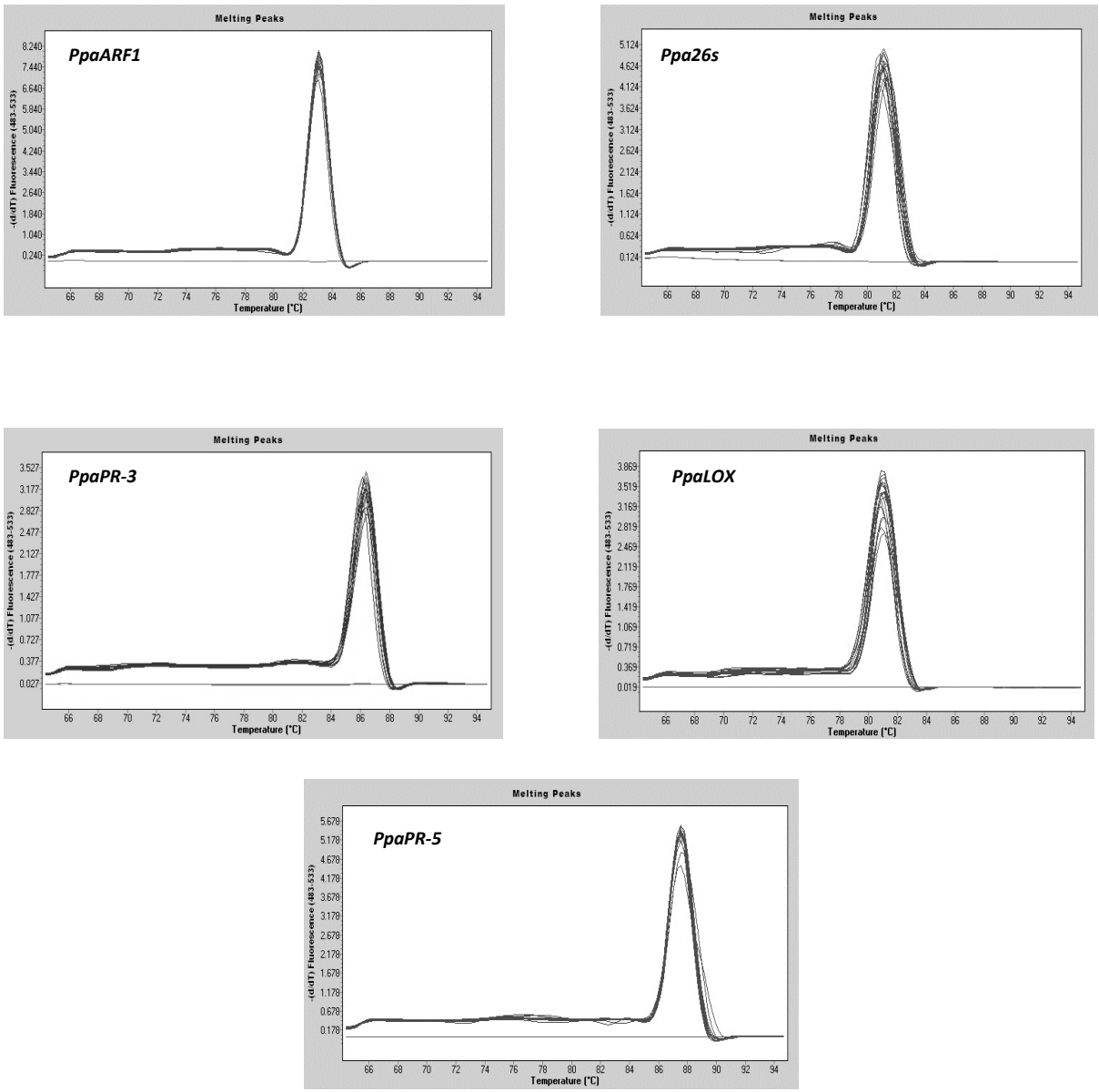

Figure S22: Melting Curves of all the targets and reference genes: These melting peaks were generated from the LightCycler® 480 Real-Time PCR system using the samples harvest 24 hrs after the first chitosan application. No amplification was obtained in the no template control reactions as is evident in the absence of a peak in the melting curves.

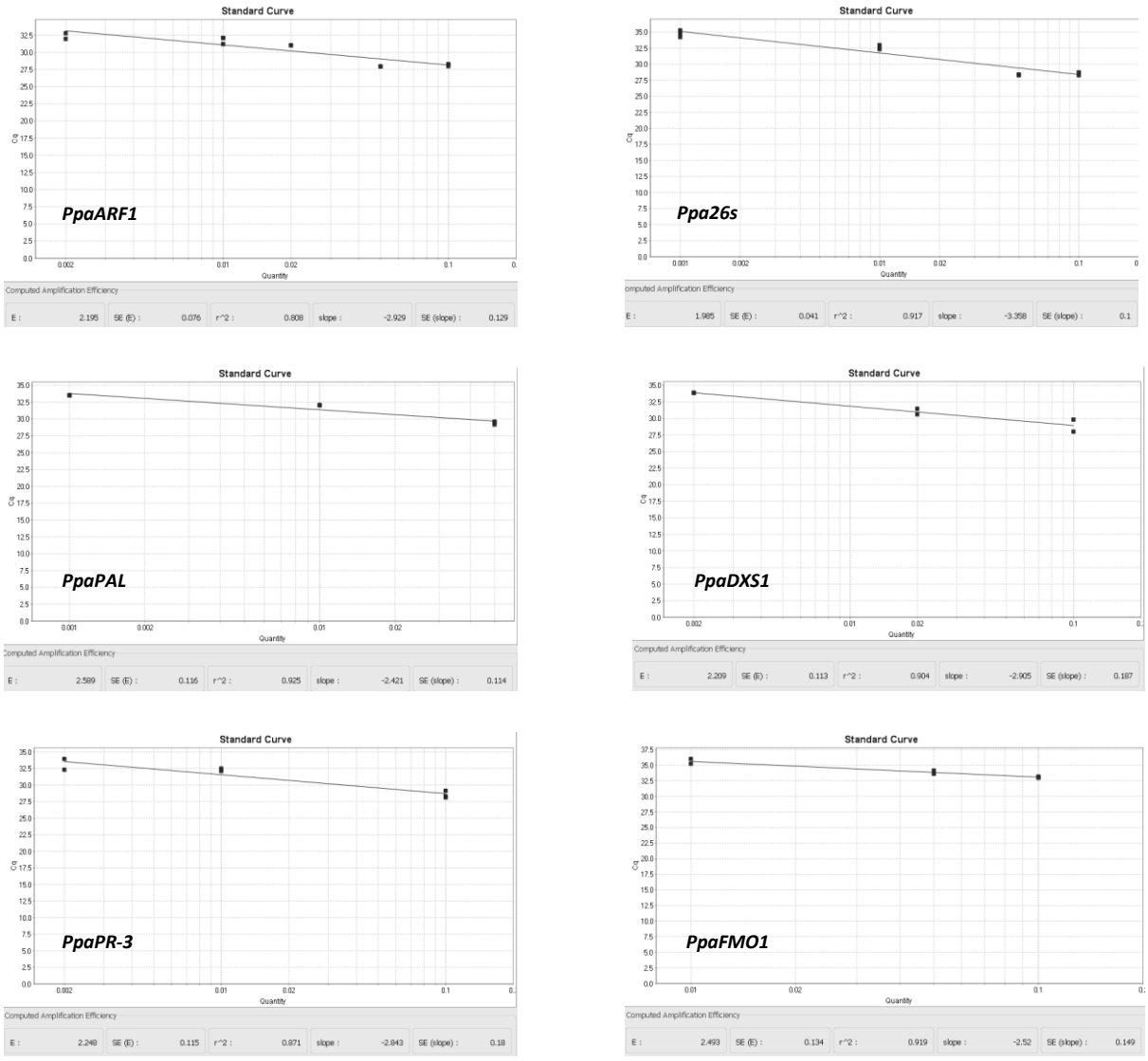

Figure S23: Standard Curves of all the targets and reference genes: These graphs were generated from the qBASE*plus* v1.0 software using the samples harvest 24 hrs after the booster chitosan application.

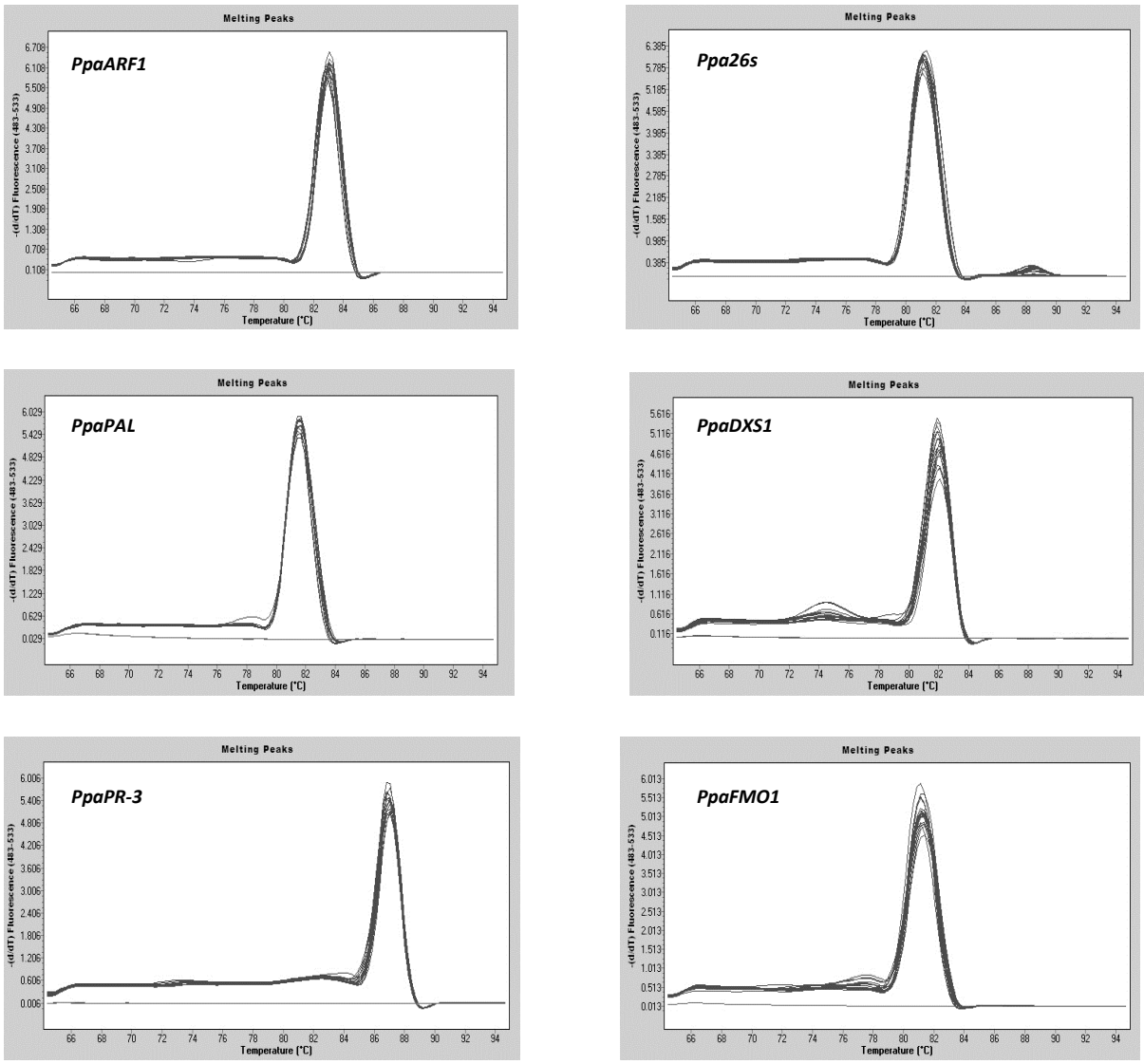

Figure S24: Melting Curves of all the targets and reference genes: These melting peaks were generated from the LightCycler® 480 Real-Time PCR system using the sample harvest 24 hrs after the booster chitosan application. No amplification was obtained in the no template control reactions as is evident in the absence of a peak in the melting curves