Transcript Profiling of the *Heterobasidion*-Conifer Pathosystem:

Host and Pathogen Responses to Biotic Stress

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Abstract

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Host–pathogen interactions have been studied mostly in agricultural crops, with very little work done in forest trees, particularly gymnosperms. The biology and genetics of one of the most economically important conifer pathogens *Heterobasidion annosum* sensu lato has been studied but knowledge about mechanisms of defence responses and resistance remains largely unexplored. A major set back for this conifer pathosystem is the lack of a suitable model system. In this thesis, a transcriptomic approach was used to analyse responses of pine seedling roots to *H. annosum* infection. The microarray profiling of *Pinus sylvestris* L. responses to infection with *H. annosum* revealed multiple overlapping strategies employed for defence purposes. Production of pathogenesis-related enzymes and antimicrobial proteins was supplemented by a major shift in primary and secondary metabolism.

As there are no avirulent strains of *H. annosum,* a follow-up microarray study used *Laccaria bicolor* (an ectomycorrhizal symbiont) and *Trichoderma aureoviride* (a saprotroph) as non-pathogen models to determine whether the observed pine responses to *H. annosum* attack were specific. The results indicated that pine was able to recognize all three fungi and specifically distinguish whether they were pathogenic, neutral or beneficial microorganisms. An additional transcript profiling study investigated whether the documented responses to *H. annosum* infection were organ specific. Comparison of transcript profiles of pine needles and roots challenged with root (*H. annosum*) and shoot specific (*Gremmeniella abietina*) pathogens, indicated that the responses were more organ-specific than pathogen-specific.

Finally, in parallel to the above studies and in the absence of any host genotype in the Pinaceae with total resistance against *H. annosum,* a molecular analysis of the mechanisms underlying the action of commonly used biocontrol agent, *Phlebiopsis gigantea* was conducted. Analysis of genes differentially expressed during the interaction of these two fungi revealed up-regulation of several *P. gigantea* genes vital for nutrient acquisition which may partly explain the observed competitive advantage over *H. annosum*.

The results of this thesis have provided further insights into the molecular basis for specificity and recognition in conifer defence and the knowledge obtained from the fungal interaction study was considered a first step towards improving the efficacy of the biological control of *H. annosum*.

Keywords: Heterobasidion annosum, *Pinus sylvestris*, defence, microarray, transcript profiling, necrotroph, saprotroph, ectomycorrhiza, biocontrol, *Phlebiopsis gigantea*.

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Appendix

Papers I-V

This thesis is based on the following papers, which are referred to by their Roman numerals:

I. Asiegbu F.O., **Adomas A**. & Stenlid J. 2005. Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. sensu lato. *Molecular Plant Pathology* 6, 395-409.

II. **Adomas A.**, Heller G., Li G., Olson Å., Chu T.-M., Osborne J., Craig D., van Zyl L., Wolfinger R., Sederoff R., Dean R.A., Stenlid J., Finlay R. & Asiegbu F.O. 2007. Transcript profiling of conifer pathosystem: response of *Pinus sylvestris* root tissues to pathogen (*Heterobasidion annosum*) invasion. *Tree Physiology,* in press.

III. **Adomas A.**, Heller G., Olson Å., Osborne J., Karlsson M., Nahalkova J., van Zyl L., Sederoff R, Stenlid J., Finlay R., Asiegbu F.O. Comparative analysis of transcript abundance in *Pinus sylvestris* roots after challenge with a pathogenic, saprotrophic or mutualistic fungus. *Submitted manuscript.*

IV. **Adomas A.** & Asiegbu F.O. Analysis of organ specific responses of *Pinus sylvestris* to shoot (*Gremmeniella abietina*) and root (*Heterobasidion annosum*) pathogens. *Submitted manuscript.*

V. **Adomas A.**, Eklund M., Johansson M. & Asiegbu F.O. (2006) Identification and analysis of differentially expressed cDNAs during nonself-competitive interaction between *Phlebiopsis gigantea* and *Heterobasidion parviporum. FEMS Microbiology Ecology* 57, 26–39.

Introduction

Background

The ability to distinguish between self and non-self is a key feature of all living organisms. It is a primary feature in maintaining integrity against potential invaders. Plants are exposed to various microorganisms, including pathogens, at every stage of their life cycle. However, disease is not a normal state and plants are able to recognize most of the pathogens and induce defence mechanisms. The molecular bases of recognition and defence have been well studied in *Arabidopsis* model and crop species (Dangl & Jones, 2001; Jones & Takemoto, 2004; Jones & Dangl, 2006) but are poorly understood in coniferous trees. Most conifers are susceptible to infection by a necrotrophic pathogen *Heterobasidion annosum* (Fr.) Bref. sensu lato (s.l.) (Woodward *et al.*, 1998). The fungus is often considered the most devastating pathogen of boreal forests in the northern hemisphere, particularly in economical terms. To control the root and butt rot caused by *H. annosum* s.l. foresters successfully use silvicultural, chemical (urea) and biological control (*Phlebiopsis gigantea* (Fr.) Jülich) (Holdenrieder & Grieg, 1998; Korhonen *et al.*, 1998; Pratt, Johansson & Hütterman, 1998). The unknown mechanism behind the biocontrol action of *P. gigantea* is another example of importance of self/non-self recognition in biological interactions. The focus of this thesis is on biotic interactions in the *H. annosum* s.l. pathosystem including the host, the pathogen and the biocontrol agent.

Plant-pathogen interactions

Due to the paucity of our knowledge about host-pathogen interactions in forest tree pathosystems, much of the information has been acquired from agricultural systems. Although gymnosperms and angiosperms diverged during evolution several million years ago, a lot can be learnt from studies conducted in crop pathosystems.

Plants are equipped with an array of defence mechanisms that include constitutive and induced mechanical and chemical barriers. Preformed defences include wax layers, cuticle, cell wall components and antimicrobial compounds, such as secondary metabolites and antifungal proteins (phytoanticipins). Timely perception of the pathogen by the plant is central to the activation of defence responses. Microbes disclose their presence to the host through pathogen-associated molecular patterns (PAMPs) (Gomez-Gomez, 2004; Jones & Takemoto, 2004; Jones & Dangl, 2006; Nurnberger *et al.*, 2004). A key feature of those molecular signatures is that they are highly conserved, functionally indispensable and present in different microorganisms. A definition of this sort does not limit the presence of such patterns to pathogens only. Consequently, a term microbe-associated molecular pattern (MAMP) was suggested (Mackey & McFall, 2006). Most importantly, PAMPs are absent in the host, and therefore typify "non-self". They might be secreted or present on the surface of the pathogen, liberated by a host enzyme, or released from the pathogen when it dies (Zipfel & Felix, 2005). Such general elicitors, involved in activation of the first

line of defence, have been isolated from various plant pathogens and include fungal cell wall constituents (chitin, glucan, proteins and glycoproteins) and bacterial lipopolysaccharides, flagellin and elongation factor (Gomez-Gomez, 2004; Gomez-Gomez & Boller, 2002; Kunze *et al.*, 2004). Microbe-associated hydrolytic enzymes have been shown to elicit plant defence through releasing fragments of plant cell wall during attempted penetration. Thus, plants are able to recognize not only exogenous pathogen-derived signals (non-self) but also endogenous structures of plant origin (modified-self) (Jones & Takemoto, 2004; Mackey & McFall, 2006). PAMP-based recognition is crucial for the fact that most plant species are resistant against the majority of potential microbial invaders, a phenomenon called non-host resistance (Heath, 2000b; Nurnberger & Lipka, 2005; Thordal-Christensen, 2003). In susceptible plants PAMP-induced defence is insufficient to stop infection (Nurnberger & Lipka, 2005).

During evolution, plant species resistance has been overcome by pathogens through the acquisition of avirulence factors (*Avr*), which enable them to interfere with plant defence mechanisms. Avr proteins are considered to be factors that contribute to host infection, although the biochemical function of most of them is unknown. Selective pressure on host plants has resulted in the co-evolution of resistance genes (*R*) which specifically recognize pathogen race-specific factors and allow for the establishment of pathogen race/plant cultivar-specific disease resistance (Dangl & Jones, 2001; Jones & Takemoto, 2004; Nurnberger & Lipka, 2005). This type of resistance conforms to the gene-for-gene hypothesis and is genetically determined by complementary pairs of pathogen encoded avirulence genes and plant resistance genes (Flor, 1971). Functional *R* genes isolated so far encode resistance to bacterial, viral, fungal, oomycete and even nematode and insect pathogens with very different lifestyles (Dangl & Jones, 2001). The most common motif found in R proteins is nucleotide binding leucine rich repeat (NB-LRR) which presumably plays a major role in recognition specificity (Takken, Albrecht & Tameling, 2006; Toyoda *et al.*, 2002).

Collectively, PAMP-induced non-host resistance, as well as Avr-induced cultivar-specific resistance, should be considered two complementary elements of plant innate immunity that have been shaped during co-evolution with pathogens (Jones & Takemoto, 2004; Nurnberger *et al.*, 2004; Nurnberger & Lipka, 2005). A simplified model illustrating plant recognition and defence mechanisms is presented in figure 1.

Induced plant defence mechanisms

Signal transduction cascades link recognition and defence responses through a network employing altered cytoplasmatic Ca^{2+} levels, reactive oxygen species (ROS) and nitric oxide (NO) as well as post-translationally regulated mitogen-activated protein kinase (MAPK) (Neill *et al.*, 2002; Toyoda *et al.*, 2002; Zhang & Klessig, 2001). The key signal molecules mediating both basal and specific defence responses are salicylic acid (SA), jasmonate (JA) and ethylene (ET) (Fig. 1). Salicylic acid is required for local and systemic acquired resistance and together with NO and ROS acts synergistically in activating defence responses (Klessig *et al.*, 2000). In turn, JA and ET are responsible for induced systemic

resistance and regulate expression of genes coding antimicrobial peptides such as thionin and defensin (Nimchuk *et al.*, 2003). Although both signalling pathways, SA- and JA/ET-dependent are mostly mutually antagonistic, the complex cross-talk between them allows the plants to fine-tune their defence program and respond to each pathogen with a mixture of defence measures (Nimchuk *et al.*, 2003).

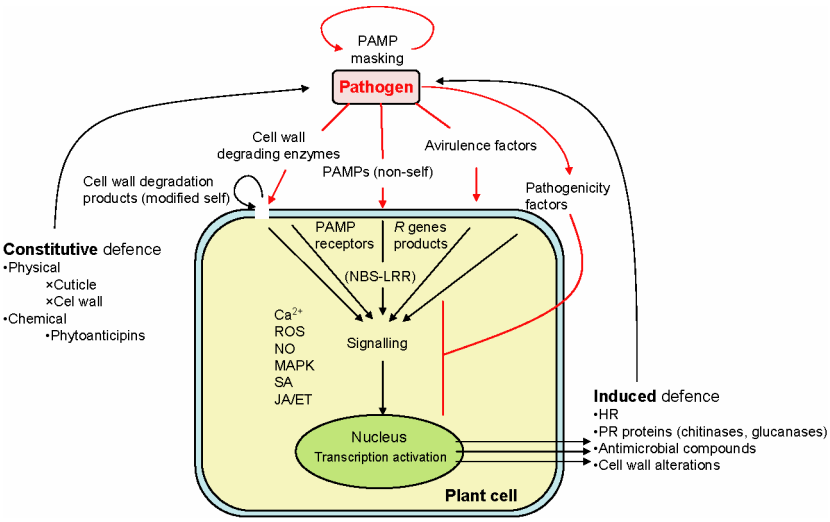

Fig. 1. A simplified model illustrating plant-pathogen interactions. A pathogen able to overcome constitutive defence mechanisms (physical and chemical) reveals its presence to the plant through general (PAMPs) and specific elicitors (avirulence factors). The host recognizes those elicitors as non-self using receptors (PAMP receptors and *R* genes products) that often share a common NBS-LRR motif. In a similar way endogenous elicitors (modified-self) are recognized; they are produced during host cell wall degradation by hydrolytic enzymes secreted by the pathogen. Recognition of the pathogen triggers multiple signalling pathways that result in transcriptional activation of pathogenesis-related (PR) genes and induce diverse defence responses. Both types of resistance: non-host (PAMP mediated) and host-specific (*R* genes mediated) share many signalling cascades and often lead to activation of similar responses. As a countermeasure to the plant defence mechanisms many pathogens evolved a way to avoid recognition by masking PAMP and/or to interfere with signalling and defence induction. Red arrows indicate pathogen strategies for infection and black arrows indicate plant defence strategies. Abbreviations: ET-ethylene, HR-hypersensitive response, JA-jasmonate, MAPK-mitogen activated protein kinase, NB-LRR-nucleotide binding leucine rich repeat, PAMP-pathogen associated molecular pattern, ROS-reactive oxygen species, SA-salicylic acid. Modified from Jones and Takemoto (2004).

Stress-signalling pathways appear to converge at specific points including not only messengers but also components further down-stream the cascade. A multitude of transcription factor genes are expressed in response to a wide variety of defence-related stimuli. Members of the *WRKY, Myb, Myc, Nac, ERF, bZIP* families and other zinc-finger factors are known to play a role in modulating defence gene expression (Jalali, Bhargava & Kamble, 2006; Nimchuk *et al.*, 2003).

The initial responses of pathogen-invaded plant cells occur in crop plants within a few minutes and are rapidly followed by local gene activation (Somssich & Hahlbrock, 1998). Transcriptional activation of defence-associated genes results in

production of pathogenesis-related (PR) proteins and lytic enzymes (chitinases, glucanases and proteases), antimicrobial proteins (defensins) and secondary metabolites, especially products of phenylpropanoid pathway (Dixon & Paiva, 1995; van Loon, Rep & Pieterse, 2006) (Fig. 1). Part of the plant response associated with pathogen defence is enzyme (particularly peroxidase) mediated cell wall reinforcement by cross-linking of cell wall components, apposition of callose and lignin and production of ROS that fulfil not only a signalling function but are also directly toxic to microbial invaders (Hotter, 1997). As a result, an extensive reprogramming of plant metabolism is initiated: primary pathways are activated to provide large carbon fluxes to the secondary ones; at the same time all non-essential cellular activities are down-regulated (Somssich & Hahlbrock, 1998).

The immediate, early defence responses of the directly invaded plant cells, starting with signal recognition and transduction, commonly lead to hypersensitive response (HR) (Kombrink & Schmelzer, 2001; Morel & Dangl, 1997). Localised cell death restricts pathogen access to nutrients and releases antimicrobial compounds stored in vacuoles. This strategy is effective against pathogens that can grow only on living host tissues (obligate biotrophs), or hemibiotrophic pathogens, but not against necrotrophic pathogens that kill host tissue during colonisation (Glazebrook, 2005). When HR occurs in hosts colonized by necrotrophs, fungal growth is accelerated rather than retarded (Mayer, Staples & Gil-ad, 2001; van Kan, 2006). Since defence against biotrophs and necrotrophs is governed by antagonistic pathways (SA- and JA/ET- dependent, respectively), it has been suggested that defence against those two groups of pathogens might likewise be antagonistic (Oliver & Ipcho, 2004).

Fungal counter-measures to plant defence

Upon pathogen invasion plants launch an array of defence strategies. Disease may result either from the failure of the recognition event or from the ability of the pathogen to avoid or overcome the resistance response (Ferreira *et al.*, 2006). Masking PAMPs could be one of the strategies used by the pathogens to avoid detection (Jones & Takemoto, 2004) (Fig. 1). Avr4 produced by the leaf pathogen *Cladosporium fulvum* has chitin-binding activity and is proposed to protect the fungus against degradation by tomato chitinases, in this way preventing both weakening of the fungal cell wall and the release of chitin-elicitors (van den Burg *et al.*, 2003). Fungal pathogens are also known to produce toxins causing cell death in particular plant genotypes, substances that suppress host metabolism and active defence, enzymes that detoxify antimicrobial compounds and plant hormones that might modulate signalling pathways (Maor & Shirasu, 2005; Toyoda *et al.*, 2002) (Fig. 1). Govrin & Levine (2000) have proposed that the cell death induced by the necrotrophic pathogen *Botrytis cinerea* is a form of the HR, and that this induction is an important component of virulence. Many of those factors are crucial for successful infection; they often govern host range and specificity and therefore act as pathogenicity determinants. While an increasing number of pathogenicity factors has been identified in pathogens posing a threat to

crop plants (Idnurm & Howlett, 2001), pathogens attacking forest trees, particularly gymnosperms, have been much less investigated.

Specificity in plant-pathogen interactions

Plants, including forest trees, are continuously exposed to a wide range of microbes including fungi, bacteria and viruses but only some of them are pathogenic. The relationships that are ultimately formed might also be beneficial or neutral, as in mutualistic or saprotrophic interactions, respectively. Plant-microbe interactions are complex and dynamic: some fungi express different lifestyles (pathogenic, mutualistic or saprotrophic) depending on the plant host and/or environment (Redman, Dunigan & Rodriguez, 2001; Vasiliauskas *et al.*, 2007). As activating the defence mechanisms is an energy consuming process, distinction between harmful and beneficial microorganisms is critical (Asiegbu, Johansson & Stenlid, 1999; Guimil *et al.*, 2005; Hahlbrock *et al.*, 2003; Weerasinghe, Bird & Allen, 2005).

The lengthy co-evolution between plants and their pathogens has generated elaborate weapon sets that determine the number of possible compatible interactions. Host-specificity of plant pathogens may range from hundreds of species, down to a single species. At the extreme, in the interactions occurring according to "gene-for-gene" manner, specificity might be restricted to one genotype of a single plant species. Another level of host specialization is the pathogen tendency to colonise only particular plant parts or organs. Stem cankers, leaf or shoot blights, root or stem rots are caused by plant pathogens in all taxonomic groups. Organ-specificity and factors determining the taxonomic range of hosts that can be infected by a specific pathogenic microbe remain a key question in plant pathology. In particular, it is poorly understood why certain pathogens preferentially infect only some organs of a generally susceptible host and not the whole plant (Hermanns, Slusarenko & Schlaich, 2003; Jansen, Slusarenko & Schaffrath, 2006; Schafer & Yoder, 1994). Unlike in crop plants, organ-specificity in pathosystems of forest trees has been little studied.

Conifer defence mechanisms

Despite the economic, environmental and ecological importance of coniferous trees, the nature of their defence responses and disease resistance is not fully understood. The long life cycle, size of the mature trees and long timescale of many of their diseases make working with these plants inherently difficult. While the ability of an herbaceous plant to halt pathogen development for a few days or weeks might be sufficient, the progress of a wood-decaying fungus might need to be restricted for decades. Other factors responsible for the lag in forest pathology research include large genome size, lack of mutant lines and lack of host material with defined genetic background: although transformation of spruce and pine has been reported, it remains a slow and tedious process (Elfstrand *et al.*, 2001; Högberg *et al.*, 1998; Wenck *et al.*, 1999). Therefore, expectations for a tree model system can not be the same as for *Arabidopsis* or other crop plants for which genetic homogeneity can be easily achieved and for which defined host-pathogen interactions are available. Moreover, until recently, trees have been

regarded more as a natural resource to be exploited, rather than as a managed crop, in which measures to reduce disease loss could be actively deployed (Pearce, 1996).

The first and usually the most effective layer of defence in conifer trees is the bark. The combination of the mechanical properties of tough lignified walls, the suberized walls that provide a hydrophobic barrier and the chemical properties of phenolics, presents a multifunctional barrier to the external environment (Franceschi *et al.*, 2005). Only few pathogens can penetrate through intact bark. Access to wood, commonly through a wound, is a pre-requisite for infection to occur (Pearce, 1996).

A major constitutive defence of particular importance in the Pinaceae is resin-producing and storing structures, effective especially against bark beetles and associated fungi (Franceschi *et al.*, 2005; Wainhouse, Rose & Peace, 1997). Inducible defence mechanisms involve cell wall alterations (lignification, suberization), production of lytic enzymes (chitinases, glucanases) and antimicrobial compounds (phenols, stilbenes, lignans, flavonoids, terpenoids) (Pearce, 1996). To date, targeted studies have identified a set of genes induced by biotic stress and encoding peroxidases (Fossdal, Sharma & Lonneborg, 2003; Nagy *et al.*, 2004a), a defensin (Fossdal *et al.*, 2003; Sharma & Lonneborg, 1996), chitinases (Nagy *et al.*, 2004a), a β-1,3-glucanase (Dong & Dunstan, 1997), a chalcone synthase (Nagy *et al.*, 2004b) and a family of dirigent (DIR) proteins (Ralph *et al.*, 2006a).

Little is known about pathogen recognition and signal transduction pathways in conifers. Liu and Ekramoddoullah (2004) cloned 67 partial plant disease resistance (*R*) gene homologues of the NBS-LRR superfamily from needles of western white pine. JA and SA induce chitinase gene expression in pine seedlings (Davis *et al.*, 2002). Methyl jasmonate (MeJa) stimulates chitinase activity and protects Norway spruce seedlings against later exposure to a root pathogen, *Pythium ultimum* Trow. (Kozlowski, Buchala & Metraux, 1999), or a bark beetle-associated fungus, *Ceratocystis polonica* (Zeneli *et al.*, 2006). Exogenous application of MeJa provokes formation of traumatic reisin ducts (Franceschi, Krekling & Christiansen, 2002) and increases resistance against large pine weevil *Hylobius abietis* (Heijari *et al.*, 2005).

Systemic responses to a pathogen infection have been documented in coniferous trees. Mechanical injury and fungal infection systemically induced traumatic resin duct development in Austrian pine (Luchi *et al.*, 2005). Conifers have also been shown to respond to pathogens in a manner consistent with systemic acquired resistance. Predisposing of Monterey pine with *Fusarium circinatum* led to a significant reduction in lesion size over a period of four to six weeks (Bonello, Gordon & Storer, 2001). Norway spruce pre-treated with *C. polonica* showed increased level of phenolic compounds and enhanced resistance to the subsequent mass-inoculations (Evensen *et al.*, 2000). Similarly, pre-inoculation of young pine trees in a nursery with avirulent nematode *Bursaphelenchus xylophilus* induced systemic resistance of trees against a subsequent inoculation with virulent *B. xylophilus* (Kosaka *et al.*, 2001).

It has been shown that, in loblolly pine, effective long-term resistance against fusiform rust can be obtained from a single qualitative resistance gene (Wilcox *et al.*, 1996). Hypersensitive reactions caused by single genes are also known to occur in needle tissues infected with white pine blister rust (Kinloch & Dupper, 2002). However, most of the conifer pathogens belong to necrotrophs whose interactions with plants do not occur according to a gene-for-gene manner. Morse *et al.* (2004) found out that genes induced by fusiform rust were not regulated by *Fusarium circinatum* and suggested that fundamental differences exist in reponses of pine to biotrophic and necrotrophic pathogens.

Heterobasidion annosum s.l. – a necrotropic fungal pathogen

The taxa within *Heterobasidion annosum* s.l. are the main cause of root and butt rot in coniferous forests in the northern hemisphere. In Europe, the *Heterobasidion* species complex comprises three taxa differing in their host specificity. *Heterobasidion annosum* sensu stricto (s.s.) (Fr.) Bref. mainly attacks pine trees, *H. parviporum* Niemelä & Korhonen primarily infects spruce and *H. abietinum* Niemelä & Korhonen attacks mainly fir species (Niemelä & Korhonen, 1998). There are no known avirulent strains of this conifer pathogen and no host genotype in the Pinaceae with total resistance.

The fungi infect fresh stump surfaces or wounds on the roots or stem by means of aerial basidiospores and further spread via root contacts from infected to healthy trees (Redfern & Stenlid, 1998). *Heterobasidion annosum* s.l. penetrates through natural openings on the root surface as well as by direct enzymatic degradation of waxes on root surface. The root rot caused by *H. annosum* s.l. is predominantly a disease of managed forests – freshly cut stumps and wounds created during thinning are the main entry point for the fungus.

As an aggressive necrotrophic wood decayer, *H. annosum* s.l. secretes a wide range of extracellular enzymes (cellulase, manganese peroxidase, laccase, pectinase, proteases) which can degrade and detoxify structural and soluble host constituents but almost nothing is known about their role in pathogenesis (Asiegbu *et al.*, 1998). Among low molecular weight compounds secreted by *H. annosum*, several toxins including fommanoxin, fommanosin and fommanoxin acid, oosponol and oospoglycol have been isolated in cultures of the pathogen (Basset *et al.*, 1967; Sonnenbichler *et al.*, 1989). Application of fommanosin to stem wounds has been shown to provoke a systemic response leading to accumulation of pinosylvin (Basset *et al.*, 1967) (see also **Paper I**).

Resistance and defence mechanisms against H. annosum s.l.

Conifer defence mechanisms against *H. annosum* s.l. consist of a diverse array of phenolic compounds, including phenylpropanoids, stilbenes, flavonoids and lignans accumulated after attack (Asiegbu *et al.*, 1998; Johansson & Theander, 1974; Johansson, Lundgren & Asiegbu, 2004; Lindberg *et al.*, 1992; Shain, 1967; Shain, 1971; Shain, 1979). Apart from lignification and suberization (Asiegbu et al., 1998; Solla *et al*., 2002), papillae formation (Asiegbu *et al*., 1998) has been implicated as a substantial barrier against penetration. In response to infection,

conifers also secrete oleoresin consisting of a mixture of volatile and non-volatile terpenes. Resin acids act as mechanical barriers, whereas the volatile compounds (monoterpenes) are fungitoxic (Johansson, Lundgren & Asiegbu, 2004; Lindberg *et al.*, 1992; Thibault-Balesdent & Delatour, 1985). Krekling *et al*. (2004) observed anatomical responses of Norway spruce to infection such as accumulation of phenolic inclusions in ray parenchyma cells, activation of phloem parenchyma cells and formation of traumatic resin ducts in the xylem.

Pathogenesis-related (PR) proteins produced in conifer tissues in response to *H. annosum* s.l. infection include a large number of enzymes: chitinases, glucanases and peroxidases (Asiegbu, Daniel & Johansson, 1994; Asiegbu *et al.*, 1995; Fossdal *et al.*, 2006; Hietala *et al.*, 2004; Johansson, Lundgren & Asiegbu, 2004). Analysis of a subtractive cDNA library of Scots pine roots led to the identification of candidate genes involved in cell rescue and defence such as peroxidase, antimicrobial peptide (*SpAMP*), thaumatin, metallothionein-like protein and genes with roles in recognition and signal transduction (*PsACRE,* leucine rich repeat [*LRR*]) (Nahalkova *et al*., 2001; Li and Asiegbu, 2004; Asiegbu *et al*., 2005).

Interestingly, Norway spruce trees pre-inoculated with *H. annosum* s.l., were more efficiently protected against a subsequent massive inoculation with pathogenic *C. polonica* (Krokene, Solheim & Christiansen, 2001).

Recent advances in transcript profiling (Asiegbu, Nahalkova & Li, 2005; Karlsson, Olson & Stenlid, 2003), genetic mapping (Lind, Olson & Stenlid, 2005) and development of a transformation system (Asiegbu, 2000; Samils *et al.*, 2006) make the *H. annosum*-pine pathosystem a candidate model for studying conifer defence responses to a necrotrophic pathogen.

Disease management

Theoretically, a root rot pathogen can be suppressed during all stages of its life cycle, starting from adhesion, early establishment and infection, through spreading inside the host, transferring between trees and prevention of the survival of its propagules. Curative measures against the root rot are not feasible since decay inside the tree cannot really be healed, although spread can be reduced in the attacked root system in order to minimise the economic losses. Prophylactic protection measures against *H. annosum* s.l. have been focused on preventing basidiospore deposition, germination and growth of the fungus. Current strategies for the control of root rot include silvicultural, chemical and biological methods (see also **Paper I**).

Biological control encompasses prophylactic stump treatment immediately after felling in an attempt to prevent infection. A number of fungal species (*Phlebiopsis gigantea, Bjerkandera adusta, Fomitopsis pinicola, Resinicium bicolor, Hypholoma* spp., *Trichoderma* spp., *Scytalidium* spp.) have been tested on the stump as competitors and antagonists against *H. annosum* s.l. (Kallio and Hallaksella, 1979; Holmer and Stenlid, 1994; 1997; Nicolotti and Varese, 1996; Holdenrieder and Greig, 1998; Nicolotti *et al*., 1999). Among these, only *Phlebiopsis gigantea* (Fr.) Jül is currently used and with very good results (Holdenrieder & Grieg, 1998; Rishbeth, 1961; Rishbeth, 1963).

However, almost nothing is known about physiological and molecular basis for the interspecific interaction between *P. gigantea* and *H. annosum* s.l. Several modes of action of biocontrol agents are documented in the literature and these include antibiosis, production of lytic enzymes, parasitism, induced resistance and competition for space and nutrients. Presently, there is no evidence of either antibiotics or toxins being secreted by *P. gigantea* (Holdenrieder & Grieg, 1998). It has been observed that *P. gigantea* hyphae changed the structure of adjacent *H. annosum* s.l. hyphae: penetration, granulation and vacuolisation of the cytoplasm and loss of opacity were documented (Ikediugwu, 1976; Ikediugwu, Dennis & Webster, 1970). This phenomenon, called hyphal interference is not uncommon in interspecific antagonistic fungal interactions and is known to have a potential in biocontrol (Boddy, 2000). Generally, antagonistic microbial agents used in biocontrol do not give any guarantee for sustainable efficacy and longevity; an appreciation of the underlying mechanism for such non-self interaction provides a good starting point for understanding and using them in a more effective and sustainable way.

Objectives

Knowledge about the molecular aspects of host-parasite interactions in the *H. annosum*-pine pathosystem is critical for understanding conifer defence mechanisms and may also serve as a basis for disease management aimed at increasing resistance through breeding or genetic engineering. The specific objectives of the work described in this thesis were:

- To review the current status of *H. annosum* s.l. research and identify directions for future studies (**Paper I**).
- To characterise the pine response to *H. annosum* s.s. infection at the gene expression level (**Paper II**). Until recently, research on the *H. annosum* pathosystem has focused on individual genes or proteins; large scale analysis of the transcriptome of infected plants could identify novel defence strategies against the pathogen.
- To use a saprotroph and a symbiotic ectomycorrhizal fungus as non-pathogen models to identify genes that may be specifically involved in recognition and regulation of interactions of *Pinus sylvestris* L. seedlings with the conifer pathogen *H. annosum* s.s. (**Paper III**).
- To use organ- (root and shoot) specific pathogens (*H. annosum* s.s. and *Gremmeniella abietina*) to investigate whether the gene machinery engaged for host defences within root tissues is the same as that employed by aerial parts of conifer trees (**Paper IV**). Investigating organ-specificity in pine defence could shed more light on the nature of the host defence.

- To investigate the molecular basis of non-self competitive interaction between the conifer pathogen *H. parviporum* and the biological control agent *P. gigantea* (**Paper V**). In the absence of any genotype in the Pinaceae with total resistance against *H. annosum,* finding ways of improving the efficacy of the only available biological control agent against the pathogen would be valuable. A major step in that direction is to understand mechanisms underlying the interaction.

Material and methods

In the papers comprising this thesis a number of techniques were employed to characterise gene expression level. Since the details of these methods are presented in the respective papers, the purpose of this section is only to outline the principles.

Biological material

Pinus sylvestris seeds (provenance Eksjö, Sweden) were surface sterilised with 33% H_2O_2 for 15 min, rinsed in several changes of sterile distilled water, sown on 1% water agar and incubated at 18°C with a photoperiod of 16h for 14 days. The fungal species: *H. annosum* s.s. (isolate FP5, courtesy of K. Korhonen, Finland), *H. parviporum* (isolate FS6, courtesy of K. Korhonen, Finland), *Laccaria bicolor* Maire (Orton) (courtesy of A. Tunlid, Sweden), *Phlebiopsis gigantea* (courtesy of K. Korhonen, Finland) and *Trichoderma aureoviride* Rifai (isolate A361, courtesy of G. Daniel, Sweden) were maintained on Hagem agar (Stenlid, 1985) at 20°C. *Gremmeniella abietina* (Lagerb.) Moralet (isolate 1:2b, courtesy of E. Stenström, Sweden) was grown on vegetable agar medium (200 ml vegetable juice, 5g glucose, 20g agar, 800 ml water).

A *Pinus sylvestris* interaction system to study pine responses to microbial colonisation

In an effort to develop a working model system for conifer-pathogen interactions (**Paper II**), *P. sylvestris* seedlings infected with *H. annosum* s.s. were used. Ten seedlings of *P. sylvestris* were transferred to wet, sterile filter paper placed on 1% water agar in a Petri dish. The roots were inoculated with 1 ml of mycelial homogenate and covered with a second set of moist sterile filter paper. Control plants were mock-inoculated with 1 ml sterile distilled water. The seedlings were then kept under a photoperiod of 16h light at 18°C. One hundred seedlings of either infected or control plants were harvested at 1, 5 and 15 days post inoculation (d.p.i.) and used for RNA extraction. There were three biological replications.

The process of infection in this system is well documented (**Paper I**): development of infection structures (germ tubes and appressoria) occurs within 24 hours, followed by direct penetration and internal colonization of the cortical tissues with the fungus reaching the endodermal region 3–7 days post inoculation

(d.p.i.); colonisation and disintegration of the vascular region take place within 9–15 days. Previous studies have shown that suberized and non-suberized roots respond in a similar way to the pathogen infection (Asiegbu, Daniel & Johansson, 1994; Johansson, Lundgren & Asiegbu, 1994). Accordingly, the *P. sylvestris* seedling-*H. annosum* s.s. system appears to be a good model to study pine defence mechanisms in terms of size, time and correlation with more advanced plant developmental stages.

The same system was used in **Paper III** to compare pine responses to pathogenic and non-pathogenic fungal species: *H. annosum* s.s. (pathogen), *T. aureoviride* (saprotroph) and *L. bicolor* (ectomycorrizal symbiont) (Fig. 2).

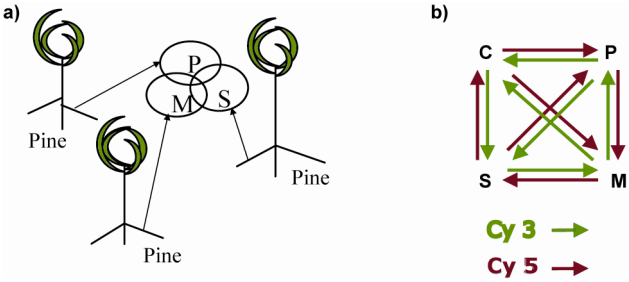

Fig. 2. a) The aim of the study described in paper II was to compare the response of pine roots to pathogenic (*H. annosum* s.s.) [P] and non-pathogenic fungal species: *T. aureoviride* (saprotroph) [S] and *L. bicolor* (ectomycorrizal symbiont) [M]. b) To achieve this, samples from the inoculated and control [C] roots harvested at 1, 5 and 15 days post inoculation were reciprocally labelled with fluorescent dyes Cy3 and Cy5 and used for microarray hybridisations.

Infection system to study organ-specificity in pine responses to pathogen attack

In **Paper IV** root and shoot specific pathogens, *H. annosum* s.s. and *G. abietina* respectively, were used to investigate whether the gene machinery engaged for host defences within root tissues is the same kind as that employed by aerial parts of conifers for resistance against necrotrophic pathogens. The roots were inoculated with 1 ml of spore suspension of $5x10^6$ spores/ml of either the root- or shoot-specific pathogen. For the shoot inoculations, 1 ml of the spore suspension of either *G. abietina* or *H. annosum* s.s. was applied on the needles. The roots were watered with 1 ml of sterile distilled water. Control plants were mock-inoculated with 1 ml sterile distilled water. The seedlings were then kept under a photoperiod of 16h light at 18°C. The roots or shoots of 100 seedlings of either infected or control plants were harvested at 7 d.p.i.

Transcript profiling of the *H. annosum* s.l. pathosystem

The transcriptome approach offers the possibility of global analysis of gene expression which is especially useful in the field of plant defence, where multiple overlapping strategies are usually employed to respond to a pathogen. A number of techniques has been used to study coniferous trees responses to pathogens: macroarrays (Morse *et al.*, 2004), differential display (Johnk *et al.*, 2005; Morse *et*

al., 2004), analysis of cDNA subtraction libraries (Asiegbu, Nahalkova & Li, 2005; Morse *et al.*, 2004) and cDNA microarrays (Myburg *et al.*, 2006; Ralph *et al.*, 2006a; Ralph *et al.*, 2006b). Differential display seems to be the least reliable method among those mentioned: Johnk *et al.* (2005) identified 36 transcripts up-regulated by spruce infected with root rot pathogen *Ceratobasidium bicorne*. However, real-time RT-PCR verification of the expression level of 18 out of those genes, confirmed induction of only nine.

Microarray profiling was used in **Papers II** and **III** and a macroarray approach was employed in **Papers IV** and **V.**

RNA amplification

As there is a threshold that defines a minimum sample concentration that must be applied in a given experiment, amplification of RNA isolated from the plant material was necessary. The cDNA was synthesised from the same amount of RNA (1 µg) using SMART™ PCR cDNA synthesis kit (Clontech, USA). SMART™ PCR is a highly efficient method for exponentially amplifying RNA but the nonlinear amplification results in a target in which sequence representation is slightly skewed compared with the original mRNA pool (Puskas *et al.*, 2002; Wadenback *et al.*, 2005). However, other authors have shown that the amplified material faithfully represents the starting mRNA population (Petalidis *et al.*, 2003; Seth *et al.*, 2003). It should also be noted that RNA amplification increases the sensitivity of microarray experiments considerably, allowing the identification of differentially expressed transcripts below the level of detection using targets prepared by direct labelling (Petalidis *et al.*, 2003).

Microarray

The high correlation of transcript level for the same tissues between *P. sylvestris* and *P. taeda* (r=0.93) (van Zyl *et al.*, 2002) permits differential screening to be done using the loblolly pine arrays with RNA obtained from Scots pine. The 2109 ESTs (expressed sequence tags) used in **Paper II** and **Paper III** were obtained from six cDNA libraries of *P. taeda* representing different developmental stages in wood formation (http://biodata.ccgb.umn.edu/) (Kirst *et al.*, 2003)**.** The target preparation, labelling**,** hybridisation and stringency washes followed the protocol from North Carolina State University (Brinker *et al.*, 2004) (Fig. 3). The experimental design involved dye swaps and comparison of inoculated versus un-inoculated sample at each time point: 1, 5 or 15 d.p.i. (see also Fig. 2b)

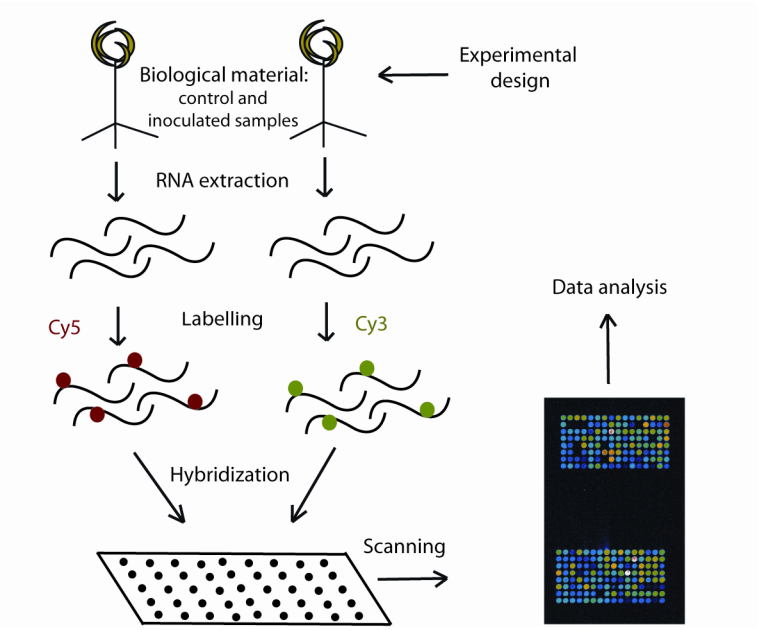

Fig. 3. Schematic illustration of microarray experiment: RNA was extracted from biological material and labelled with fluorescent dyes. Samples from inoculated and control roots were mixed and hybridised with cDNA spotted on glass slides. After stringency washes the slides were scanned and obtained images were used for calculating relative abundance of transcript levels in each sample.

Significance in transcript abundance changes was estimated using two successive mixed models as described by Wolfinger *et al*. (2001) and Jin *et al*. (2001). Many transcript abundance expression changes less than two-fold were statistically significant (Jin *et al.*, 2001); moreover, some compression in these estimates was likely, as shown in later comparison with real-time RT-PCR. To conservatively ensure a false positive rate of 0.01, a p-value cutoff was set at the Bonferroni value of $0.01/2109 = 4.5 \times 10^{-6}$, as suggested by Wolfinger *et al*. (2001).

Verification of expression of selected genes was performed using real-time quantitative RT-PCR. The transcript abundance was detected by the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Sweden) using SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's recommendations. Transcript levels were calculated from three technical replicates using the standard curve method (User Bulletin #2, ABI Prism 7700 Sequence Detection System, Applied Biosystems) and normalized against total amount of RNA (Hashimoto, Beadles-Bohling & Wiren, 2004; Silberbach *et al.*, 2005).

From mRNA to a protein - cellular localization of the antimicrobial peptide (AMP)

Both microarray and real-time RT-PCR analyses revealed that *P. sylvestris* root tissues in response to infection with pathogen *H. annosum* had elevated transcript levels of genes coding for antimicrobial peptide (AMP) (**Paper II**). To confirm

accumulation of the protein and determine its cellular localisation at 15 d.p.i., an immunocytochemical study was performed using specific antibody. The AMP peptide sequence used for raising polyclonal antibody corresponded to amino acids within the conserved region of the gene (Asiegbu *et al.*, 2003). Polyclonal antibody was raised against the synthetic peptide using rabbits at the Statens Veterinärmedicinska Anstalt (SVA, Uppsala, Sweden). The procedures for embedding, tissue fixing and immunocytochemical labelling have previously been described (Asiegbu, Daniel & Johansson, 1994). The sections were examined using a Philips EM201 transmission electron microscope operated at 60kV.

Macroarray

In **Paper IV** a total of 384 cDNAs was selected from a *P. sylvestris* cDNA subtraction library (HASP) containing genes differentially expressed during interaction with *H. annosum* s.s. (Asiegbu, Nahalkova & Li, 2005) and *P. taeda* cDNA library (Kirst *et al.*, 2003). The clones were manually transferred onto Hybond® N^+ nylon membranes (GE Healthcare, Sweden). The membranes were used for hybridisation with cDNA probes labelled according to the manufacturer's instruction (AlkPhos Direct™ labelling kit, GE Healthcare, Sweden). Signal generation and detection were done with CDP-Star (GE Healthcare). The arrays were wrapped in plastic foil and exposed to ECM film (GE Healthcare). The films were scanned and used for further analysis with the help of Quantity One software (Bio-Rad, http://www.biorad.com).

The data analysis was modified from the procedures published by Duplessis et al. (2005). Data quality assessment was performed using analysis of variance. The *t*-test was done using a Microsoft Excel data analysis tool − *t*-Test: Two Sample Assuming Unequal Variance.

To verify the expression pattern of selected genes virtual northern blot was done according to the manufacturer's instructions (Clontech, USA).

Experimental model system to study *H. parviporum-P. gigantea* interaction

In **Paper V** an experimental model consisting of dual cultures grown at 18°C on Hagem agar was used to examine pathogen–biocontrol agent interactions. Agar plugs of actively growing cultures of either *H. parviporum* or *P. gigantea* were inoculated as paired cultures on agar plates at a distance of 30 mm from each other. The paired cultures were grown either as self interactions (*H. parviporum-H. parviporum* and *P. gigantea-P. gigantea*) or non-self interactions (*H. parviporum-P. gigantea*). Total RNA was extracted from either pure cultures, the barrage zones formed by interacting fungi or mycelia outside the barrage zones during the self- or non-self interactions at different periods ranging from 5 to 28 days. cDNA was synthesized with SMART™ PCR cDNA kit (Clontech, USA).

The cDNA probes were used for screening of *P. gigantea* and *H. parviporum* cDNA libraries.

Construction and differential screening of P. gigantea (GIGA) cDNA library and H. parviporum (HAGS) cDNA library

Phlebiopsis gigantea was grown in liquid Hagem medium at 18°C in static conditions. After 14 days the total RNA was extracted (Chang, Puryear & Cairney, 1993). The cDNA library was constructed using Creator™ SMART™ cDNA Library Construction kit (Clontech). The library (GIGA) consisted of 2,636 clones. The clones were stored in 96-well plates at -80°C. A total of 716 clones randomly selected from GIGA cDNA library were manually transferred onto Hybond® N^+ nylon membranes.

The cDNA library for *H. parviporum* germinated spores (HAGS) was similarly constructed and the recombinant cDNA clones (3,072) stored in 384-well microtitre plates, as previously described (Abu, Li & Asiegbu, 2004). A total of 3,072 clones from *H. parviporum* cDNA library (HAGS) were replicated onto Hybond N^+ membranes (Amersham Pharmacia Biotech, USA) using a Q-bot automated workstation (Genetix, USA).

The constructed macroarrays were used for differential screening with probes made from self and non-self interactions of *P. gigantea* and/or *H. parviporum.* The labelling, hybridisations and signal detection were done as outlined above.

A parametric empirical Bayesian approach (Lönnstedt & Speed, 2002) was used to identify differentially expressed genes.

To verify the expression pattern of selected genes virtual northern blot was done.

Results and discussion

Conifer responses to pathogen invasion

While knowledge about regulation of defence and resistance in agricultural and horticultural crops is well established, the nature of defence responses and disease resistance in pines and other gymnosperms remains largely unexamined. In **Paper II**, an array consisting of 2109 ESTs was used to document changes in gene expression following *P. sylvestris* infection with a necrotrophic fungal pathogen *H. annosum* s.s. at 1, 5 and 15 d.p.i. Mixed model analysis identified 164 unique genes differentially expressed and noted a general tendency of increase over time in number of genes differentially regulated.

The delay (5-15 d.p.i.) before detection of substantial plant response contrasts with the prompt hypersensitive reaction related symptoms usually visible within hours or even seconds after infection in angiosperms (Heath, 2000a). Gymnosperm pathosystems tend to respond in a slower manner to a pathogen attack (Hietala *et al.*, 2004; Nagy *et al.*, 2004a; Pearce, 1996). The small number of genes differentially expressed at the early stage of interaction may reflect a lack of information a plant possesses about the nature of the attack during the initial phase of infection (Franceschi *et al.*, 2005). It is also possible that the pathogen has

evolved a strategy to mask detection by the conifer root. For example, masking chitin and glucan molecules that may act as potential elicitors could delay plant responses until the pathogen has established itself in the host.

In the experiment, at 1 d.p.i. two genes coding for proteins with antimicrobial properties were found to be significantly up-regulated: thaumatin and antimicrobial peptide (AMP). Antimicrobial peptides have been detected in a wide variety of agricultural plant species and have been implicated in resistance of such plants to microbial infections (Broekaert *et al.*, 1997). A similar role has been suggested for conifer defensins (Fossdal *et al.*, 2003; Pervieux *et al.*, 2004). Immunocytochemical localization of AMP revealed substantial accumulation of the peptide in the cell wall region at 15 d.p.i. (Fig. 4). Abundance of the antimicrobial peptide on the cell surface may indicate a direct role of AMP in defence against invading fungal hyphae.

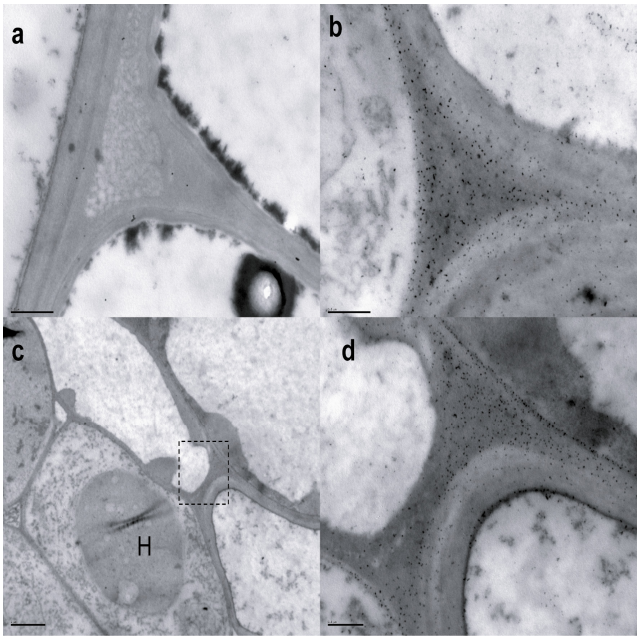

Fig. 4. Transmission electron microscope photographs of *P. sylvestris* un-infected and *H. annosum* infected roots showing immuno-localisation of antimicrobial peptide (AMP) at 15 d.p.i.: a) cell wall region in the control root and corresponding region in the b) infected root illustrate increased accumulation of the AMP (black dots - gold-labelled secondary antibodies); c) hyphae (H) infecting plant cell; neighbouring cell wall region (dashed square) is enlarged in d). Bar represents 0.2µm (a, b, d) and 1µm (c).

Many of the genes induced during more advanced stages of *H. annosum* s.s. infection were involved in secondary metabolism. Numerous chemicals are produced by enzymes active in the phenylpropanoid pathway, starting with phenylalanine ammonia lyase and branching into lignin, stilbene and flavonoid biosynthesis (Dixon & Paiva, 1995). A class III secretory peroxidase was induced by pine challenged with *H. annosum* s.s. throughout the whole infection process. Peroxidases have been associated with plant defence and resistance, particularly with lignin and suberin synthesis, but also with cross-linking phenolic compounds

into papillae and production of toxic compounds (Asiegbu, Daniel & Johansson, 1994; Fossdal, Sharma & Lonneborg, 2003; Takahama & Oniki, 2000).

Successful resistance to pathogens depends not only on genetics – the ability to produce certain antimicrobial compounds; but also on physiology – ability to compensate high costs of investing into constitutive defence mechanisms and later on into the inducible ones. The metabolic shift into increased production of secondary compounds initiated at 5 d.p.i. was intensified at 15 d.p.i. and completed by induction of enzymes involved in primary metabolism (methionine metabolism, shikimate pathway) and energy acquisition.

One of the first macroscopically visible symptoms of pathogen invasion was a necrotic browning reaction. The question arises whether it was solely due to the necrotrophic activity of the pathogen, or whether host controlled programmed cell death (PCD) was engaged. Occurrence of several hallmarks of PCD (differential expression of BAX inhibitor, caspase-like protease, lipases and cyclin dependent kinase) indicates an active role of the plant, although induction of the process by the pathogen cannot be ruled out. Unfortunately, host controlled cell death can only prevent the growth of biotrophic pathogens but not necrotrophs like *H. annosum* s.s. (Glazebrook, 2005; Morel & Dangl, 1997). Since PCD is connected with production of reactive oxygen species, it could lead to uncontrolled damage at the tissue level (Morel & Dangl, 1997). Potential protective mechanisms include anti-oxidants, like glutathione-S-transferase, metallothionein and thioredoxin up-regulated by *P. sylvestris*, or flavonoid compounds. Induction of genes implicated in oxidative processes seems to be a common feature of conifer responses to root pathogens like *H. annosum* or *Rhizoctonia* sp. (Johnk *et al.*, 2005) and insects (Ralph *et al.*, 2006b).

The full extent of the overall reprogramming of the infected plant functioning comprises not only positive but also negative regulatory mechanisms. The significance of gene repression during pathogen defence is probably associated with the down-regulation of all non-essential cellular activities and mobilization of the gained resources to cope with the challenge (Somssich & Hahlbrock, 1998).

Plant responses to pathogenic, saprotrophic or mutualistic interactions

In **Paper III** transcript profiling of *P. sylvestris* roots challenged with a pathogen (*H. annosum* s.s.) (**Paper II**) was compared to transcript profiles from pine roots interacting with two other fungi, a saprotroph *Trichoderma aureoviride* and an ectomycorrhizal fungus (*Laccaria bicolor*) (Heller et al., in preparation). The most striking feature of the global expression pattern at 1 d.p.i. was the clear response to the mycorrhizal fungus (Fig. 5a). The expression profile of control pine tissues was most closely related to the saprotroph challenged roots. The host reaction to the pathogen was not specific, except for a number of genes belonging to cluster 6 (Fig. 5a). The global expression pattern at 5 d.p.i. was remarkable due to the clear distinction between all three interactions (Fig. 5b). At 15 d.p.i. there was a shift in the global expression pattern towards a pine response that was specific to the pathogen and fairly unspecific to the saprotroph and mycorrhiza. Only a small

number of up-regulated genes were exclusively associated with the mycorrhiza treatment (cluster 21) (Fig. 5c).

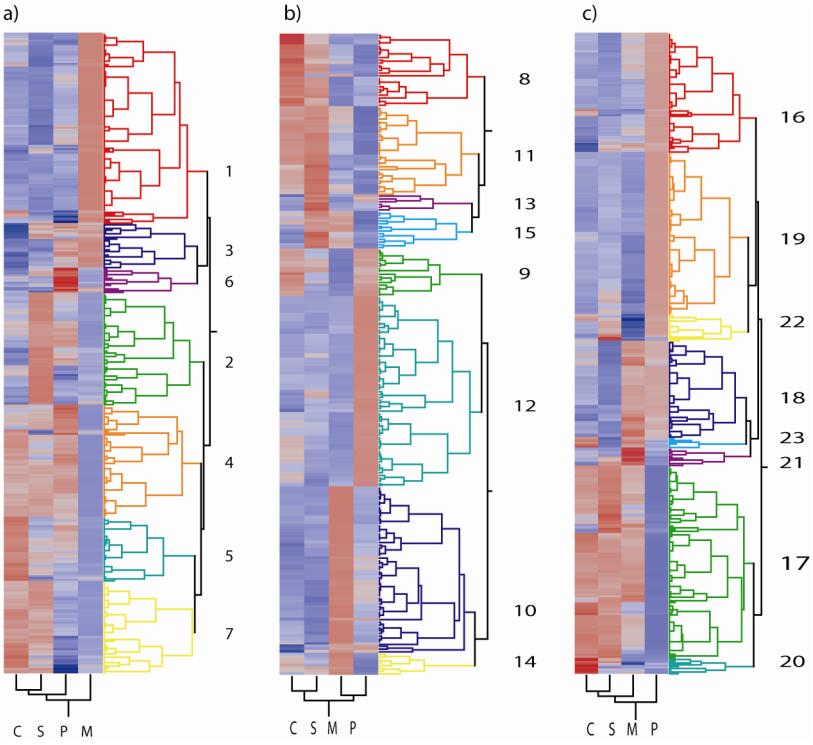

Fig 5. Hierarchal clustering illustrating groups of *P. sylvestris* genes coordinately expressed in response to pathogen *H. annosum* (P), ectomycorrhiza *L. bicolor* (M) or saprotroph *T. auroviride* (S) infection at a) 1, b) 5 and c) 15 d.p.i. as compared to un-inoculated control (C). Each row illustrates expression profile of each of significantly expressed genes (identified by mixed model analysis) (red-blue colour - high-low expression). All the differentially expressed genes were divided into 23 regulatory patterns, indicated by the numbers 1-23.

Comparing pathogenic interaction with non-pathogenic models, it was expected that pine roots would initially respond more dramatically to the pathogen than to the other fungi. Instead, a lower number of genes was found to be differentially expressed in the pathogenic interaction at the early stage of interaction, compared to mycorrhizal and saprotrophic associations. The delayed plant response to the pathogen infection may be attributed to a lack of information possessed by the plant about the nature of the attack during the initial phase of infection (**Paper II**). Since the timing of the host response seems to be crucial, the ability of *H. annosum* s.s. to avoid immediate recognition during the initial stages of interaction may allow it to penetrate the host tissues before the defence response can be induced. This can be seen as a part of the strategy of a successful pathogen.

In spite of their suggested common evolutionary origin (Hibbett, Gilbert & Donoghue, 2000), the saprotrophic and ectomycorrhizal interactions investigated in the study displayed very little overlap in terms of differential gene expression. Furthermore, there were only four genes regulated in the same way after challenge

with the pathogen or the saprotroph; interestingly, two of them had defence-related functions. Although there is a general lack of cellular interaction between the saprotroph and the root, the plant was able to recognise the presence of *T. aureoviride*, mount some defence reaction, presumably as a preventive measure, and then largely ignore the fungus.

The existence of a common pattern of response to microbial colonisation was suggested by Güimil et al. (2005). There are genes in legumes that affect symbiosis with both eukaryotic arbuscular mycorrhiza (AM) and prokaryotic *Rhizobia* spp. indicating conservation of symbiotic mechanisms (Ane *et al.*, 2004; Levy *et al.*, 2004). Strikingly, *Rhizobia* and root knot nematodes invoke similar morphological effects in *Lotus japonicus* and elicit common signal transduction events, indicating recruitment of symbiotic pathways by the pathogens (Weerasinghe, Bird & Allen, 2005). Güimil et al. (2005) showed a significant overlap (43%) between AM-specific rice genes and those responding to infection with fungal pathogens. The much smaller overlap observed in our study between the pathogen and mycorrhiza treatment, may stem from differences between the hosts (*L. japonicus*, rice, pine), the kind of pathogen investigated (nematode, necrotrophic fungus) or the symbiotic strategies of ECM and AM fungi. In addition, the results of our study do not fully reflect a mycorrhiza-specific pine response, as at 15 d.p.i. the symbiosis was not fully established and a later time point would be necessary for a full comparison of symbiosis- and pathogenesis-related genes.

The existence of a common pattern of response to fungal colonisation is supported by the results of real-time RT-PCR analysis of antimicrobial peptide expression. AMP was induced at 1 d.p.i. in all three interactions. While the up-regulation continued in the roots infected with the pathogen at 5 and 15 d.p.i., in both non-pathogenic systems the expression level was considerably lower. Apparently, pine AMP represents a broad defence mechanism which might be employed against a wide range of organisms and activated even before recognition mechanisms identify the nature of the microorganism as beneficial, neutral or harmful.

Considering the different trophic strategies and distant taxonomic relation of the three fungal species used in this study, the small overlap detected between plant responses to the microbes is not surprising. Instead, the results suggest that there are specific regulatory patterns of transcriptional responses of conifer trees to colonisation by either ectomycorrhizal symbionts, saprotrophs or pathogens.

A model illustrating the pine response mechanisms in the three different interactions is presented in figure 6. The saprotroph, as an example of an organism not interacting directly with the plant, caused only very little change in gene expression, with an initial peak symbolizing recognition of the presence of the fungus. The ectomycorrizal symbiont triggered a striking initial response and induction of defence related genes that subsequently declined. The response and induction of defence against the pathogen was delayed and the magnitude increased over time. This has led to a supposition that pathogen, saprotroph, or ectomycorrhizal symbiont associated specific molecular patterns may have much to do with gene expression levels and separation in time rather than the absolute uniqueness of the individual genes that are differentially regulated by the host.

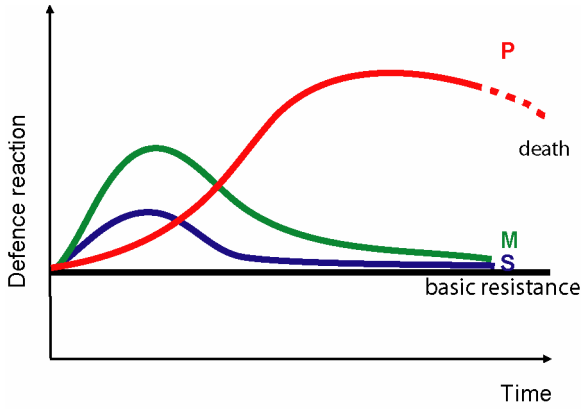

Fig. 6. A model illustrating the observed time-dependent changes in pine response to challenge with a pathogen (P), an ectomycorrhizal symbiont (M) or a saprotroph (S). The saprotroph provokes very weak reaction that declines when it is recognised as neutral microorganism. The ectomycorrhizal fungus induces transient expression of defence-related genes that diminishes over time. On the other hand, the induction of defence in response to the pathogen invasion is initially delayed and increases rapidly with prolonged infection.

Organ specificity in the *H. annosum* s.s – conifer pathosystem

Root and foliar pathogens differ in their ecology, epidemiology, life cycles, pathogenesis and infection. The knowledge of host-pathogen interactions is based mostly on foliar pathogens, the mechanisms of resistance to necrotrophic root pathogens are less well understood. *H. annosum* s.s. and *G. abietina* are forest pathogens with overlapping host range but differing in organ-specificity: while *H. annosum* causes root and butt rot, *G. abietina* infects shoots and needles. In **Paper IV** pine shoots and roots were challenged with both pathogens. Macro- and microscopic observations revealed that *H. annosum* s.s. was able to cause necrosis not only on the root but also on the needles. In this way, *H. annosum* s.s. could be a second example after *Magnaporthe grisea* (Sesma & Osbourn, 2004) of a pathogen capable of infecting other tissues apart from the organ it has been typically reported to attack.

The gene expression level of pine shoots and roots infected with either pathogen was investigated at 7 d.p.i. The root response to *H. annosum* s.s., namely up-regulation of genes with functions related to metabolism and defence, correlates with results obtained in **Paper II**. Although defence related genes constituted one of the main functional categories up-regulated by the shoot challenged with *H. annosum* s.s., gene expression profile of both organs infected with the same pathogen was very different (Fig. 7). A similar situation occurred when needles or roots were infected with the shoot specific pathogen, *G. abietina.* Interestingly, defence related genes, like peroxidases were differentially expressed in the root but not in the shoot.

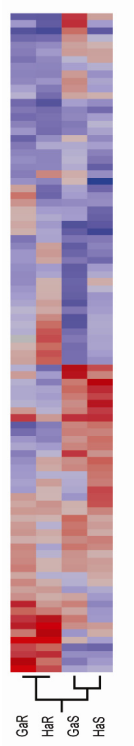

Fig. 7. Hierarchical clustering showing coordinated expression of genes differentially regulated by *P. sylvestris* shoots and roots inoculated with *H. annosum* s.s. or *G. abietina* at 7 d.p.i. as compared to un-inoculated control shoots or roots (red-blue: up- and down-regulation, HaR-*H. annosum* s.s. infected roots, GaR-*G. abietina* infected roots, HaS-*H. annosum* s.s. infected shoots, GaS-*G. abietina* infected shoots).

Surprisingly, challenging the pine root with a shoot specific pathogen that did not cause any visible symptoms led to differential expression of a higher number of genes compared to infection with the root specific pathogen, *H. annosum* s.s. Pathogens able to engage in compatible interactions leading to disease often possess mechanisms suppressing host defence (Okubara & Paulitz, 2005) which may result in a lower number of differentially expressed genes.

The relative similarity of the expression profile of pine root (or shoot) infected with two pathogens with different organ specificity points towards organ-specific rather than pathogen-specific defence. The differences in defence strategies employed by the different plant organs have been demonstrated before in *Arabidopsis* model and crop plants, mostly responding to the pathogen attack in a gene-for-gene manner (Hermanns, Slusarenko & Schlaich, 2003; Jansen, Slusarenko & Schaffrath, 2006; Schafer & Yoder, 1994).

The differences in responses of the various organs may also be partly related to the biological functions of the tissues and how their development is impacted by the invading pathogen. Biologically roots and aerial parts of the plant perform different functions; the leaves or needles are responsible for photosynthesis and roots maintain water and mineral uptake. The different tasks are performed in different surroundings: roots are anchored in soil, full of microorganisms which are not always hostile. Hence, the necessity to develop recognition mechanisms that might not be required in the leaves or needles and stems.

Self and non-self interactions between *H. parviporum* and its biocontrol agent *Phlebiopsis gigantea*

The interaction between *H. parviporum* and *P. gigantea* is known to be a deadlock or overgrowth rather than antibiosis or parasitism commonly observed with other biocontrol agents such as *Trichoderma* spp. (Viterbo *et al.*, 2002). In the *Trichoderma* spp. biocontrol system, secretion of chitinase, a major enzyme involved in breakdown of hyphal cell walls, has been implicated in the success of the biocontrol process (Haran, Schickler & Chet, 1996; Viterbo *et al.*, 2001). On the other hand, in the *P. gigantea* – *H. parviporum* interaction, preferential expression of genes encoding a diverse range of proteins, including those important for efficient substrate utilization and nutrient acquisition (fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, endo-galacturonase, glutamine synthetase) were documented. The results of the virtual northern blot analysis suggest that the increased transcript level of fructose bisphosphate aldolase in the barrage zone could be due to strong expression by *H. parviporum* whereas high levels of glutamine synthetase and glyceraldehyde-3-phosphate dehydrogenase (GADPH) most likely originated from *P. gigantea* mycelia (Fig. 8). It is however difficult to explain whether the expression of these genes is specific for the interaction or perhaps a response to stress or nutrient starvation.

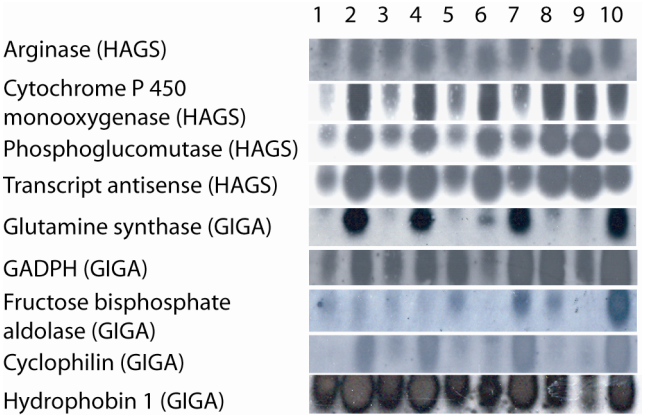

Fig. 8. Virtual northern blot verified expression level of selected genes isolated from *H. parviporum* (HAGS) or *P. gigantea* (GIGA) cDNA library in self and non-self interactions of *P. gigantea* (Pg) and *H. parviporum* (Hp). GADPH - glyceraldehyde-3-phosphate dehydrogenase. Descriptions of numbers:1= Pre-contact interacting *H. parviporum* hyphae (5 d.p.i.) , 2= Pre-contact interacting *P. gigantea* hyphae (5 d.p.i.), 3= Hyphae contact zone for self-interaction of *H. parviporum* (Hp-Hp) (9 d.p.i.), 4= Hyphae contact zone for self-interaction of *P. gigantea* (Pg-Pg) (9 d.p.i.), 5= *H. parviporum* domain outside the barrage zone during non-self interaction with *P. gigantea* (9 d.p.i.) 6= *P. gigantea* domain outside the barrage zone during non self interaction with *H. parviporum* (9 d.p.i.), 7= Barrage zone during non-self interaction of *P. gigantea* and *H. parviporum* at either 9 d.p.i. , 8= Monoculture of *H. parviporum* (28 d.p.i.), 9= Monoculture of *P. gigantea* (28 d.p.i.), 10= Barrage zone for non-self interaction of *P. gigantea* and *H. parviporum* (28 d.p.i.).

Two other interesting genes encoding cyclophilin and hydrophobin 1 were up-regulated in the barrage zone but their role in the interaction remains unclear.

Cyclophilin is a highly conserved protein that regulates a variety of cellular processes, including the response to environmental stresses, cell cycle control, the regulation of calcium signalling and the control of transcriptional repression. Cyclophilin has also been identified in another biological control agent - *Trichoderma harzianum* (Grinyer *et al.*, 2004). Hydrophobins are small secreted multifunctional proteins that have been detected in ascomycetes and basidiomycetes (Wessels, 1997; Whiteford & Spanu, 2002). They possess ability to self-assemble at hydrophilic-hydrophobic interfaces into highly amphipathic films. Hydrophobins have also been implicated in cell-wall assembly and in pathogenic interactions where the monomers could act as toxins and elicitors (Tucker & Talbot, 2001). Changes in hyphal hydrophobicity might lead to sealing-off of the hyphal boundaries at the interaction interface and formation of the barrage zone (Rayner, Griffith & Wildman, 1994). The results of the virtual northern blot further suggests that the up-regulation of hydrophobin 1 and cyclophilin in the barrage zone is most likely due to increased expression by the interacting *P. gigantea* mycelia (Fig. 8).

Four other genes (phosphoglucomutase, arginase, cytochrome P450 monooxygenase, transcript antisense to ribosomal RNA) isolated from *H. parviporum,* when used for heterologous hybridisation, were found to be strongly expressed by *P. gigantea* (Fig. 8). Several authors studied the changes in mRNA levels of genes involved in amino acid biosynthesis and observed that most of these genes are expressed at significantly higher levels during periods of isotropic growth or in response to nutritional shifts (dEnfert, 1997; Sachs & Yanofsky, 1991). Cytochrome P450 monooxygenase on the other hand has been implicated in pathogenesis, antifungal resistance, biosynthesis of secondary metabolites and toxin detoxification (van den Brink *et al.*, 1998). Transcript antisense mRNAs (mitochondrial protein, Tar1p) have been shown to regulate mRNA transcription, processing, translation and DNA replication (Coelho *et al.*, 2002).

These four genes described above were slightly down-regulated in the barrage zone, and stronger expression was documented within *P. gigantea* mycelia located outside the barrage zone (Fig. 8). This suggests that the signals emanating from barrage zone formation could lead to increased expression of some key genes in other regions of the hyphae during non-self combative interaction.

Moreover, under varying nutrient sources *P. gigantea* had a higher growth rate than *H. annosum* s.l. and it also possessed the ability to secret significant levels of wood degrading enzymes (Adomas and Asiegbu, unpublished data). Consequently, the ability of *P. gigantea* to efficiently produce many of the functionally important enzymes necessary for nutrient acquisition and other metabolic processes presumably confers some competitive advantage during colonization of available niche on a suitable substrate and contributes to the success of *P. gigantea* as a biocontrol agent.

Application of transcript profiling to study conifer defence mechanisms

Both, microarrays and macroarrays proved to be useful techniques to elucidate molecular defence mechanisms mounted by pine in response to microbial colonisation. Large scale analysis of gene expression facilitates the identification of novel strategies and metabolic pathways used in the defence of conifer trees against fungal infection. This technology also allows for comparative analysis of responses to different microorganisms or different forms of stress. Certainly, using arrays with larger numbers of genes will in future enhance the value of such studies.

However, while performing transcript profiling, it should be remembered that defence-related genes commonly occur as families of closely related homologues whose mRNAs may cross-hybridise. Besides, annotation of genes in newly studied plant species is usually inferred on the basis of homology to an arbitrary member of the family without knowledge of whether and where the gene is expressed. An additional challenge in conifer systems is posed by low similarity of many pine or spruce genes with previously annotated angiosperm genes (Kirst *et al.*, 2003; Ralph *et al.*, 2006b). Under these circumstances, in many cases, the correlation between genes/mRNAs and proteins might be far from clear. Therefore, a focus should also be placed on gene expression at the protein level. To date, there have been few proteomics studies of conifers focusing on development (Lippert *et al.*, 2005) and defence mechanisms (Lippert *et al.*, 2007).

Comparing the microarray results with real-time RT-PCR revealed that the latter was a more sensitive technique but limited in terms of scale.

Conclusions

The microarray profiling of *P. sylvestris* responses to infection with fungal pathogen *H. annosum* s.s. (**Paper II**) revealed multiple overlapping strategies employed for defence purposes. Production of pathogenesis-related enzymes and antimicrobial proteins was supplemented by a major shift in primary and secondary metabolism. A wide array of oxidative stress protection mechanisms was documented, possibly related to programmed cell death. The similarity in the expression profiling pattern observed in this pathosystem to those documented in crop pathology suggests that both angiosperms and gymnosperms might use similar genetic programs for responding to invasive growth of microbial pathogens. The differences in response of the two plant groups might hinge on the spatial and temporal pattern of the gene regulation.

Analysis of *P. sylvestris* genes expressed during interaction with either a pathogenic, saprotrophic or mutualistic fungal species (**Paper III**) indicates that pine was able to recognize all three species and specifically distinguish whether they were pathogenic, neutral or beneficial microorganisms. The response and induction of defence against the pathogen was delayed and the magnitude

increased over time (**Paper II**). The saprotroph caused only very little change in gene expression. The ectomycorrizal symbiont triggered a striking initial response and induction of defence related genes that subsequently declined. Subtle differences in the timing and amplitudes of transcriptional activation, rather than profound qualitative differences in global expression patterns, may account for specificity of plant responses in those different interactions.

Gene coding for pine antimicrobial peptide (AMP) was shown to be transcribed and translated in response to infection with the necrotrophic pathogen *H. annosum* s.s. (**Paper II**). AMP transcription was also induced during non-pathogenic interactions with either a saprotroph or ectomycorrhizal fungus, although the expression level was considerably lower (**Paper III**). Apparently, pine AMP represents a broad defence mechanism which might be employed against a wide range of organisms and activated even before recognition mechanisms identify the nature of the microorganism as beneficial, neutral or harmful.

Comparison of differentially expressed genes from pine tissues challenged with either root (*H. annosum* s.s.) or shoot specific (*G. abietina*) pathogens (**Paper IV**) indicates that the responses were more organ-specific than pathogen-specific: transcript profiles of roots infected with *H. annosum* s.s. were more similar to roots challenged with *G. abietina* than to profiles of shoots exposed to *H. annosum* s.s.

The ability of *P. gigantea* to efficiently produce many of the functionally important enzymes necessary for nutrient acquisition and other metabolic processes (**Paper V**) presumably confers some competitive advantage during colonization of available niches on a suitable substrate and contributes to the success of *P. gigantea* as a biocontrol agent of *H. annosum* s.l.

Future perspectives

The ultimate goal is to exploit knowledge about *H. annosum*-conifer interactions in disease management. Comparing host responses of trees with various levels of resistance to the pathogen could aid breeding programs and facilitate the selection of naturally occurring genotypes with higher resistance against the root rot.

Future research should focus on unveiling the signal transduction pathways following perception of the pathogen by the host and the complex ways in which they interact. Increasing our knowledge of signalling pathways that activate pine defence responses would provide a basis for understanding the defence mechanisms of gymnosperms in general. How defence mechanisms are regulated and maintained over longer time periods and large physical distances in trees as compared to short-lived annuals and crop plants is poorly understood but of potential ecological and biotechnological significance.

It would be of general interest to study the plant recognition of pathogenic and non-pathogenic microorganisms in a more detailed way. The kinds of receptors involved, the signalling pathways triggered and the factors determining switching

off of the defence responses in case of non-pathogenic microbes remain unknown in coniferous trees.

The mechanisms responsible for the mode of action of *P. gigantea* remain unexplained and poorly investigated. The mechanistic basis for the antagonism is still largely hypothetical. The competitive advantage based on nutrient acquisition seems plausible but requires further investigations. A major concern is a build-up of selection pressure in either fungus, potentially leading to *H. annosum* s.l. strains resistant against the biocontrol agent or *P. gigantea* strains developing pathogenicity against conifers. Future studies geared towards these aspects will be valuable.

Increasing the number of available conifer ESTs will facilitate performing large scale analyses of defence mechanisms, employing not only transcriptomic approaches, but proteomics as well. Recent developments in metabolomics mean a new, wider approach to biochemical analysis has become possible. Technologies for extract preparation, component separation and accurate mass detection combined with automated strategies for data collection, handling and analysis could provide a broader insight into the biochemical composition of phenolics and terpenoids produced by conifers and their respective roles in pathogenic interactions.

For full comprehension of the host-pathogen interactions, factors determining *H. annosum* s.l. pathogenicity should be investigated. Of particular interest would be to examine strategies used by the fungus to avoid recognition by the host; the possibility of active suppression of host defence mechanisms; and the pathogen's role in triggering host cell death. The genome sequence of *H. annosum* that will be available shortly will greatly ease the study of virulence factors in this necrotrophic pathogen.

Finally, improving efficiency of transformation techniques in both the host and the pathogen would enable developing mutant lines and functional studies with candidate genes identified in this thesis as defence related.

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List of abbreviations

AM – arbuscular mycorrhiza
AMP – antimicrobial peptide
ECM – ectomycorrhizal
EST – expressed sequence tag
ET – ethylene
HR – hypersensitive response
JA – jasmonate
MAMP – microbe associated molecular pattern
MAPK – mitogen activated protein kinase
MeJA – methyl jasmonate
NBS-LRR – nucleotide binding leucine rich repeat
PAMP – pathogen associated molecular pattern
PCD – programmed cell death
PR – pathogenesis related
Real-time RT-PCR – real-time reverse-transcription polymerase chain reaction
ROS – reactive oxygen species
SA – salicylic acid

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