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ORIGINAL ARTICLE

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Use of Scots pine seedling roots as an experimental model to investigate gene expression during interaction with the conifer pathogen *Heterobasidion annosum* (P-type)

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Abstract The root-rot fungus *Heterobasidion annosum* is a major pathogen of woody trees in temperate regions of the world. In this study, seedling root of Scots pine was used as an experimental model to investigate gene expression in conifer trees during challenge with *H. annosum*. Initial cellular and histochemical studies have established the systems and indicated the key sequence of events during the infection process. Also, to correlate histochemical observations with the time-dependent pattern of events in host gene expression, a transcriptome profiling of a selected set of host genes from a pine-root subtraction cDNA library was conducted. Differential screening of the subset of genes arrayed on nylon membrane with cDNA probes made from seedling roots infected for 1, 3, 7 and 15 days revealed a number of up-regulated genes [disease-resistance gene analog, antimicrobial peptide (AMP) gene homolog etc.] following inoculation. The results also showed strong expression of genes involved in cell defense and protein synthesis at the early stages of the infection (3–7 days) with a decline at late stages of infection (15 days). The decline in expression of key defense genes at late stages of infection correlated well with the period of vascular colonization and subsequent loss of root turgor. Northern analyses with two of the major induced genes (AMP homolog and disease-resistance gene analog) indicated a several-fold increase in host gene expression following infection. In addition, a particular single gene (thaumatin-like protein) was consistently expressed throughout the four sampling periods of the experiment. BlastX analyses revealed that the Scots-pine thaumatin-like gene shared 51–77% sequence homology with other thaumatin-like proteins in GenBank. The importance of these results in tree defense and use of conifer seedling root in host–parasite interaction in forest trees is discussed.

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Introduction

Root-rot disease of conifer trees is the most economically important tree disease in the northern hemisphere (Hodges 1969). The disease is caused by the root-rot pathogen, *Heterobasidion annosum*. Several conifer species (Norway spruce, Scots pine and Douglas fir) serve as host to the three forms of *H. annosum* S-, P- and F-types respectively. Pretreatment of stumps with chemicals and the biocontrol agent *Phlebiopsis gigantea* are considered the only feasible means of controlling the disease in forest plantations. In recent years, interest in phenotypic selection for resistance and breeding among recognized resistant trees have increased. However, the molecular basis of the host–pathogen interaction in root-rot infection is still not fully understood. The root-rot fungus (*H. annosum*), which has been used as a model organism to investigate various aspects of the host–pathogen interaction appears to have been more intensively studied than the host. Ecological and biochemical factors regulating spore dispersal, germination and host penetration in this fungus have received much more attention (Redfern and Stenlid 1998; Asiegbu 2000) but very few papers have been published regarding genes involved in resistance and pathogenicity such as those that may be differentially expressed during pathogenesis in conifer pathosystems. Several reasons may be cited for the secondary place of biochemical and molecular studies in conifer pathosystems; mature trees do not lend themselves to simple, laboratory based studies on account of their large size and long life. In addition their size places severe constraint on environmental control within the experimental area. The need for a functional model system for genomic studies in the *H. annosum* pathosystem is of critical importance for understanding variations in natural resistance and also as basis for increasing resistance through selection or molecular biology (Asiegbu et al. 1998).

Recent cytological and biochemical techniques may help towards a better understanding of the mechanisms that regulate plant–fungus interactions. The outcome also involves a series of molecular and cellular recognition processes between the plant and pathogen (Kroj et al. 2003). Furthermore, to obtain an overall view of all the processes that are occurring during fungal invasion, a novel genomic method may be necessary to identify as many as possible of the genes that show induced expression during the interaction. The initial strategy of gene discovery by sequencing of large numbers of cDNAs to identify expressed genes has only been applied to a few studies (Adams et al. 1991; Tagu and Martin 1995; Cooke et al. 1996). Although the large-scale expressed sequence tag (EST) technique has been available since 1991, most of the projects applying this methodology in plant biology have concentrated on identifying genes expressed during developmental processes (Wyrich et al. 1998) and plant responses to environmental cues (Umeda et al. 1994; Liu et al. 1995; Lim et al. 1996; Kwak et al. 1997; Lee et al. 1998). The application of ESTs to characterize genes expressed during symbiotic interaction was pioneered by Tagu and Martin (1995). Similarly, very few papers have been published relating to the application of ESTs to identify genes involved in host–pathogen interactions (Botella et al. 1997; Rauyaree et al. 2001).

To compare results of histochemical observation with the expression pattern of selected host defense genes during conifer tree–pathogen interaction, we have used seedling roots of Scots pine as an experimental model.

Materials and methods

Host material and pathogen isolate

Scots pine (*Pinus sylvestris*) seeds were purchased from Assidomain, Sweden. *Heterobasidion annosum* (FP5) was obtained from K. Korhonen (Finland) maintained on Hagem agar at 20 ± 1°C. Mycelium of *H. annosum* used for inoculation was obtained from cultures cultivated in liquid Hagem medium (glucose 5 g/l, NH_4NO_3 0.5 g/l, KH_2PO_4 0.5 g/l, $MgSO_4$ 0.5 g/l, malt extract 5 g/l) for 10 days under static conditions.

Root-rot inoculation

Ten seedlings of *Pinus sylvestris* (at 10 days after germination) were transferred to sterile filter paper pre-laid on 1% water agar per Petri dish. The root regions of the seedlings were inoculated with 1 ml homogenized mycelia of *Heterobasidion annosum* pre-grown in Hagem liquid medium. Control seedlings were mock inoculated with sterile distilled water. After inoculation, seedlings were exposed to a 16-h photoperiod and used for RNA extraction.

Tissue preparation for histochemistry

Samples (approx. 5 roots per sample) were collected 1–7, 9–11, 13 and 15 days post inoculation (p.i.). Root regions (first 10 mm) were fixed for 3.5 h in 0.1 M sodium phosphate buffer (pH 7.2) containing 3% v/v glutaraldehyde, dehydrated in ethanol, infiltrated using a series of ethanol/Technovit mixtures (2:1, 1:1, 1:2, 0:1) and finally embedded in a mixture of 15 ml Technovit and 1 ml hardener II. Sections (4 μm) were cut using an ultratome (LKB 4804). Sections were stained with aqueous toluidine blue.

Subtractive cDNA library construction

A subtraction cDNA library was constructed (Asiegbu et al. 2003) using a Smart Subtraction PCR kit according to manufacturer's instruction (Clontech, USA). Briefly, total RNA was extracted from 6-day-old infected and uninfected Scots pine seedling roots following the procedures of Chang et al. (1993). The mRNA was prepared by the oligo-dT cellulose-column protocol (Gibco BRL, Life Technologies, USA). Double stranded cDNA was synthesized by reverse transcriptase (Clontech). Excess cDNA from the uninfected seedling roots was used for the subtraction in a two-round hybridization and PCR reaction with diluted amounts of adaptor-ligated cDNA from the infected seedling roots. During secondary PCR amplification, the background is reduced and differentially expressed genes are further enriched (Clontech). Secondary PCR product from the subtraction was cloned into the pT-Adv vector. The recombinant cDNA clones (12,288) were stored in microtiter plates and also replicated onto Hybond N^+ nylon membranes using a Q-bot automated workstation (Genetix, USA) as described by Zhu et al. (1997).

Random sequencing of cDNA from subtractive library and sequence analyses

The plasmid DNA template purification was performed using 96-well filter plates from 2 ml of Terrific broth (TB) cultures. For DNA sequencing, each reaction was performed with 2 μl Big dye terminator chemistry (Perkin-Elmer) in a 10 μl reaction with T7 primer using an ABI Prism 3700 96-well capillary automated DNA sequencer. Nucleotide sequencing and data analysis were done after deleting the vector sequence. cDNA sequences were compared with GenBank database sequences using BlastX. Sequences for which no match was found were classified as unknown. A subset of interesting clones relevant in host defense and other cellular function was selected for differential screening (Table 1). The sequences of antimicrobial peptide (AMP) homolog (BI416519, BI416764, BI416814, and BI416868), disease-resistance gene analog (BI416767) and thaumatin-like protein (BI416916) described in this study have been deposited at GenBank with the accession numbers indicated.

Differential hybridization

About 82 clones were selected (Table 1) for differential hybridization. The majority of the clones selected have high

Table 1 Functional category of genes used in the macroarray differential screening

Description	Number
Cell rescue, defense and virulence	
Antimicrobial peptide (AMP)	8
Catalase	1
Translationally controlled tumor-protein homolog	1
Cytochrome P450 monooxygenase	12
Expansin	1
Hydroxyproline-rich glycoprotein	9
Intracellular PR-protein	1
Peroxidase	2
Disease-resistance gene analog	1
Thaumatin-like protein	1
Total for cell rescue, defense and virulence	37
Metabolism	
Calmodulin mutant SYNCAM15	1
Calmodulin	1
Carboxyltransferase	1
CDHI-D	1
Cinnamyl dehydrogenase	1
Cystein proteinase	1
Galactosidase	1
Glutamine synthase	1
Gluthathione S-transferase	1
Lipoamide dehydrogenase	1
Methionine synthase	1
Monodehydroascorbate reductase ($NADH_2$)	1
Non-functional folate-binding protein	1
Phospholipid hydroperoxide	1
Proprotein convertase	1
Protease	1
Steroid 22-alpha-hydroxylase	1
Total for metabolism	17
Transcription, protein synthesis and protein fate	
40 S Ribosomal protein	3
Polyubiquitin	1
Ribosomal protein S12	1
Ribosomal protein S14	1
RNA binding protein	1
RNA polymerase	1
Senescence protein	4
Ubiquitin	1
Total for transcription, protein synthesis and protein fate	13
Signal transduction, cellular transport and transport mechanisms	
Ethylene-inducible protein	1
GTP-binding protein	2
MAP kinase	1
Membrane-intrinsic protein	2
Serine/threonine kinase	1
Total for signal transduction, cellular transport and transport mechanisms	7
Transport facilitation	
Vacuolar ATP synthase	1
Unclassified proteins	
Hypothetical protein	1
Unknown protein	6
Total for unclassified proteins	7

homology to plant genes. EST clones of some genes such as AMP homolog and cytochrome P450 monooxygenase, represented several times on the array, have significant homology to the same gene in the GenBank database. The inserts of these EST clones with multiple copies on the array were considered to be either part of the same gene or in a gene family. The clones were picked and arrayed on nylon membrane filter and allowed to grow overnight on LB-ampicillin medium. After overnight growth, the membrane was treated as described by Zhu et al. (1997). There were three replicates for each clone on separate membranes. For standardization, the cDNA used as a probe was made from same amount of total RNA. The processed membrane was hybridized with a cDNA probe made from Scots pine roots infected for 1, 3, 7 and 15 days with *Heterobasidion annosum*. As control, a cDNA probe made from roots mock-inoculated with sterile water was used. To minimize background problems due to difference in growth of the bacterial clones on the membrane (Fig. 3), the same nylon membranes containing the gene clones were used in hybridization assay for each pair of probes (made from either infected or mock-inoculated roots). With the help of the software Quantity One (Bio-Rad), the hybridization signals on nylon membrane were analyzed and the volume-intensity analysis reports were compared and contrasted to find the relative change of gene expression.

Northern hybridization analyses

For northern hybridizations, 30 μg RNA from Scots pine roots infected for 7 days with *Heterobasidion annosum* was subjected to electrophoresis on 1.2% formaldehyde-agarose gel. The RNA was transferred to Hybond nylon membranes (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Gene-cleaned disease-resistance gene analog and AMP gene homolog PCR products were used as probes. The probes were labelled and hybridized with the AlkPhos direct Labelling Kit (Amersham Pharmacia Biotech). Blots were hybridized at 55°C and post-hybridization stringency washes were done according to manufacturer's instructions. Signal generation and detection were done with CDP-Star (Amersham Pharmacia Biotech).

Results and discussion

Histochemistry

A schematic representation of the infection process during pathogenesis of Scots pine root by P-type *Heterobasidion annosum* is shown in Fig. 1. Histologically, the first signs of epidermal and cortical penetration were observed 48 h and 72 h p.i., respectively (Fig. 2A–C). Extensive disintegration of cortical cells was recorded 6–9 days p.i. (Fig. 2D–E). At this point in time, the pathogen has reached the endodermal region and the vascular region in the process of invasion. At 10–15 days, disintegration of meristematic tissues and vascular system of some of the root tissues examined was visible (Fig. 2F). These results are in line with our previous observation with a related conifer species, Norway spruce (Asiegbu et al. 1994). During development, the non-suberized conifer root mainly containing epidermis, cortex

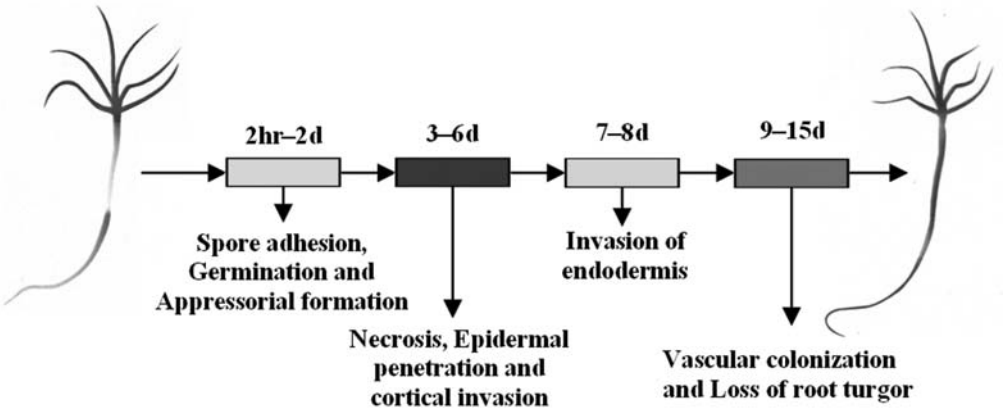

Fig. 1 Schematic representation of the infection process of Scots pine seedling roots by *Heterobasidion annosum*

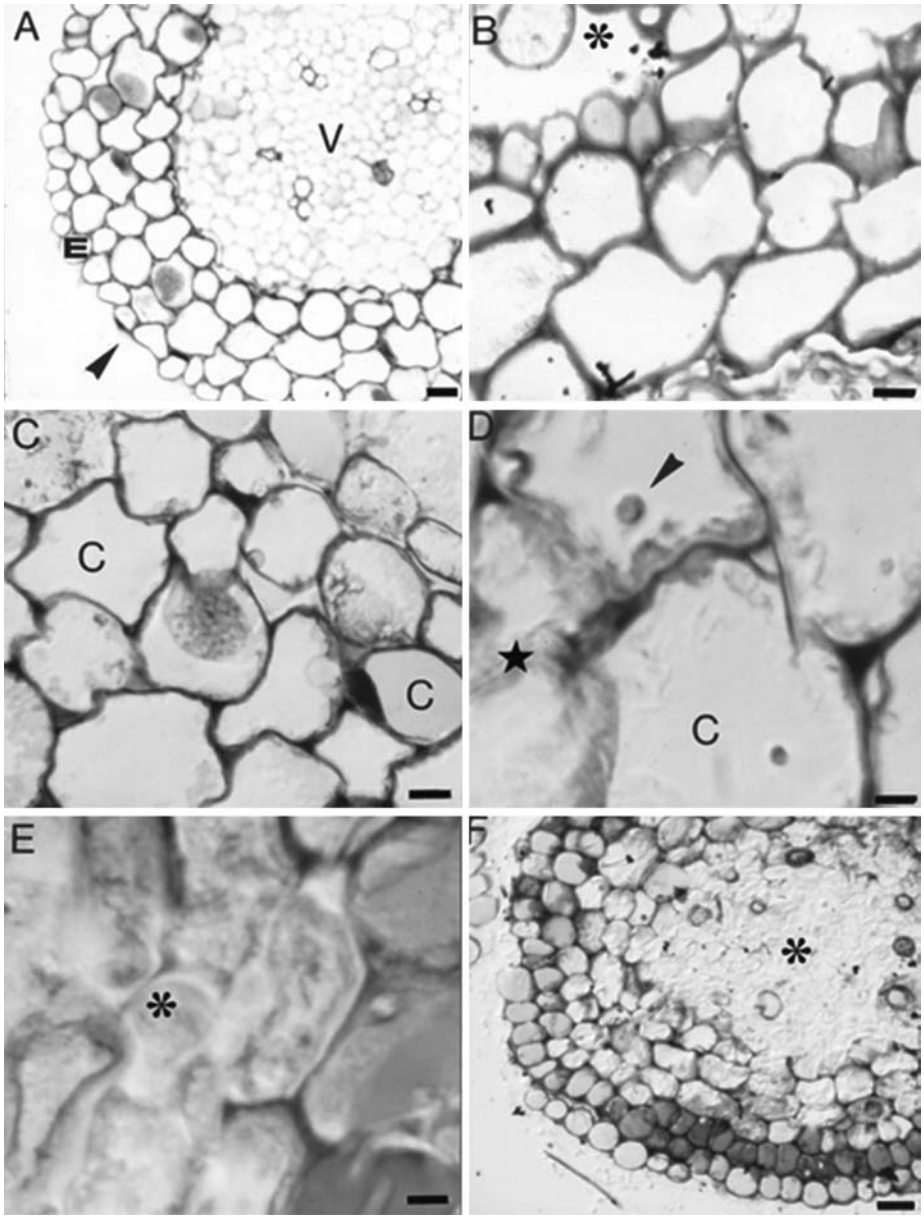

Fig. 2A–F Micrographs showing cellular responses of pine root tissues following challenge with pathogen. **A** Hyphal materials of the pathogen (*arrowhead*) were seen on the periphery of epidermal cells at day 2 post inoculation (p.i.). Note that vascular region (*V*) and epidermis (*E*) are intact. *Scale bar* 5 µm. **B** Disintegration of epidermal cells (*asterisk*) at 3 days p.i. *Scale bar* 2.5 µm. **C** Initial stage of cortical invasion (**C**) at 4 days p.i. *Scale bar* 2.5 µm. **D** Invasion and disintegration (*star*) of cell wall region of cortical cells (**C**). Note presence of hyphal cells (*arrowhead*) at 6 days p.i. *Scale bar* 1 µm. **E** Extensive damage of cortical cells (*asterisk*) at 9 days p.i. and progression of invasion into endodermis. *Scale bar* 1 µm. **F** Disintegration of meristem and initial vascular colonization (*asterisk*) at 10 days p.i. *Scale bar* 5 µm

meristem and stele undergoes several morphological and physiological transformations and is successively suberized, the cortex disappears and the secondary xylem in the vascular region becomes dominant. Later the epidermis, cortex and endodermis are replaced by the rhytidome, phelloderm and phloem layers (Asiegbu et al. 1994). However, our recent studies have shown that the root-rot fungus is capable of infecting conifer tissues of all ages (Asiegbu et al. 1998).

Subtraction Scots pine root cDNA library and sequence analyses

In this experimental model, the seedlings were grown under uniform sterile conditions and used at an age when their genetic differences are only faintly expressed. The subtractive-hybridization cDNA library approach (Asiegbu et al. 2003) was used in order to obtain cDNA clones enriched with host defense genes. A similar approach has been used by other authors (Wang and Brown 1991). The infected root samples used for the subtraction cDNA library construction were harvested at 6 days p.i. At this point in time, necrotic symptoms on the roots were becoming visible as well as cortical colonization (Fig. 2E–F). Sequence analyses of a random set of 480 ESTs from the library (Asiegbu et al. 2003) showed that several kinds of genes were identified as involved in disease resistance, signal transduction, metabolism and other cellular functions. In this study, genes on the array from the subtraction Scots pine root cDNA library were selected to represent various functional categories (Table 1). Among the selected clones, preference was given to genes involve in cell defense, disease resistance and signal transduction, although all categories were represented, including unidentified genes.

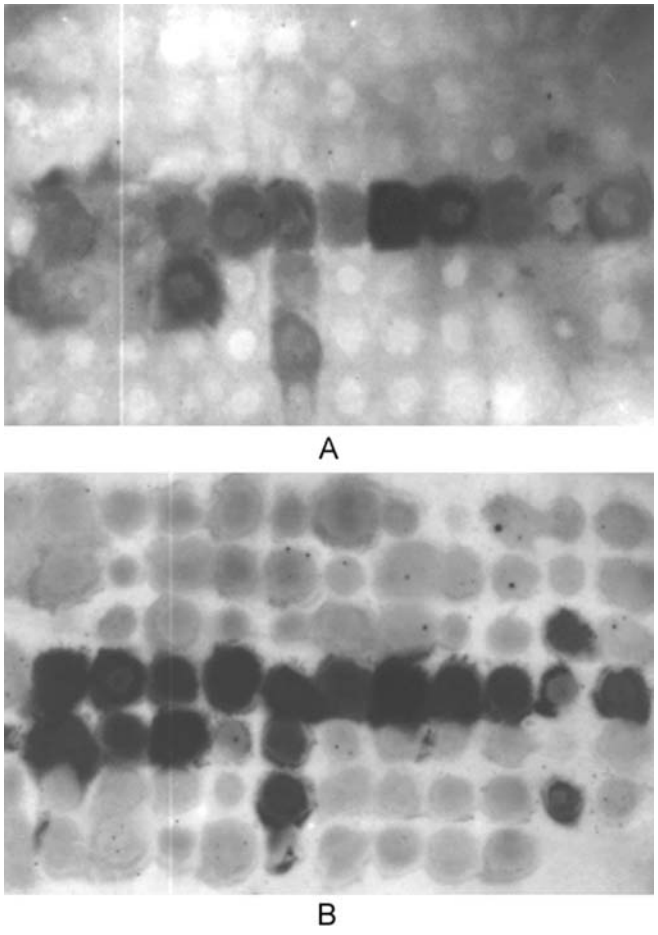

Fig. 3A, B A view of the macro-arrays. **A** Membrane hybridized with a cDNA probe made from Scots pine roots mock-inoculated with sterile water for 1 day. **B** Membrane hybridized with cDNA probe made from Scots-pine roots infected for 1 day with *Heterobasidion annosum*

Identification and expression pattern of differentially expressed cDNA clones from the Scots pine root cDNA array

A subset of interesting genes representing various functional categories from the cDNA library were selected (Table 1) and arrayed on nylon membrane for differential hybridization (Fig. 3). The membrane was hybridized with cDNA probe made from Scots pine roots infected for 1, 3, 7, and 15 days with *Heterobasidion annosum*. As control, a cDNA probe made from roots mock-inoculated with sterile water was used. Differential hybridization using cDNA derived from a root that had been infected for 7 days as a probe revealed that AMP gene homolog, serine/threonine kinase, cytochrome P450 monooxygenase, GTP-binding protein and thaumatin-like protein were among the genes induced during the infection. The relative changes of the gene expression at 7 days p.i. are shown in Table 2. There were 12, 19, 30 and 6 up-regulated gene clones at 1, 3, 7 and 15 days p.i., respectively. Clones that distinctly hybridized to cDNA probes made against a specific stage (3, 7 and 15 days p.i.) were selected and used to construct Venn diagrams (Fig. 4). Analysis of the up-regulated clones revealed that only one clone was consistently up-regulated throughout the sampling periods. BlastX analyses of the partial sequence data for this clone showed homology to thaumatin-like protein (GenBank accession no. BI416916). Alignment of amino acid sequence of Scots-pine-thaumatin-like protein with proteins from other species revealed a 51–77% sequence homology (Fig. 5, Table 3). Several other thaumatin-like proteins known to be related to pathogenesis-related protein group 5 (PR-5) have been described (Walden et al. 1999). Walden et al. (1999) indicated that these thaumatins have antifungal properties and act by permeabilizing the fungal hyphae by forming a pore or a channel that leads to the release of cytoplasmic content. Furthermore, at 15 days p.i., one of the few up-regulated genes was interestingly found to be an unknown protein. The expression of this unknown protein could probably be related to the infection of *H. annosum*. The result also showed strong expression of genes involved in cell defense and protein synthesis at the early stages of the infection (3–7 days) with a decline at late stages of infection (15 days). The decline in expression of key defense genes at late stages of infection correlated well with the period of vascular colonization and subsequent loss of root

Table 2 List of induced genes at 7 days post inoculation

Induced genes and gene function	Relative change of gene expression
Cell rescue, defense and virulence	
Antimicrobial peptide (AMP) (P80915)	3.0
AMP (P80915)	2.0
AMP (P80915)	3.0
AMP (P80915)	2.0
Catalase (P29756)	1.3
Cytochrome P450 monooxygenase (T02955)	2.0
Cytochrome P450 monooxygenase (T02955)	2.0
Cytochrome P450 monooxygenase (T02955)	2.0
Cytochrome P450 monooxygenase (T02955)	1.3
Cytochrome P450 monooxygenase (T02955)	1.7
Cytochrome P450 monooxygenase (T02955)	1.5
Hydroxyproline-rich glycoprotein (AJ242540)	1.5
Hydroxyproline-rich glycoprotein (AJ242540)	1.5
Hydroxyproline-rich glycoprotein (AJ242540)	1.5
Hydroxyproline-rich glycoprotein (AJ242540)	2.0
Hydroxyproline-rich glycoprotein (AJ242540)	2.0
Intracellular PR-protein (AF038949)	2.0
Thaumatin-like protein (AJ131731)	2.0
Metabolism	
Cinnamyl dehydrogenase (T16995)	1.5
Glutamine synthase (P08282)	1.5
Lipoamide dehydrogenase (AF295339)	2.0
Transcription, protein synthesis and protein fate	
40 S Ribosomal protein (P49203)	1.5
40 S Ribosomal protein (AC011664)	1.5
RNA-binding protein (AP000370)	2.0
RNA polymerase (AW689548)	3.0
Senescence protein (BAB33421)	1.5
Senescence protein (BAB33421)	2.0
Signal transduction, cellular transport and transport mechanisms	
GTP-binding protein (P54766)	2.0
Serine/threonine kinase (Q07099)	2.0
Transport facilitation	
Vacuolar ATP synthase (O82702)	2.0

Table 3 Amino acid sequence identity (%) of thaumatin-like protein from Scots pine (*Pinus sylvestris*) and other species

Other species	GenBank accession nos.	Amino acid sequence identity (%)
Arabidopsis thaliana	NP_173365	60%
Cestrum elegans	AB031870	56%
Castanea sativa	Q9SMH2	59%
Hordeum vulgare	AAK55326	52%
Juniperus ashei	P81295	67%
Malus × domestica	P83336	55%
Oryza sativa	T04165	57%
Pseudotsuga menziesii	CAA10492	77%
Prunus persica	P83332	57%
Pyrus pyrifolia	O80327	63%
Sambucus nigra	AAK59278	51%
Triticum aestivum	AAM15877	54%
Vitis vinifera	AAF82264	59%
Zea mays	JS0646	58%

turgor (Fig. 6). It is also possible, due to the cyclical pattern in gene regulation, that the specific induction of some of these genes may have been missed at the particular sampling point.

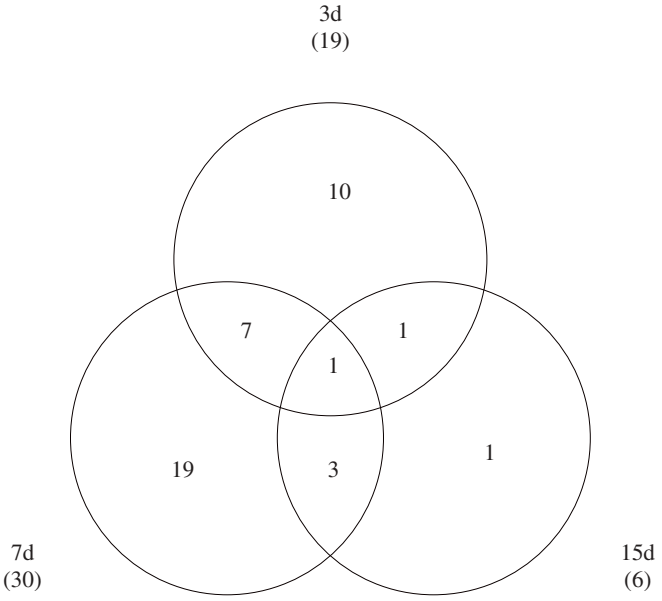

Fig. 4 Overlapping and unique cDNA clones identified by cDNA probes prepared from Scots-pine roots at 3, 7 and 15 days p.i. *Numbers within brackets* indicate the number of cDNA clones associated with a particular time period. *Number within intersects* of circles represent the number of clones common to particular combinations of time period

Fig. 5 Alignment of deduced amino acid sequence of thaumatin-like protein (*black frames* indicate the different amino acids of *Pinus sylvestris* compared with the consensus.). *At Arabidopsis thaliana* (GenBank accession no. NP_173365); *Ce Cestrum elegans* (GenBank accession no. AB031870); *Cs Castanea sativa* (GenBank accession no. Q9SMH2); *Hv Hordeum vulgare* (GenBank accession no. AAK55326); *Ja Juniperus ashei* (GenBank accession no. P81295); *Md Malus × domestica* (GenBank accession no. P83336); *Os Oryza sativa* (GenBank accession no. T04165); *Pm Pseudotsuga menziesii* (GenBank accession no. CAA10492); *Pp Prunus persica* (GenBank accession no. P83332); *Py Pyrus pyrifolia* (GenBank accession no. O80327); *Sn Sambucus nigra* (GenBank accession no. AAK59278); *Ta Triticum aestivum* (GenBank accession no. AAM15877); *Vv Vitis vinifera* (GenBank accession no. AAF82264); *Zm Zea mays* (GenBank accession no. JS0646); *Ps Pinus sylvestris* (GenBank accession no. BI416916)

Northern hybridization analyses of AMP homolog and disease-resistance gene analog

Another interesting gene identified is disease-resistance gene analog (GenBank accession no. BI416767). In plants

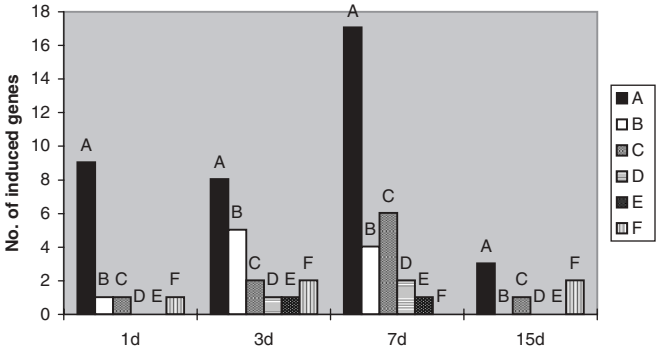

Fig. 6 The relationship of gene expression with period of the pathogen inoculation. *A* Cell rescue, defense and virulence; *B* metabolism; *C* transcription, protein synthesis and protein fate; *D* signal transduction, cellular transport and transport mechanisms; *E* transport facilitation, *F* unclassified protein

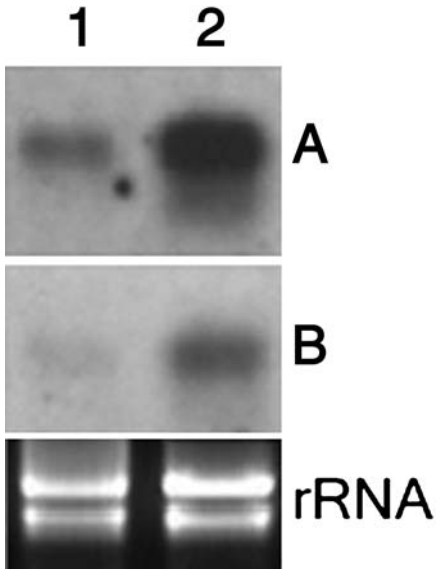

Fig. 7 Northern hybridization of antimicrobial peptide (AMP) gene homolog (*A*) and disease-resistance gene analog (*B*). Total RNA was extracted from Scots pine roots infected for 7 days with *Heterobasidion annosum* (*2*) and mock-infected with water (*1*). A 30-µg aliquot of total RNA was subject to electrophoresis on 1.2% formaldehyde-agarose gel. The RNA was transferred onto a Hybond-nylon membrane. AMP gene homolog and disease-resistance gene analog PCR products were used as probes labeled and hybridized with AlkPhos direct Labelling Kit (Amersham Pharmacia Biotech)

a number of disease-resistance genes that confer resistance to a variety of plant pathogens have been cloned and characterized (Hulbert et al. 2001) and the majority of the disease-resistance genes described in the literature seem to be involved in signal transduction. Northern analyses using AMP gene homolog and disease-resistance gene analog cDNA as probes further confirmed that the genes were induced upon infection with the root-rot fungus (Fig. 7). The functional significance of several of these defense and disease-resistance gene homologues as well as those implicated in signal transduction during the host–pathogen interaction and their potential role in the host resistance deserves to be further investigated. Other authors have also identified similar disease-resistance genes in crop pathosystems such as downy-mildew-resistance protein (Parker et al. 1997) and wheat-leaf-rust resistance gene (Feuillet et al. 1997). BlastX analyses using the AMP cDNA showed strong homology (*e* value of 7×10^{-25}) to a similar peptide from *Macademia integrifolia* (MiAMP) (Marcus et al. 1999). AMPs have been grouped into several families such as lipid-transfer proteins, thionins, plant defensins, heveains and knottin type proteins. The structure of MiAMP is unique and forms a new class called β-barrellins. The structure of MiAMP is, however, similar to a yeast-killer toxin from *Willopsis mraki*, and such structural similarity might reflect a similar mode of antimicrobial action as *W. mraki* toxin inhibits α-glucan synthesis in other yeasts, thereby weakening their cell walls. Marcus et al. (1999) also reveal that the AMP peptide is capable of inhibiting the growth of a variety of fungi and gram-positive bacterial phytopathogens. Other genes also observed to be induced during the interaction include cytochrome P450 monooxygenase. In plants, cytochrome P450 monooxygenases are implicated in normal plant development, growth and defense including the biosynthesis of gibberellins, jasmonates, lignin, fatty acids, alkaloids, phytoalexins, phenylpropanoids and terpenoids (Durst and O'Keefe 1995; Chou and Kutchan 1998). Persans et al. (2001) have also reported the induction of cytochrome P450 monooxygenase in maize seedlings infected with bacterial pathogens. Furthermore, the absence of genes involved in signal transduction within the first day of infection (Fig. 6) could be due to the few number of such genes represented in the cDNA arrays. However, the expression of the two signal-transduction genes (MAP kinase, GTP-binding protein) used in the analyses at this point in time were at levels comparable to control. The lack of induction recorded at this point in time could also be due to the sensitivity of the technique, or detection level and it could also be that gymnosperm tissues respond much more slowly to pathogen invasion when compared to crop plants. Furthermore, a possible evidence of the low resolution and sensitivity of the Quantity One analyses (BioRad) is the comparable expression levels documented for disease-resistance gene analog of both infected and control seedlings at 7 days p.i. on the cDNA arrays. However, the results of the northern analyses revealed increased levels of the disease-resistance gene expression following infection at the same point in time (7 days p.i).

Finally, although genes from the subtraction cDNA library originated from both the host and pathogen, a large proportion of the genes on the cDNA array were associated with host defense responses against the invading fungus. However, it is also possible that a very small proportion of the genes on the array identified as either unclassified or having homology to non-plant or fungal genes could have originated from either the pathogen or host. Further characterization of this small minority of unclassified genes will help to unravel their origin as well as their potential role in the interaction. In conclusion, the correlation between histochemical observation and expression pattern of key host-

defense and cell-rescue genes further confirms that conifer tissues, even at an immature stage, could very well serve as a good experimental model system for investigating gene expression in the *Heterobasidion annosum*–conifer pathosystem.

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