

**Transcriptional Responses during the
Pathogenic Interaction between
Heterobasidion Annosum s. l. and
Conifers**

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Abstract

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This thesis summarizes the results of four studies regarding transcriptional changes of *Heterobasidion annosum* (Fr.) Bref. s. l. and conifers in response to their pathogenic interaction.

The diversity of expressed genes in *H. annosum* during the pathogenic interaction with Scots pine seedlings were studied by construction of a cDNA-library and generation of expressed sequence tags. Contig analysis identified 318 unigenes, of which 62 were repeatedly sampled. A putative function was assigned to 223 unigenes (70 %) that showed high or moderate similarity to protein sequences in public databases. The unigenes encoded proteins of metabolic functions with putative involvement in the pathogenic process, such as secondary metabolism, degradation of host material, signal perception and transduction and membrane transport.

The transcriptional responses of several genes were studied with realtime-PCR during fungal infection of conifer material. Genes with a putative involvement in secondary metabolism, protection against oxidative stress and degradation/detoxification of host material were shown to be differentially expressed. A cytochrome P450 gene displayed sequence similarities towards genes encoding proteins involved in secondary metabolism and was highly expressed during fungal growth in Norway spruce bark. Up-regulation of a superoxide dismutase gene during early infection of Scots pine seedlings was detected. Transcript profiles of a laccase and two glutathione S-transferase genes showed transient expression during a time-course experiment of fungal infection of Norway spruce tissue cultures. A putative arabinase gene was exclusively expressed during infection of Scots pine seedlings. Up-regulation of Norway spruce genes phenylalanine ammonia lyase, peroxidase, glutathione S-transferase and class II and IV chitinases were detected upon fungal colonization of spruce callus tissue.

Keywords: *Heterobasidion annosum*, *Heterobasidion parviporum*, *Pinus sylvestris*, *Picea abies*, expressed sequence tags, gene expression, cytochrome P450, glutathione S-transferase, arabinase.

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Karlsson, M., Olson, Å. & Stenlid, J. 2003. Expressed sequences from the basidiomycetous tree pathogen *Heterobasidion annosum* during early infection of Scots pine. *Fungal genetics and biology*, 39, 51-59.

II. Karlsson, M., Stenlid, J. & Olson, Å. Identification of a superoxide dismutase gene from the conifer pathogen *Heterobasidion annosum* and its infection-related regulation. (Submitted manuscript).

III. Karlsson, M., Elfstrand, M., Stenlid, J. & Olson, Å. Differential gene expression during the interaction between *Heterobasidion annosum* sensu lato and its conifer hosts. (Manuscript).

IV. Karlsson, M., Hietala, A., Kvaalen, H., Olson, Å., Solheim, H., Stenlid, J. & Fossdal, C. Infection-related transcriptional responses from the interaction of Norway spruce callus tissue and the fungal pathogen *Heterobasidion parviporum*. (Manuscript).

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Introduction

Fungi are eukaryotic, typically filamentous heterotrophic organisms, with absorptive mode of nutrition. A broad distinction of fungi can be made according to how they obtain nutrients – from a living host through mutualistic or parasitic symbiosis, or from non-living materials as saprotrophs. The majority of fungi are saprotrophs, less than 10 % of the known fungal species are able to infect plants and an even smaller fraction of these cause disease (Knogge, 1996). Many of these fungal pathogens are, nevertheless, causing serious economic losses in both agriculture and silviculture. It is therefore important to determine the unique factors that differentiate pathogens from saprotrophic fungi and enables a fungus to cause disease in host plants. Different levels of specialization can be observed in the interactions between fungal pathogens and their host plants (Scheffer, 1991). Opportunistic pathogens require wounds or a weakened host to be able to infect. More specialized are the pathogens that rely on living plants to grow but are able to survive outside their hosts. Obligate pathogens are dependent on a living host to complete their lifecycle. The strategies employed by fungi in the pathogenic process vary in different types of interactions (Agrios, 1988). Necrotrophic pathogens typically grow through plant tissues as hyphae while secreting a variety of enzymes and toxins that kill and degrade plant tissues. Biotrophic pathogens rely on living plant cells for their nutritional needs. Typically in biotrophic interactions, the fungus penetrates the cell wall, but not the plasma membrane, and develops a specialized feeding structure, the haustorium. Pathogens that are capable of infecting healthy tissues of plants under non-stressed conditions typically have narrow host ranges as they must circumvent, tolerate or overcome the specific resistance factors of a particular host (Deacon, 1997).

Annosum root rot

The basidiomycetous fungus *Heterobasidion annosum* (Fr.) Bref. sensu lato (s.l.) is a common, necrotrophic pathogen on forest trees in coniferous forests in the northern hemisphere (Korhonen & Stenlid, 1998). The fungus produces sporocarps on stumps, roots, logs and dead or diseased trees (Greig, 1998; Redfern & Stenlid, 1998) and vast amounts of spores are produced during the growing season (Redfern & Stenlid, 1998). These basidiospores are responsible for the primary establishment of the disease in a forest stand, by infection of freshly exposed woody tissues such as wounds (Isomäki & Kallio, 1974) or stumps of cut trees (Rishbeth, 1951; Redfern & Stenlid, 1998). The fungus then spreads in the stand by mycelial growth in the root system and infects healthy trees through root-root contacts (Rishbeth, 1949, 1951). In Scots pine the fungus causes root death, growth losses and mortality (Stenlid & Redfern, 1998). In Norway spruce the fungus can cause extensive stem decay that results in growth losses and decreased timber value (Bendz-Hellgren & Stenlid, 1995, 1997). Annual losses for forest owners due to decayed wood and reduced growth inflicted by *H. annosum* s.l. are

estimated to €790 millions in Europe alone (Woodward *et al.*, 1998), of which Sweden accounts for €54 millions (Bendz-Hellgren *et al.*, 1998).

Heterobasidion annosum s.l. is a species complex consisting of several different species and intersterility (IS) groups. In Europe, three species with partly overlapping distributions and host specificities have been identified (Korhonen, 1978; Capretti *et al.*, 1990), *H. annosum* sensu stricto (s.s.), *H. parviporum* Niemelä & Korhonen and *H. abietinum* Niemelä & Korhonen, with a preference for pine, spruce and fir, respectively (Niemelä & Korhonen, 1998). *H. annosum* s.s. mainly attacks *Pinus* species, although it has a broad host range including species of *Picea*, *Larix*, *Betula*, *Alnus* and *Juniperus* (Korhonen & Stenlid, 1998). In contrast, *H. parviporum* mainly attacks *Picea abies* and its distribution follows that of the host, although *Abies sibirica* and *Pinus sylvestris* occasionally get infected (Stenlid & Swedjemark, 1988). *H. abietinum* mainly infect *Abies* species in their geographic range in Europe (Capretti *et al.*, 1990). Two IS-groups of *H. annosum* s.l., P and S, have been found in North America, where the host range of the S-group includes species of *Picea*, *Abies*, *Pseudotsuga*, *Tsuga* and *Sequoiadendron*, while the P-group is mostly associated with *Pinus*, *Juniperus* and *Calocedrus* (Garbelotto *et al.*, 1996).

***Heterobasidion annosum* s. l. pathogenesis**

The attachment of basidiospores to host tissues constitute the initiation of the infection process of *H. annosum* s.l. Spore adhesion has been studied using both non-suberized conifer seedling roots and fine roots of 15 year old Sitka spruce, showing adhesion of conidia and mycelia after 1-2 hours (Snape, Preston & Woodward, 1993; Asiegbu, 2000). This adhesion can be reduced by pre-treatment of spores with acid, potassium hydroxide, diethyl ether or by boiling. The effect of diethyl ether indicates that the adhesive spore component is a lipid or bound to a lipid (Asiegbu, 2000). The adhesion of mycelia to fine roots can be inhibited by pre-treatment of the roots with substances capable of complexing with and denaturing proteins, such as Cu^{2+} or HAsO_4^{2-} (Snape, Preston & Woodward, 1993).

On conifer seedling roots, *H. annosum* s.l. readily forms infection structures such as appressoria and infection pegs (Asiegbu, Daniel & Johansson, 1993), although the importance of these structures has not been fully determined (Figure 1). In fine roots, the meristem appears to be susceptible to *H. annosum* s.l. infection, while the endodermis constitutes a major barrier for the fungus (Asiegbu, Daniel & Johansson, 1994; Heneen *et al.*, 1994), although penetration into the stele through the endodermis has been observed (Asiegbu, Daniel & Johansson, 1995). During infection *H. annosum* s.l. is challenged with defence reactions from the plant, including necrosis, formation of structural barriers and formation of antifungal enzymes and metabolites (Karjalainen, Ernst & Woodward, 1998). Extensive formation of papillae and lignification can partially restrict invasion of the fungus, although it can seldom completely stop the infection (Asiegbu, Daniel &

Johansson, 1993; Heneen *et al.*, 1994), which indicates that *H. annosum* s.l. has efficient degradation and detoxification systems (Figure 2 and 3).

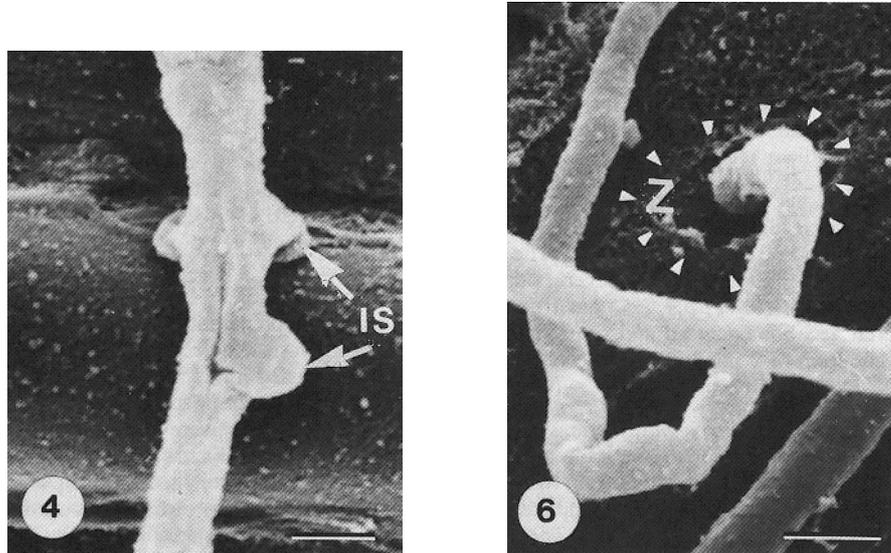


Fig. 1. Scanning electron micrographs of initial stages of *H. parviporum* infection of Norway spruce seedling roots. 4. Formation of multiple infection structures (IS) 48 hours post infection. 6. Penetration of hyphae by enzymatic hydrolysis and weakening of epidermal cell walls. Formation of clear zones (Z) on the root cell surface. Pictures kindly provided by F. Asiegbu with permission from NRC Research Press.

H. annosum s.l. secretes a range of polysaccharide-degrading enzymes. Cellulase, mannanase, xylanase, aryl- β -glucosidase and β -glucosidase have been identified although their role in pathogenesis is still not thoroughly investigated (Asiegbu *et al.*, 1998). β -glucosidase enables *H. annosum* s.l. to use the energy in the glucosidic bond of cellobiose, an enzyme system that appears to be rare in white-rot fungi (Hutterman & Volger, 1973). A higher number of polygalacturonase and pectin esterase isozymes are present in *H. annosum* s.s. than in *H. parviporum* (Karlsson & Stenlid, 1991). Additionally, the total pectin-degrading capabilities of *H. annosum* s.s. are higher than in *H. parviporum*, which has been hypothesised to account for the greater host range of *H. annosum* s.s. (Johansson, 1988; Comparini *et al.*, 2000).

Lignin and other phenolic compounds are part of the chemical and structural defense of conifers (Philips & Croteau, 1999). *H. annosum* s.l. is a white-rot fungus, thus capable of degrading lignin. Three enzymes involved in lignin degradation have been detected in *H. annosum* s.l., laccase, lignin peroxidase (LiP) and manganese-dependant peroxidase (MnP) (Asiegbu *et al.*, 1998; Majjala,

Harrington & Raudaskoski, 2003). LiP and MnP are proposed to be the primary lignin-decomposing enzymes, subsequently followed by oxidation and demethylation reactions by laccase (Leonowicz *et al.*, 1999). The aromatic phenol rings are finally cleaved by dioxygenases (Leonowicz *et al.*, 1999). Both MnP and laccase genes have been identified in *H. annosum* s.l. (Maijala, Harrington & Raudaskoski, 2003; Asiegbu *et al.*, 2004). Laccase activity is induced by phenols, which are subsequently oxidized by the enzyme (Haars & Hutterman, 1980; Haars, Chet & Hutterman, 1981). Laccase was also shown to detoxify lignans occurring in the reaction zone of *H. annosum* s.l. infected Norway spruce (Popoff, Theander & Johansson, 1975). There is also evidence that *H. annosum* s.s. have higher laccase activity than *H. parviporum* (Johansson, Lundgren & Asiegbu, 1998; Johansson, Denekamp & Asiegbu, 1999).

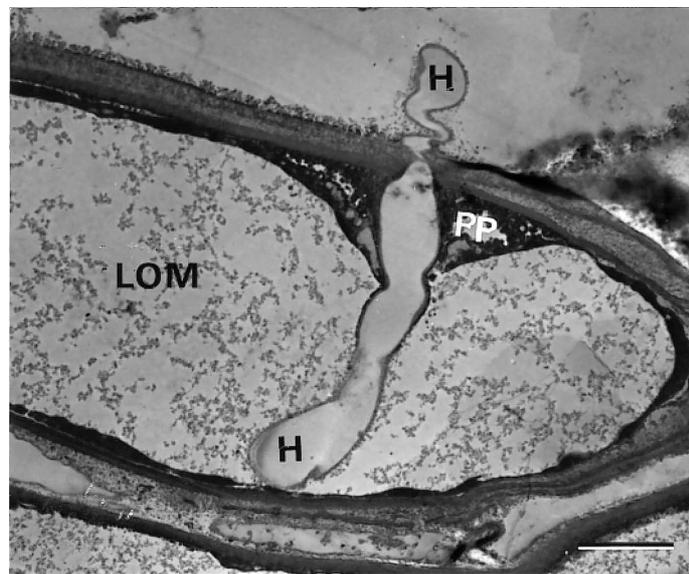


Fig. 2. Transmission electron micrograph from *H. parviporum* infected Norway spruce seedling roots. Direct penetration of fungal hyphae (H) through cell walls and papillae (PP). LOM = loss of electron-dense material. Picture kindly provided by F. Asiegbu with permission from NRC Research Press.

H. annosum s.l. is known to produce at least 12 different toxins including fomannosin, fomannoxin, fomannoxin acid, oosponol and oospoglycol (Asiegbu *et al.*, 1998). These compounds are produced both in axenic cultures and during interaction with plants and other fungi. Fomannosin was the first toxin that was isolated from *H. annosum* s.l. and hypothesized to be involved in pathogenesis by Basset and co-workers in 1967, although they were not able to detect the compound from infected *P. taeda* stems or roots, nor from *H. annosum* s.l. cultures on pine sapwood shavings (Basset *et al.*, 1967). Fomannosin was only produced on media containing high sugar levels, associated with the declining growth phase of the fungus. Fomannosin, and related compounds FD86, FD56 and

FD32 (Sonnenbichler *et al.*, 1989), was recently proposed to be produced through the isoprenoid pathway with farnesyl-pyrophosphate as an intermediate (Yang, 2003). Application of fomannosin to stem wounds in pine seedlings causes needle browning and death, and *de novo* synthesis of pinosylvin (Basset *et al.*, 1967). Another toxin produced by *H. annosum* s.l. is fomannoxin, which have a 100 fold greater toxicity to plant cells than fomannosin (Hirotsu *et al.*, 1977). This toxin has been isolated from *H. annosum* s.l. infected Sitka spruce stem wood (Heslin *et al.*, 1983). Uptake of fomannoxin by Sitka spruce seedlings resulted in rapid browning of the roots accompanied by chlorosis and progressive browning of needles (Heslin *et al.*, 1983). This, and the production of fomannoxin by actively growing hyphae, suggests a role for fomannoxin during pathogenesis (Heslin *et al.*, 1983). Compounds like fomannoxin, FD56, oosponol, fomannosin and FD32 shows varying degrees of toxicity to both plant, fungal and bacterial cells (Sonnenbichler *et al.*, 1989). *H. annosum* s.l. also produce plant growth inhibitory substances which was hypothesised to cause physiological disorders and to induce a local nutrient sink activity in the area of infection to initiate nutrient flow from the host plant (Hoque, 1984).

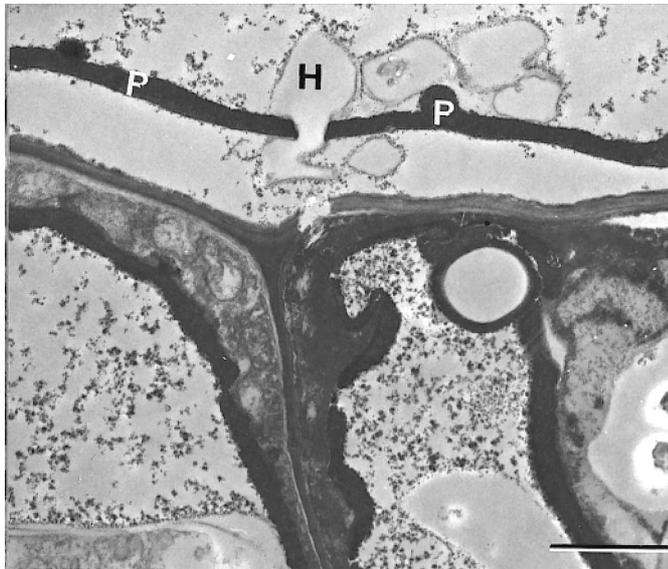


Fig. 3. Transmission electron micrograph from *H. parviporum* infected Norway spruce seedling roots. Penetration of fungal hyphae (H) through a phenolic structural barrier (P). Picture kindly provided by F. Asiegbu with permission from NRC Research Press.

Aims of the study

The overall aim with this study was to expand the current knowledge on factors that enables *H. annosum* s.l. to infect and cause disease in conifers.

More specifically, the objectives were:

- to study the diversity of expressed genes in *H. annosum* s.l. during the interaction with Scots pine seedlings and to assist future research on *H. annosum* s.l. by providing collections of expressed sequence tags
- to test whether genes with a putative involvement in secondary metabolism are differentially expressed in *H. annosum* s.l. during the interaction with Scots pine and Norway spruce
- to test whether genes with a putative involvement in protection against oxidative stress are differentially expressed in *H. annosum* s.l. during the interaction with Scots pine and Norway spruce
- to test whether genes with a putative involvement in degradation and detoxification of host material are differentially expressed in *H. annosum* s.l. during the interaction with Scots pine and Norway spruce
- to test whether defense-genes are differentially expressed in Norway spruce during the interaction with *H. annosum* s.l.

Materials and Methods

Biological material and infection systems

Both the North American, P-group *H. annosum* isolate TC 32-1 (Chase, 1985) and the European *H. parviporum* isolates Rb 175 and 87-257/1 (Stenlid, 1987; Norwegian Forest Research Institute culture collection) were used in this work. The North American P-isolate was used because it was previously characterized with respect to its intersterility alleles, thus enabling fertile crosses with North American S isolates. This was a prerequisite for the production of a mapping population with the aim to construct a genetic map over the *H. annosum* genome in a project parallel to the work described in this thesis (Lind, Olson & Stenlid, 2005). The *H. parviporum* isolates were used in order to extend the results from the North American P-form to a species of greater economic importance for Swedish forestry. The fungal isolates were maintained on Hagem medium (Stenlid, 1985) or S-medium (Hietala *et al.*, 2003).

Three different infection systems were used in this work. Infection of aseptically grown, two-week old *P. sylvestris* seedlings by isolate TC 32-1 was used for construction of cDNA-library and expression studies (I, II and III). This system can be used as a model of fine root infection. Clonal, 10-year old *P. abies* trees with high resistance (1900) and low resistance (1412) against *H. parviporum* (Swedjemark, Stenlid & Karlsson, 1997) were inoculated with isolate Rb 175 for expression studies in spruce bark under natural conditions (II and III). This is aiming at reflecting the conditions for fungal infection through the periderm and phloem layers of larger roots, with constitutive and induced defence mechanisms that restrict fungal access to the sapwood. Tissue cultures of *P. abies* have the potential to be used as a model of fine root infection and invasive growth. Callus material of *P. abies* tree clones, characterised as high resistant (589) and low resistant (409) (Kvaalen & Solheim, 2000; Hietala *et al.*, 2003; Hietala *et al.*, 2004) was infected with isolate 87-257/1 for expression studies on both fungal and host genes (IV). In addition, a time series experiment was conducted with isolate TC 32-1 growing in MMN-medium (Marx, 1969) with different N concentrations (4 mM and 40 mM).

cDNA-library construction

Studying fungal pathogenicity factors can be done with a multitude of different methodologies (Gold, 2001), and with different underlying ideas about preconceived assumptions. The choice of cDNA-library construction and expressed sequence tag (EST) analysis was based on the need to investigate several different biological functions in one experiment. *H. annosum* isolate TC 32-1 was exposed to two-week old *P. sylvestris* seedling roots for 6 and 72 hours, before the roots were removed and the mycelia used for RNA extraction. The subsequent construction of a *H. annosum* cDNA-library was done using the SMART cDNA Library Construction Kit, including oligo(dT) primed reverse transcription of total RNA, directional cloning into a lambda phage vector and conversion to a plasmid vector equivalent by cre/lox recombination, according to the manufacturer's recommendations (Clontech, Palo Alto, CA).

Sequence analysis

Gene homologies originate from the evolutionary history of a particular gene and can offer clues about the function and cellular localization of the corresponding protein. Sequences were determined with a CEQ 2000 using the Dye Terminator Cycle Sequencing Chemistry (Beckman Coulter, Fullerton, CA) or with a ABI 310 Genetic Analyzer using the BigDye Terminator cycle system (Applied Biosystems, Foster City, CA). Sequencing reactions were performed according to the manufacturer's protocols, except that the total reaction volume was 10 µl with 3 µl PCR product as template. Sequence similarity searches were performed using the BLAST algorithm (Altschul *et al.*, 1997) to sequence data in public databases.

Neighbor-joining analysis of protein sequences was conducted using PAUP version 4.0 b10 (Swofford, 2002), with 1000 bootstrap resamplings.

Gene expression measurements

Genes coding for proteins that have an active role in pathogenicity has to be distinguished from those that have other functions and one way to do this is by identifying genes that are up-regulated during the infection process. Additional information from transcription patterns can provide indications about regulatory factors for the individual genes. The choice of studying transcript levels was also based on the fact that the same methodology can be used for all different kind of genes. Real-time PCR was the choice because of the possibilities to achieve specific amplification of either fungal or host transcripts in mixed samples, thus minimizing problems with cross-hybridisations between similar genes from the different organisms (van Zyl *et al.*, 2002), or from within a single organism. Transcript levels were quantified by real-time quantitative RT-PCR using the SYBR Green PCR Master Mix Kit, according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Cycle threshold (Ct) values for the PCR product growth curves were determined. The corresponding number of transcripts was deduced from individual standard curves for each gene in a serial dilution (10^6 , 10^5 , 10^4 , 10^3 , 10^2 and 10^1 molecules). The relative expression levels for each gene in relation to α -tubulin were calculated from the Ct values and the primer amplification efficiencies by using the formula described by Pfaffl (2001).

Results and Discussion

The rationale behind this work was to take a holistic approach to study *H. annosum* s.l. pathogenicity by looking at the general pattern of gene expression during the pathogenic interaction with conifers. This was followed by a more atomistic approach where individual genes were studied in more detail.

Infection-related expressed sequence tags

One factor that is limiting the research about *H. annosum* s.l. pathogenesis is the lack of coding sequence information. In the year 2000 there was only one coding sequence from *H. annosum* s.l. available in GenBank, a laccase gene (Y16951). Therefore, the start of this work was to produce sequence data from *H. annosum* s.l. by generating ESTs. This method is a fast and reliable way of providing information on uncharacterised genomes and has been successfully employed in other plant pathogenic fungi, as *Sclerotinia sclerotiorum* (Li *et al.*, 2004), *Ustilago maydis* (Nugent, Choffé & Saville, 2004), *Gibberella zeae* (Trail *et al.*, 2003) and

Verticillium dahliae (Neumann & Dobinson, 2003) among others. A cDNA-library from *H. annosum* isolate TC 32-1 after exposure to pine seedling roots was made and EST sequencing and contig analysis identified 318 unigenes, of which 62 were repeatedly sampled. A putative function was assigned to 223 unigenes (70 %), based on high or moderate similarity (E-value $\leq 10^{-5}$) of their translation products to protein sequences from public databases (Figure 4).

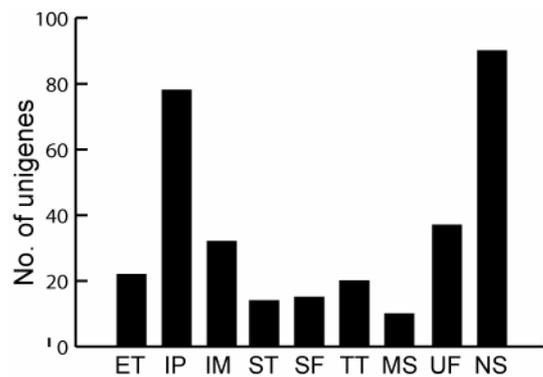


Fig. 4. Functional distribution of *H. annosum* unigenes. The 223 unigenes with significant (E-value $\leq 10^{-5}$) similarity to proteins in public databases were assigned to a functional group according to the Metabolic Pathway database functional directory. 95 unigenes had no significant similarity to any protein. ET = electron transport, IP = information pathways, IM = intermediate metabolism, ST = signal transduction, SF = structure and function of the cell, TT = transmembrane transport, MS = miscellaneous, UF = unknown function, NS = no significant similarity.

The unigenes are encoding proteins involved in a wide range of metabolic functions with putative importance in the pathogenic process, as secondary metabolism, degradation of host material, signal perception and transduction and membrane transport (Table 1).

This collection of sequence data forms the necessary starting material for the continuation of this project, and will assist future research on *H. annosum* s.l. It is interesting that 30 % of the unigenes do not have any similarity to any known proteins and 16 % have similarity only with proteins with unknown functions. This is a representative number of unknown unigenes for other fungal EST sequencing projects (Skinner, Keon & Hargreaves, 2001) and highlights a lack of sequence information on fungi. It can also be an indication of high gene diversity in fungal pathogens, reflecting the many different ways that fungi have evolved to complete the complex process of pathogenesis.

Table 1. Excerpt of *H. annosum* unigenes with similarity to other proteins

Unigene	Best match	E-value ¹	Organism
Contig 163	Cytochrome P450	-18	<i>Coprinus cinereus</i>
Contig 223	Cytochrome P450	-13	<i>Lentinula edodes</i>
Contig 340	Farnesyl-pp synthase	-27	<i>Lactarius chrysorrheus</i>
Contig 41	Cytochrome b5	-23	<i>Rhizopus stolonifer</i>
Contig 77	Arabinase	-11	<i>Streptomyces coelicolor</i>
Contig 172	Manganese peroxidase	-9	<i>Coriolus versicolor</i>
Contig 131	Peroxidase	-21	<i>Termitomyces albuminosus</i>
Contig 181	Quinone oxidoreductase	-58	<i>Gloeophyllum trabeum</i>
Contig 107	Cellulase	-32	<i>Schizosaccharomyces pombe</i>
Contig 82	Glutathione-S-transferase	-30	<i>Paxillus involutus</i>
Contig 335	Glutathione-S-transferase	-15	<i>Oryza sativa</i>
Contig 139	Transcription factor	-29	<i>Saccharomyces cerevisiae</i>
Contig 274	Rac1 protein	-46	<i>Schizophyllum commune</i>
Contig 193	Ras1 protein	-29	<i>Schizophyllum commune</i>
Contig 259	G-protein	-38	<i>Schizosaccharomyces pombe</i>
Contig 184	Ser/Thr protein kinase	-14	<i>Schizosaccharomyces pombe</i>
Contig 5	Protein transport protein	-17	<i>Schizosaccharomyces pombe</i>
Contig 313	Amino acid transporter	-28	<i>Amanita muscaria</i>

¹E-value exponent resulting from the best BlastX similarity search.

The next part in this work was to identify individual genes encoding putative pathogenicity factors. This was done by identifying genes that have high transcript levels during infection stages as compared to other treatments, and by studying sequence similarities with proteins that has a characterised role in pathogenesis in other systems. It is important to obtain this kind of background data before starting more thorough investigations with heterologous protein expression or mutant constructs, as these methods are both expensive and labour intensive.

Infection and secondary metabolites

One feature of *H. annosum* s.l. pathogenicity is the multitude of toxins that are produced by the fungus. Therefore, unigenes displaying sequence similarities to genes with putative involvement in biosynthesis of secondary metabolites, including cytochrome P450s (*CPM1* and *CPM2*), cytochrome b5 (*CYB1*) and farnesyl-pyrophosphate synthase (*FPS1*), were chosen for an expression study. Cytochrome P450 enzymes are catalyzing the oxidation of many chemicals by introducing an oxygen atom originating from molecular oxygen (Guengerich, 2001). In fungal pathogenesis they have important roles in biosynthesis of toxins such as dothistromin by the forest pathogen *Dothistroma pini* (Bradshaw *et al.*, 2002) and sirodesmin by *Leptosphaeria maculans* (Gardiner *et al.*, 2004), or in detoxification of phytoalexins such as pisatin demethylation by *Nectria haematococca* (Wasmann & VanEtten, 1996). Cytochrome b5 is part of

cytochrome P450 systems as a reductase protein that can provide necessary electrons (Sakuradani, Kobayashi & Shimizu, 1999). Farnesyl-pyrophosphate synthase is the responsible enzyme for production of farnesyl-pyrophosphate, which is an intermediate in the isoprenoid biosynthesis pathway. Several different classes of isoprenoid compounds are produced by plant-pathogenic fungi such as the plant hormone-like gibberellins by *Gibberella fujikuroi* (Johnson & Coolbaugh, 1990) and toxins like the trichothecenes by *Fusarium* spp. (Hestbjerg *et al.*, 2002).

Expression data showed a down-regulation of *CPM1*, *CPM2*, *FPS1* and *CYB1* 72 hours post inoculation (hpi) in *H. annosum* using a Hagem-based pine seedling *in vitro* infection system (Figure 5) (III), although only the change for *CPM1* and *CPM2* were statistically significant (Student's *t*-test, $p = 0.021$, $p = 0.014$, respectively).

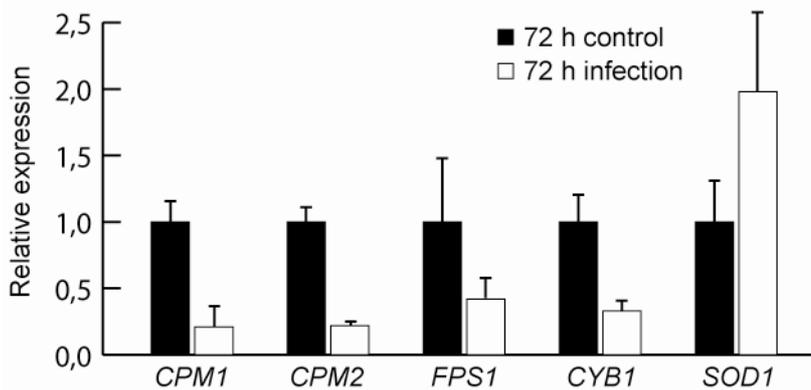


Fig. 5. *Heterobasidion annosum* gene expression. Transcript levels of *H. annosum* genes were determined with realtime-PCR and normalized against α -tubulin expression after 72 hpi of contact with pine seedling roots or water agar plugs. Expression levels are displayed in relation to the 72 hpi control sample (= 1x). Error bars represent the standard error of three independent samples.

There was a 749 times higher α -tubulin normalised expression of *CPM2* in *H. parviporum* growing in spruce bark for 20 days as compared to growth on S-medium, thus indicating a function of *CPM2* in *H. parviporum* during *in planta* growth (III). *CPM2* was also induced during the early stages of spruce callus colonization by *H. parviporum* ($p < 0.0001$; Figure 6f) (IV). This was in contrast to *CPM1*, *CYB1* and *FPS1* which had no detectable expression in *H. parviporum* growing in spruce bark (III).

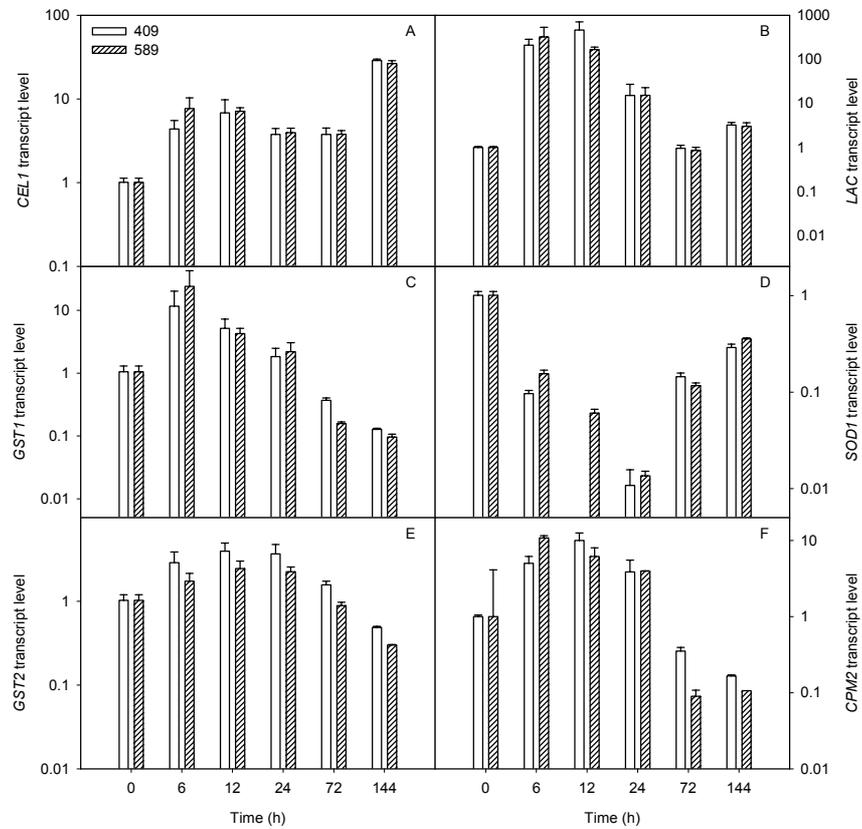


Fig. 6. *Heterobasidion parviporum* gene expression. Relative transcript levels of *H. parviporum* genes, *CEL1*, *GST1*, *GST2*, *LAC*, *SOD1* and *CPM2*, at the time of inoculation (0 hpi) and during colonization of callus tissue from Norway spruce trees characterized as high (589) and low (409) resistant. Expression levels are displayed in relation to the 0 hpi sample. Error bars represent the standard error of three independent samples.

The general expression levels for *CPM1* was lower in modified MMN-medium than during growth in Hagem-medium (pine seedling *in vitro* system control) (Figure 7a), while *CPM2* showed higher expression than in Hagem-medium (Figure 7b) (III).

The expression was declining with time for both genes in high nitrogen media (Figure 7). The same trend was evident also in low nitrogen media, except for a peak of higher expression at 4 days of growth for *CPM1* and at 7 days of growth

for *CPM2*. These expression peaks were significantly different from the corresponding expression in high nitrogen media (Student's *t*-test, $p = 0.025$, $p = 0.001$, respectively) (III).

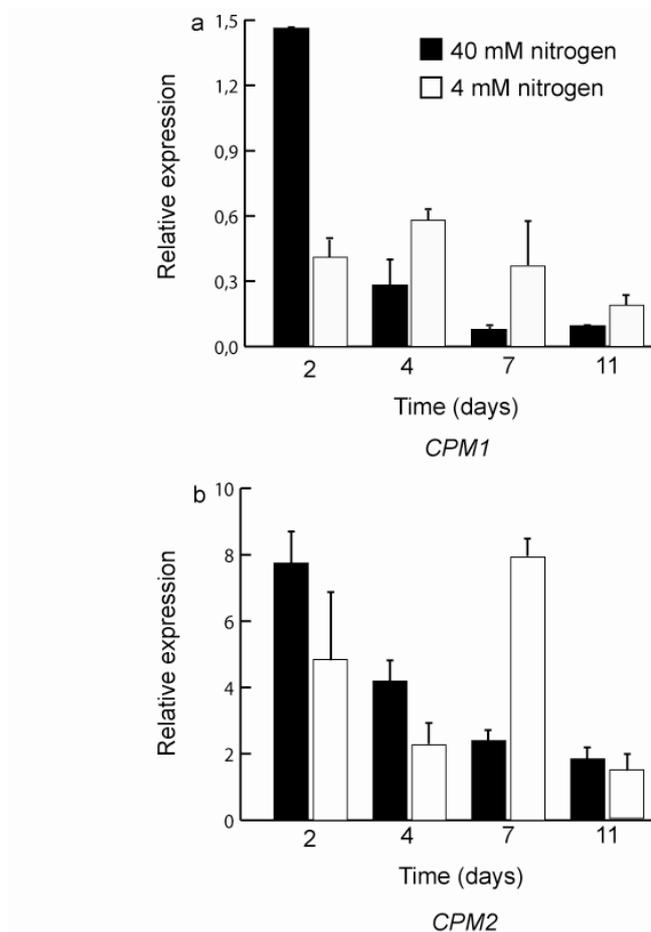


Fig. 7. Effect of time and nitrogen on transcript levels of *CPM1* and *CPM2*. Transcript levels were determined with realtime-PCR and normalized against α -tubulin expression after growth in low (4 mM_N) or high (40 mM N) nitrogen-containing liquid media supplied with 2.5 g l⁻¹ Glucose for 2, 4, 7 or 11 days. Expression levels are displayed in relation to the 72 hpi pine seedling infection system control sample (= 1x, Figure 5). Error bars represent the standard error of three independent samples.

Farnesyl-pyrophosphate synthase was suggested to be involved in the biosynthesis of fomannosin by Yang (2003) and the expression of *FPSI* in carbon rich media (pine seedling *in vitro* system control) (III) correlates with the production of fomannosin in high sugar containing media (Basset *et al.*, 1967). Furthermore, fomannosin was never isolated from *H. annosum* s.l. infected *P. taeda* stems or

roots, nor from *H. annosum* s.l. cultures on pine sapwood shavings (Basset *et al.*, 1967) which could provide an explanation for the inability to detect *FPS1* transcripts from *H. parviporum* infected spruce bark in this work (III). The similar transcriptional profile of *CPM1* and *CYB1* to that of *FPS1* could indicate that their gene products are functioning in the same biosynthetic pathways, although no conclusive information is available (III).

The high expression of *CPM2* in *H. parviporum* during growth in spruce bark and callus (III and IV) qualifies this gene for further studies as a potential pathogenicity gene. In addition, the generally higher expression levels of *CPM2* in MMN-medium as compared to Hagem-medium might indicate that *CPM2* is induced by low carbon levels, as the MMN-medium contains only 2.5 g l⁻¹ glucose as compared to 5 g l⁻¹ in Hagem. Alternatively, as Hagem is a complex medium there is a potential for other differences in nutrient content that might account for the differential regulation of *CPM2*. The peak in expression of *CPM1* at 4 days of growth in low nitrogen media and of *CPM2* at 7 days of growth have been observed for other fungal cytochrome P450s, where it was hypothesised to be related to the onset of secondary metabolism (Yadav *et al.*, 2003). Conifer root compounds can inhibit the function of *H. annosum* s.l. extracellular enzymes (Johansson, Popoff & Theander, 1976). It is possible the down-regulation of *CPM2* in the pine seedling system might be caused by a similar effect on transcription, especially as most hyphae in this system are growing extracellularly, diluting the expression from invasive hyphae.

The predicted *cpm2* protein displays the characteristic domains for cytochrome P450 proteins including heme binding, I- and K-helix motifs and have similarities to a fruit body specific cytochrome P450 from *Lentinula edodes* (Score 370, E=1e-101) (Hirano, Sato & Enei, 2004) and the *ord1/ordA* O-methylsterigmatocystin oxidoreductases from *Aspergillus flavus/A. parasiticus* (Score 306, E=1e-81), responsible for catalysing the final step in aflatoxin biosynthesis (Prieto & Woloshuk, 1997). The high similarity of *cpm2* with the *ord1* protein in aflatoxin biosynthesis classified it as a subgroup to CYP64 (*ord1/ordA*) named CYP697A1 by the Cytochrome P450 Nomenclature Committee (D. R. Nelson, personal communication).

Because of the high diversity of reactions catalyzed by cytochrome P450s it is impossible to determine the function of this particular cytochrome P450 only based on expression and sequence data. The classification of *cpm2* as a subgroup to CYP64, and the clustering of CYP64 to other cytochrome P450s involved in secondary metabolism (Yadav *et al.*, 2003) imply that *cpm2* might be involved in biosynthesis of toxins or other plant-growth inhibiting compounds that are produced by *H. annosum* s.l. (Heslin *et al.*, 1983; Hoque, 1984; Sonnenbichler *et al.*, 1989). Other possible functions could be in detoxification of plant-produced, toxic compounds as in the case of pisatin demethylation by *N. haematococca* (Wasmann & VanEtten, 1996). Related to this is the function of metabolising plant derived non-toxic compounds like alkenes and fatty acids (Yadav *et al.*, 2003), although the down-regulation of *CPM2* during the later stages of spruce callus colonisation makes this explanation less likely (IV).

Infection and oxidative stress

There are several proposed functions for reactive oxygen species (ROS) in plant-pathogen interactions, including signalling, antimicrobial activity, cell wall strengthening and biosynthesis of antimicrobial metabolites (Mehdy, 1994; Baker & Orlandi, 1995; Tenhaken et al., 1995; de Pinto, Tommasi & De Gara, 2002). Some necrotrophic fungal pathogens are capable of tolerating high levels of ROS (Govrin & Levine, 2002), by so far unknown mechanisms but it is likely that the mechanisms involve enzymes capable of inactivating ROS, such as superoxide dismutase (SOD), catalase, peroxidase and polyphenol oxidase (Mayer, Staples & Gil-ad, 2001).

A unigene with similarity to SODs (*SOD1*) was chosen for a neighbor-joining analysis and an expression study because of the potential involvement in protecting *H. annosum* s.l. against infection-related oxidative stress. The hypothesis being that SOD, which is dismuting superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), is up-regulated during infection to protect the fungus against infection-related oxidative stress.

In a phylogenetic analysis (I) the *SOD1* gene was clustering with manganese-dependent SODs (Mn-SOD). An additional neighbor-joining analysis of the amino acid sequence of *sod1* with 29 other fungal Mn-SODs and three animal Mn-SODs was performed in order to investigate whether any indications about protein localisation could be drawn (Figure 8).

Interestingly, many ascomycetous species have two Mn-SOD encoding genes, although all basidiomycetes examined have one with the exception of *Ustilago maydis* which have two. Furthermore, the neighbor-joining analysis divides the ascomycete proteins into two major clades, one consisting of proteins predicted to be localized to the mitochondria and one not (Figure 8). Such separation is not seen among the basidiomycetes. All homobasidiomycetes examined contain only one Mn-SOD encoding gene and none of the proteins contain a mitochondrial targeting peptide. A cytoplasmic localisation of the *SOD1*-encoded protein is suggested by the lack of a transport signal peptide in the *sod1* sequence.

The expression of *SOD1* in *H. annosum* was higher after 72 hpi on pine seedlings than in the corresponding control (Student's *t*-test, $p = 0.005$; Mann-Whitney, $p = 0.04$; Figure 5) (II). The expression of *SOD1* during growth of *H. parviporum* on spruce callus showed the highest activity in the 0 hpi control (S-medium), followed by a rapid decrease in activity until 72 and 144 hpi where a slight increase were detected ($p < 0.0001$; Figure 6d) (IV). In addition, the expression of *SOD1* in *H.*

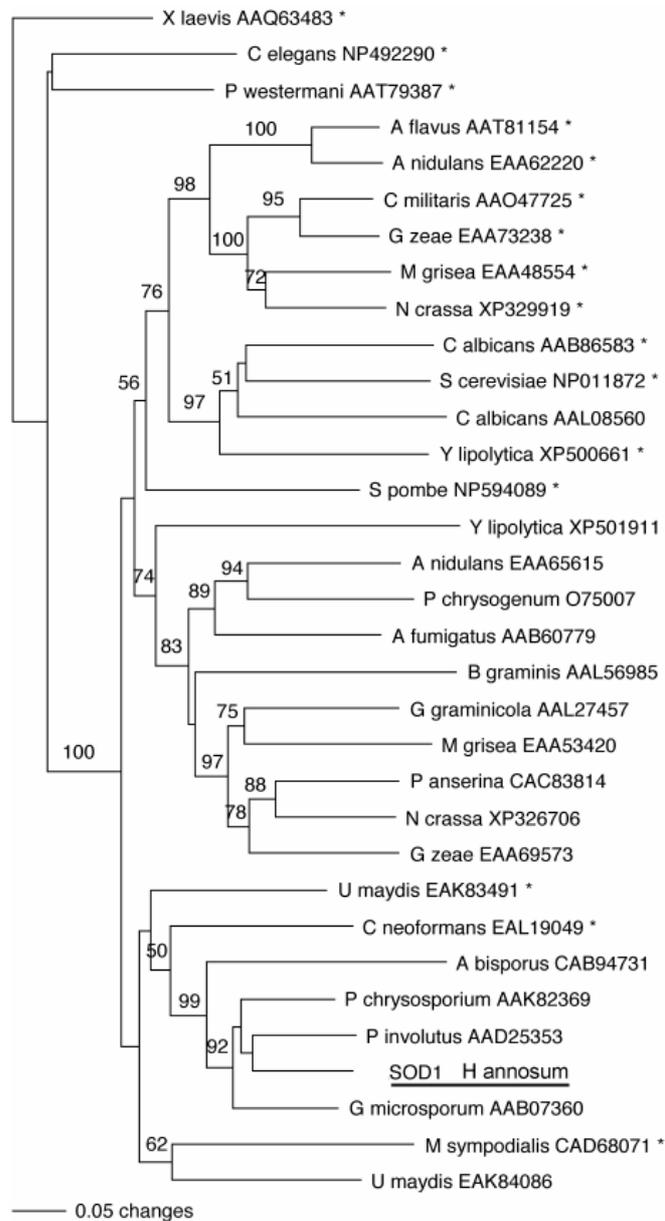


Fig. 8. Neighbor-joining analysis of fungal Mn-SOD protein sequences. A sequence alignment of the translated *SOD1* and selected fungal Mn-SOD homologous protein sequences, accession nos. indicated in the figure, were used. Neighbor-joining tree constructed with minimum evolution method estimated with mean character difference, values above 50 from 1000 bootstrap iterations are given above the lines. Asterisks indicate presence of mitochondrial targeting peptide and *SOD1* is marked by underline.

parviporum growing in spruce bark for 20 days was 3 times higher as compared to growth on S-medium.

One explanation for these, apparently contradicting results, could be that the primary function for *sod1* is in protecting the fungal cell against endogenously produced O_2^- , produced as a byproduct from oxidative metabolism, thus the activity should correlate with the nutritional status of the fungus which, in the callus system, were high during growth on S-medium and during growth on dead host cells in the later stages of infection. Both control and infection stages of the pine seedling system would represent high nutrient conditions as most hyphae are growing in Hagem-medium without root contact. An additional effect generated by host-derived oxidative stress would then be masked by the low levels of oxidative stress caused by low nutritional status in the callus system while it can be detected under high nutrient conditions, such as the pine seedling system.

An alternative explanation to the low expression of *SOD1* during the early stages of callus colonization was provided by Cho and coworkers (2002) that presented data suggesting that SOD activity is low when the activity of ligninolytic enzymes, peroxidase and laccase, are high, possibly because radicals are fully involved in biodeterioration processes under ligninolytic conditions. Further experiments are needed to distinguish between these alternative hypotheses, taking into account the problems arising from the multitude of reactions and enzymes involved both in generating and using/detoxifying ROS.

Infection and degrading/detoxifying enzymes

After depleting easily available nutrients, the pathogenic fungus degrades structural polysaccharides of plant cell walls to retrieve carbon. Destruction of structural elements of the plant cell wall also interferes with the normal cellular functions of the host, thus contributing to the pathogenic process. Conifers also produce a multitude of toxic substances that prevent growth of an invading fungus, such as resins and phenolics (Philips & Croteau, 1999). There are reports that *H. annosum* s.l. is capable of detoxifying many of these compounds (Popoff, Theander & Johansson, 1975; Haars, Chet & Hutterman, 1981; Johansson & Stenlid 1985; Woodward & Pierce, 1988) which indicate that the detoxification systems of the fungus are putative pathogenicity factors. Unigenes with similarity to cellulases (*CEL1*), arabinases (*ARA1*), two glutathione-S-transferases (*GST1* and *GST2*) and a laccase gene (*LAC*) were chosen for an expression study because of their potential involvement in pathogenesis.

CEL1 expression was increasing with time and reached a maximum level at 144 hpi in the *H. parviporum* / Norway spruce callus system ($p < 0.0001$; Figure 6a). This suggest that levels of easily available nutrients such as glucose, which is known to repress the transcription of cellulases in fungi (Maijala, Fagerstedt & Raudaskoski, 1991; Tomme, Warren & Gilkes, 1995), are low during colonization

and the pathogenic fungus degrades structural polysaccharides of plant cell walls to retrieve carbon.

The *ARAI* gene was exclusively expressed during infection of pine seedling roots (Ct-value = 35.47) but had no expression in the corresponding control (III). The transcriptional activation of *ARAI* during host contact is interesting as arabinose is a constituent of structural components of plant cell walls such as xylan and pectin (de Vries *et al.*, 2000), and the pectin-degrading capabilities of *H. annosum* s.l. have been implicated to be of importance for the ability to grow through the bordered pits of conifer tracheids, thus spreading efficiently in the tree (Johansson and Asiegbu, 1994). Alternatively, arabinase activity could be important for the fungus to quickly establish on stump surfaces by metabolising easily available sugars, as small amounts of arabinose are found on freshly cut stump surfaces of both Scots pine and Norway spruce (Asiegbu, 2000a). This explanation appears to be less probable though, as arabinose is poorly utilized by *H. annosum* s.l. (Asiegbu, 2000b) which supports the role of *ara1* in degrading structural polysaccharides for efficient spread, rather than for nutrient acquisition.

Laccase transcription was increased rapidly upon host contact using the spruce tissue culture system ($p < 0.0001$; Figure 6b) (IV), which is consistent with a function in detoxification of phenolics (Haars & Hutterman, 1980; Haars, Chet & Hutterman, 1981; Johansson, Lundgren & Asiegbu, 1998). *H. annosum* s.l. laccase was also shown to detoxify lignans occurring in the reaction zone of infected conifers (Popoff, Theander & Johansson, 1975). Although polymerisation of phenolic monomers by laccase commonly occurs during *in vitro* growth of *H. annosum* s.l. (Haars & Hutterman, 1980; Hutterman, Herche & Haars, 1980), this polymerisation have never been observed under more natural conditions in the presence of cellulose (Westermarck & Eriksson, 1974; Haars, Chet & Hutterman, 1981; Kurek *et al.*, 1998). It should be noted that increased *LAC* expression could be a general response to interspecific interactions as increased laccase activity has been observed in white-rot fungi in response to other fungi and bacteria (Iakovlev & Stenlid, 2000; Baldrian, 2004).

Additional indications of the importance of detoxification mechanisms in *H. annosum* s.l. pathogenicity come from the rapid induction of the putative fungal glutathione-S-transferase genes, *GST1* and *GST2*, at the early stages of spruce callus colonization by *H. parviporum* ($p < 0.0001$; Figure 6c and 6e) (IV). Several fungal GSTs have been shown to participate in detoxification processes (Pocsi, Prade & Penninckx, 2004) and recent investigations have revealed a multitude of putative GSTs in fungal genome sequences (McGoldrick, O'Sullivan & Sheehan, 2005). The observed expression pattern could be explained by a role of *gst1* and *gst2* in detoxification of spruce compounds, by covalent conjugation of the toxic metabolite to glutathione. The subsequent down-regulation of *GST1* and *GST2* during the later stages of colonization might then be an indication of successful fungal detoxification of the particular host derived compounds that constitute the substrates for the enzymes.

Spruce defense responses

Transcriptional responses of spruce defense genes were monitored in parallel with fungal genes in the *H. parviporum*/spruce callus infection experiment (IV). Phenylalanine ammonia lyase (*PaPAL*), peroxidase (*PaPOX*), glutathione-S-transferase (*PaGST*) and class II and IV chitinases (*PaCHI2* and *PaCHI4*) have all been shown to possess a role in plant defense upon pathogen infection and wounding in other studies (Collinge *et al.*, 1993; Kombrink and Somssich, 1995). These genes all showed significantly higher transcript levels in inoculated callus than the control tissues ($p \leq 0.02$; Figure 9). The relative transcript levels of the included non-defense gene, *PaCHI1*, were decreasing in the inoculated tissues, except for an increase at 144 hpi ($p < 0.001$), which is in conjunction with its down-regulation upon *H. parviporum* infection in mature Norway spruce trees (Hietala *et al.*, 2004).

The rapid induction of *PaPOX* in inoculated tissues could be explained by the need of peroxidase-mediated oxidative cross-linking of structural proteins that makes the plant cell wall more resistant to digestion by pathogen enzymes (Brisson *et al.*, 1994). These rapid modifications may enhance the cell wall effectiveness as a barrier to slow down pathogen spread. As *PaPOX* had a significantly higher expression in clone 589 than in clone 409 ($p < 0.002$) during the early stages of interaction, this is compatible with a scenario of a fast activation of peroxidase-mediated defense reactions that can be related to Norway spruce resistance against pathogens. In addition to its role as an electron-accepting substrate for a wide variety of peroxidases, H_2O_2 also acts as a diffusible signal for the induction of genes encoding cellular protectants such as glutathione-S-transferases (Tenhaken *et al.*, 1995). The induction of spruce *PaGST1* as a response to pathogen colonisation could be explained by the need to detoxify products of lipid peroxidation and from oxidative DNA injury resulting from generation of ROS upon pathogen attack (Frova, 2003).

Transcription levels of *PaCHI4* were increasing throughout the experiment in the low resistance clone 409, while in the high resistance clone 589 the transcript levels did not increase after 12 hpi ($p < 0.0001$). As class IV chitinases, highly similar to *PaCHI4*, have been proposed to stimulate programmed cell death (PCD) during somatic embryogenesis of Norway spruce (Wiweger *et al.*, 2003), this could indicate a more substantial cell death in the low resistance clone which is supported by recent work by Nagy *et al.* (2005). The fact that PCD and hypersensitive response facilitates host infection by necrotrophic pathogens (Govrin & Levine, 2000) might partly explain the low resistance phenotype of clone 409. *PaCHI2* was also up-regulated in inoculated spruce tissues, which imply a role of the corresponding protein in defense against pathogens. This gene was recently shown to be up-regulated in bark of mature Norway spruce trees *H. parviporum* infection

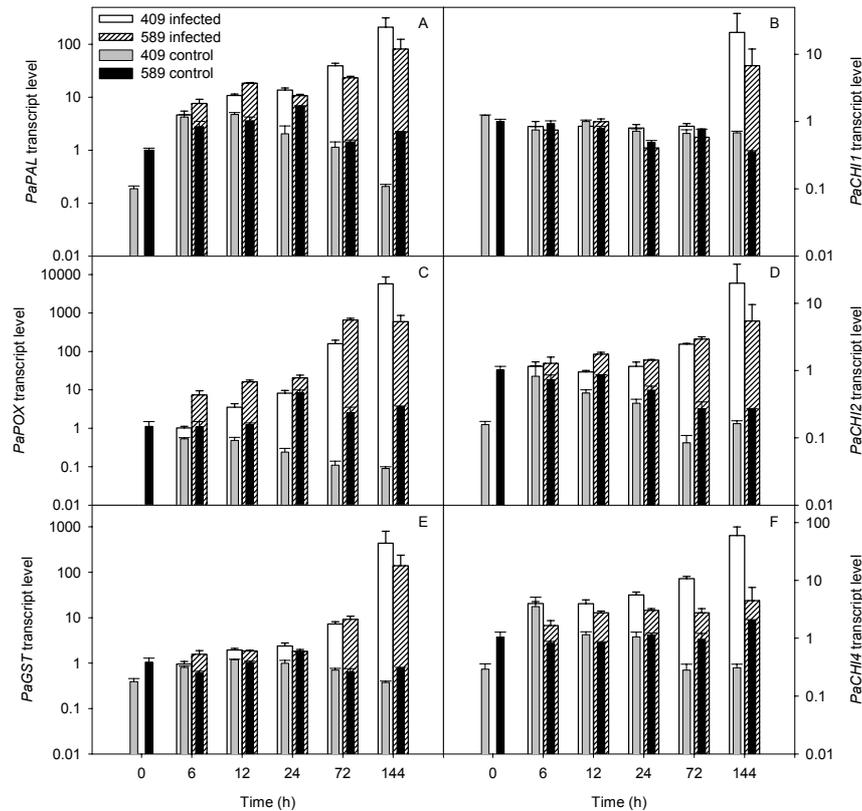


Fig. 9. *Picea abies* gene expression. Relative transcript levels of *P. abies* genes, *PaPAL*, *PaPOX1*, *PaGST1*, *PaCHI1*, *PaCHI2* and *PaCHI4*, in control tissues and following inoculation with *H. parviporum* of callus tissue from Norway spruce trees characterized as high (589) and low (409) resistant. Expression levels are displayed in relation to the 589 0 hpi sample. Error bars represent the standard error of three independent samples.

(Hietala *et al.*, 2004) and another member of this class, the *PsCHI4* gene from *P. elliotii* var. *elliotii*, was induced by the fungal pathogen *Fusarium subglutinans* f.sp. *pini* (Davis *et al.*, 2002).

The up-regulation of *PaPAL* in response to fungal colonization indicates *de novo* synthesis of secondary metabolites in spruce, as pal catalyses the conversion of L-phenylalanine to cinnamic acid which is first reaction in the phenylpropanoid biosynthetic pathway. This pathway provides precursors not only for formation of lignin but also for a diverse group of secondary metabolites, including flavonoids, styrenes, stilbenes and suberin (Dixon & Paiva, 1995; Baucher *et al.*, 1998).

Evaluation of infection systems

Three different infection systems were used in this work, aseptically grown, two-week old *P. sylvestris* seedlings (I, II and III), 10-year old *P. abies* trees (II and

III) and tissue cultures of *P. abies* (IV). Some inconsistencies in transcript levels between pine seedling infection and spruce bark/callus infection were recorded, high expression of *CPM2* in bark/callus but low in pine seedlings and low expression of *SOD1* in callus but high in pine seedlings. This could possibly be reflecting regulatory differences between *H. annosum* and *H. parviporum*, but also be a consequence of the different infection systems used. A high proportion of fungal mycelia were not in direct contact with the pine seedling roots, thereby diluting the expression changes in the interacting mycelia. During fungal growth in spruce bark all hyphae are surrounded by host tissues, therefore this system should provide the biologically most relevant data. A high proportion of invasive fungal hyphae are present during colonisation of spruce callus tissue, although some non-interacting hyphae can also be present. Nutritional status of the fungus at the time of inoculation and during infection should also be considered, as high nutrient levels might interfere with the regulation of pathogenicity genes. Again, fungal growth in spruce bark would provide biologically relevant data as a minimum of nutrients are available for the fungus. Although very small amounts of nutrients are available for the fungus during the initial stages of spruce callus infection, the possibility of nutrient import from the S-medium agar plate that constitute the inoculum in this system should not be underestimated. The high nutrient levels from the Hagem-medium used in the pine seedling system can potentially interfere with the regulation of pathogenicity genes, although this and the spruce callus system can represent a situation where the fungus is established in a large substrate, such as a stump, from where it infects a living root.

Conclusions

H. annosum s.l. is the economically most important fungal pathogen on conifers in Sweden and research directed to understand the basis of this pathogenicity is important. Although biochemical studies of this fungus have a long history, the molecular genetic research offers the possibility of new approaches like genome-wide transcript profiling and genetic transformation techniques. This work was started with the aim to provide a platform for continued research on molecular genetics of the *H. annosum* s.l. /conifer pathosystem.

This work has provided a collection of ESTs that constitute the first survey into *H. annosum* gene diversity. This sequence data will greatly assist future research in this area. Individual genes with a putative function in secondary metabolism, protection against oxidative stress and degradation/detoxification of host material were shown to be differentially expressed during infection of conifer material. The transcript profiles of some of these genes qualifies them for further research as putative pathogenicity factors, e.g. cytochrome P450, arabinase, glutathione-S-transferases and laccase. Norway spruce defense genes, including phenylalanine ammonia lyase, peroxidase, glutathione-S-transferase and class II and IV chitinases were shown to be up-regulated upon fungal colonization of spruce callus tissue.

Future prospects

Future research should focus on determining the exact role of the identified genes and their encoded proteins in *H. annosum* s.l. pathogenesis. Amount and localization of the gene products can be investigated with immuno-based methodology or gene transformation technology. The first steps are taken to develop an efficient transformation methodology for *H. annosum* s.l. and this work should have high priority, as this will provide researchers with a powerful tool to address the role of individual proteins for the phenotype of the organism. Continued generation of ESTs will provide the basis of the construction of both fungal and conifer gene microarrays. The current situation with increasing EST collections, development of an efficient transformation methodology and the construction of a genetic linkage map is promising to the ongoing efforts to elucidate the mechanisms behind *H. annosum* s.l. pathogenicity.

A lot of focus in today's research in the area of pathogen/plant interactions is put upon gene expression profiling. This is important, but research should also be directed to understand the mechanisms that do not include transcriptional regulation. Preformed defense structures and metabolites as well as protein level regulation of interactive mechanisms are all overlooked by transcript profiling.

The most important means for reducing damage by annosum root rot are currently to avoid the establishment of the disease in the forest stand by practicing forestry management techniques like short rotations, few thinnings and winter-logging or stump treatment, and these operations will remain to be the main control strategies. However, tree breeding for resistance against root rot is another possible strategy and the results from this work will contribute with identifying key processes in *H. annosum* s.l. pathogenesis which can be exploited in breeding programs or to facilitate the selection of naturally occurring tree genotypes with high resistance against root rot.

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