Analysis of organ specific responses of *Pinus sylvestris* to shoot (*Gremmeniella abietina*) and root (*Heterobasidion annosum*) pathogens

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Abstract

Using root and shoot specific pathogens *Heterobasidion annosum* sensu stricto (Fr.) Bref. and *Gremmeniella abietina* (Lagerb.) Morelet, respectively, as experimental model, we investigated whether the gene machinery engaged for host defences within root tissues is the same kind as genes employed by aerial parts of conifers for resistance against necrotrophic pathogens. Inoculations with the shoot pathogen (*G. abietina*) on its preferred host tissue (pine needles) led to necrotic browning reaction but corresponding infection of the root with the same pathogen did not provoke any host reaction. Interestingly both shoot and root tissues infected with the root pathogen *H. annosum* responded with strong necrosis. Host responses were further characterised using mRNA profiling technology for identification of genes differentially expressed. A macroarray containing 384 individual pine cDNA's representing a range of transcripts expressed during different stages of development was examined. Hierarchical clustering of the differentially expressed genes from pine tissues challenged with *H. annosum* or *G. abietina* suggests that the responses were more organ-specific than pathogen-specific: transcript profile of roots infected with *H. annosum* was more similar to roots challenged with *G. abietina* than to shoot response to *H. annosum*. The results of the macroarray analysis were further verified and confirmed by virtual northern blot analysis. Overall many transcripts of defence related genes preferentially accumulated in the infected roots in comparison to the shoot. The differences in defence strategies employed by the different plant organs are discussed with reference to *Arabidopsis* model and crop plants.

Key words: organ specificity, *Heterobasidion annosum, Gremmeniella abietina*, pine, plant defence.

Introduction

Plant pathogens are characterized by various degrees of host-specificity that may range from hundreds of species, 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 specialisation is the pathogen preference 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. Host specificity and factors determining the taxonomic range of hosts that can be infected by a specific pathogenic microbe remain key question in plant pathology. Particularly, it is poorly understood why certain pathogens preferentially infect only some organs of a generally susceptible

host and not the whole plant. Although a few studies have been done on *Arabidopsis* model or crop plants (Hermanns, Slusarenko & Schlaich 2003; Jansen, Slusarenko & Schaffrath 2006; Schafer & Yoder 1994), organ-specificity in pathosystems of forest trees has not been investigated.

It is possible that the defence mechanisms might be different in aerial parts of the plant as compared to the roots. It has been shown that certain defence-related proteins preferentially accumulate in un-infected roots as compared to the shoots (Broekaert, Terras & Cammue 2000; Asiegbu, unpublished). Contrary to that, gene-for-gene interactions, which very actively arrest pathogen infections on leaves, do not operate with the same efficiency in the rice roots (Jansen et al. 2006). Similarly, expression of resistance (*R*) genes and downstream components of the signalling cascade is not sufficient for the induction of avirulence gene-mediated defense mechanisms in *Arabidopsis* roots (Hermanns et al. 2003). The reason for differences in defence strategies of plant organs might have evolutionary context. The co-evolution of free-living fungi and ancestral plants resulted in formation of mycorrhizal symbioses that occurred concurrently with the first colonisation of land by plants 450-500 million years ago (Cairney 2000). Although mutualistic associations also exist between fungi and aerial parts of grasses, these appear to have evolved relatively recently (Clay 1988). Therefore, roots in contrast to shoots and leaves have been exposed to a range of microbes, beneficial and harmful and had to develop mechanisms to be able to distinguish between them.

Plant-pathogen interaction is a complex and dynamic situation as both host and pathogen struggle for survival and existence. To parasitize a plant successfully, a pathogen must posses a set of pathogenicity factors that will allow it to breach the host defence system. Organ-specificity in plant-pathogen interactions is probably a consequence of both, differences in defence mechanisms of roots and aerial parts of the host and corresponding specialisation of attack strategies employed by the invader. However, the common practise of classifying fungal plant pathogens as root- or aerial parts-infecting may need revision. Recently, it was reported that the fungus *Magnaporthe grisea*, casual agent of rice blight, typically considered as a leaf pathogen, is also able to infect roots (Sesma & Osbourn 2004).

Many examples of organ-specificity can be found in forest pathology: *Heterobasidion annosum* s.l., *Phellinus weirii, Phytophthora lateralis, Armillaria ostoyae* cause major root diseases; *Gremmeniella abietina, Lophodermium* spp. and *Mycosphaerella dearnessii* are responsible for conifer foliage diseases.

Heterobasidion annosum (Fr.) Bref. sensu lato is a causative agent of a root and butt rot disease of coniferous trees. The fungus is widely regarded as the most economically important forest pathogen in temperate regions of the northern hemisphere (Asiegbu, Adomas & Stenlid 2005a). *Heterobasidion* species complex comprises taxa preferentially infecting pine (*H. annosum* sensu stricto), spruce (*H. parviporum*) or fir (*H. abietinum*). 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). The ecology of the disease spread has been intensively studied and transcriptomic

approach has been recently employed to investigate the genetics and molecular aspects of the host response to the pathogen (Adomas, unpublished).

Gremmeniella abietina (Lagerb.) Morelet (anamorph *Brunchorstia pinea* (P. Karst.) Höhn.) has been responsible for the destruction of many conifer plantations in North and Central Europe, North America and Japan in recent decades (Kaitera & Jalkanen 1992). The pathogen causes stem canker and shoot dieback on more than 40 coniferous species in seven genera. The mode of infection has been investigated on a few occasions (Patton, Spear & Blenis 1984; Ylimartimo et al. 1997). Under favourable conditions, the life cycle of *G. abietina* takes two years to complete and infected trees can remain undetected for several years before manifesting visible symptoms (Hellgren & Barklund 1992). Very little is known about the resistance mechanisms to this disease.

The aim of the study was to use organ-specific pathogens to investigate whether the gene machinery engaged for host defences within root tissues is the same kind as genes employed by aerial parts of conifer trees for resistance reactions. Transcriptomic approach enables analysis of the expression level of numerous genes in a single experiment. Macroarrays have been successfully used to study biotic interactions (Adomas et al. 2006; Moran et al. 2002). Here we report the use of cDNA macroarrays for identification of genes differentially expressed during organ-specific interaction of *P. sylvestris* roots and shoots with *H. annosum* s.s. or *G. abietina*.

Material and methods

Fungal species, maintenance of cultures and spore harvest

Heterobasidion annosum s.s. (isolate FP5, obtained from K. Korhonen, Finnish Forest Research Institute, Finland) was cultured and maintained on Hagem agar medium (Stenlid 1985) at 20^{o}C. Petri plates of *H. annosum* were grown for 20–40 days in the day light for abundant conidia formation. The conidiospores were aseptically harvested and washed twice with sterile distilled water. *Gremmeniella abietina* (European race, isolate 1:2b obtained from Elna Stenström, Swedish University of Agricultural Sciences, Sweden) was grown on vegetable agar medium (200 ml vegetable juice, 5g glucose, 20g agar, 800 ml water) at 20^{o}C for 14 days in the dark. For spore production, plates were transferred to the light at room temperature. The spores were harvested after 3-4 weeks and suspended in sterile distilled water. The number of spores of both fungal species was counted using haemocytometer. The spores were diluted to the final concentration of $5x10^{6}$ spores/ml.

Experimental model system for investigating organ specific response of *P. sylvestris*

To study organ specific response of pine to pathogen infection a model system was developed. *Pinus sylvestris* seeds (provenance Eksjö, Sweden) were surface

sterilized with 33% H_2O_2 for 15 min, rinsed in several changes of sterile distilled water, sown on 1% water agar and left to germinate under a photoperiod of 16h light at 18°C. After 14 days, the seedlings were used for inoculation. Ten seedlings of *P. sylvestris* were transferred to wet, sterile filter paper placed on 1% water agar in Petri dishes. The roots were inoculated with 1 ml of the spore suspension of $5x10^6$ spores/ml of either root specific (*H. annosum*) or shoot specific (*G. abietina*) pathogen and covered by a second set of moist sterile filter paper. For the shoot inoculations, 1 ml of the spore suspension of either *G. abietina* or *H. annosum* was applied on the needles. The roots were watered with 1 ml of sterile distilled water and covered by a second set of moist sterile filter paper. Control plants were mock-inoculated with 1 ml sterile distilled water. The plates were sealed with parafilm and the region of the dish containing the roots was covered with aluminium foil. The seedlings were then kept under a photoperiod of 16h light at 18°C. Macro- and microscopic observations of infected roots and shoots were carried out at 1, 3, 5, 7, 10, 15 and 42 days post inoculation (d.p.i.). The roots and shoots of 100 seedlings of either infected or control plants were harvested at 7 d.p.i., homogenised in liquid nitrogen and stored at -80°C until RNA extraction. There were three biological replications.

Use of PCR to confirm presence of *G. abietina* on the infected seedlings

To test presence of the *G. abietina* on the shoots and roots, 100 seedlings with either roots or shoots infected with the pathogen were harvested. The seedlings were washed by dipping in 95% ethanol for 30s and then wiping with a tissue paper pre-soaked in ethanol. The shoots or the roots were cut off, ground with liquid N_2 and used for DNA extraction. High molecular weight genomic DNA was extracted from the shoots and the roots using a CTAB protocol modified from http://darwin.nmsu.edu/~fungi/protocols/genomic_DNA_isolation.php and Moller et al. (1992). *Gremmeniella abietina* specific primers (courtesy of Elna Stenström, Swedish University of Agricultural Sciences, Sweden) were tested using the following PCR reaction conditions: 400 ng of genomic DNA, 10µM primers G5 (sequence CTC CCA CCC GTG CCT ATA TTA CTC) and G18 (sequence CTC CCG AGC CCT GTA GCG) and Advantage 2 Synthesis kit reagents (BD Biosciences, Bedford, MA) were mixed in total volume 25 µl and run for 35 cycles in a thermal cycler using the program: 94°C, 30 s; 62°C, 30 s; 68°C, 2 min. As a positive control, *Gremmeniella abietina* genomic DNA was used. The PCR products were electrophoresized on a 1.1% agarose gel and visualised with ethidium bromide staining.

Macroarray procedure

A total of 384 cDNAs were selected from *P. sylvestris* cDNA library (HASP) containing genes differentially expressed during interaction with *H. annosum* (Asiegbu, Nahalkova & Li 2005b) and *P. taeda* cDNA library (Kirst et al. 2003). Sequences of the ESTs (expressed sequence tags) are available in the GenBank or in the database at http://biodata.ccgb.umn.edu/. Functional designation for the cDNAs included on the array was based on homology to the inferred gene

sequence following blastx analysis (http://www.ncbi.nlm.nih.gov/). The clones were manually transferred onto Hybond® N^+ nylon membranes (GE Healthcare, Sweden) as described previously (Adomas et al. 2006).

Total RNA isolation, cDNA synthesis and differential hybridisation

The roots and shoots of *P. sylvestris* challenged with *G. abietina* or *H. annosum* as well as uninfected controls were harvested 7 d.p.i. and immediately frozen in liquid N_2 followed by RNA extraction (Chang, Puryear & Cairney 1993). Because of the low amounts of RNA, it was necessary to amplify cDNA to generate enough probes for macroarray hybridisations. cDNA was synthesized and further amplified by long-distance PCR using the SMART™ PCR cDNA synthesis Kit (BD Biosciences, Sweden). The cDNA arrayed nylon membranes were hybridised with the 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, Sweden). There were a total of three biological replications based on RNA extracted from the pine tissues on three separate occasions and two technical replicates.

Data collection and statistical analysis

Detection and quantification of the 384 signals representing hybridised DNA were performed using the 'volume tools' of the Quantity One software, version 4.4.1 (Bio-Rad; http://www.bio-rad.com/). Each spot was defined by manual positioning of volume circle over the array image and density of each spot was determined. Volume analysis reports were exported to Microsoft Excel software. Net signal was determined by subtraction of the local surrounding background from the intensity for each spot. Spots deemed unsuitable for accurate quantification because of array artefact were flagged and excluded from further analysis. For each treatment five replicates out of six available were selected for further analysis (see Supplementary Table S1). The data analysis was modified from the procedures published by Duplessis et al. (2005). To take account of experimental variations in specific activity of the cDNA probe preparations or exposure time that might alter the signal intensity, the raw data obtained from different hybridisations were normalized by the global normalization method (Baldi & Hatfield 2002). 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. Based on the statistical analysis, a gene was considered significantly up- or down-regulated if it met all four criteria: (1) *t*-test *P*-value ≤ 0.05; (2) inoculated vs control fold change ≥ 1.3 or ≤ -1.3; (3) the trend (up- or down-regulation) was consistent in at least four data sets; and (4) there were significant fold changes in at least four data sets. For the final analysis, fold changes of genes significantly differentially expressed were averaged. Fold changes were supplied to JMP™ (The Statistical Discovery Software™, version

5.1) for clustering. The experimental design is illustrated in Supplementary Figure S1.

Virtual northern blot analysis

To verify the expression pattern of seventeen selected genes (antimicrobial peptide (CK928060), ascorbate peroxidase (CA305546), auxin induced cell wall protein (CK927853), basic blue protein (CA305451), cationic peroxidase (EH628377), cell wall associated hydrolase (CK909801), choline-phosphate-cytidylyltransferase (NXCI_005_H04), protein containing similarity to transfactor (EH628391), intracellular pathogenesis related (CK928055), late embryogenic abundant protein (CK927826), monodehydroascorbate reductase (CA305445), nuclear DNA binding protein (NXCI_057_B04), purple acid phosphatase (NXCI_005_G03), putative auxin induced transcription factor (CA305460), serine/threonine protein phosphatase (NXCI_057_E03), ubiquinol-cytochrome C reductase (NXCI_006_F01), vacuolar ATPase (CA305565)), virtual northern blot was done according to the manufacturer's instructions (Clontech, USA) and earlier published methods (Adomas et al. 2006; Hammerle et al. 2003). Equal amounts of cDNA (0.5 µg) used for the macroarray screening from inoculated and control *P. sylvestris* roots and shoots were separated on an agarose gel, denatured and subsequently blotted onto nylon membrane. For preparation of the DNA probes, plasmids of interest were extracted with QIAprep® Spin Miniprep kit (Qiagen, Sweden). The inserts were amplified using the following PCR reaction conditions: 50 ng of plasmid DNA, 10 µM primers (T7 forward and M13 reverse for ESTs from *P. sylvestris* cDNA library and M13 forward and reverse for ESTs from *P. taeda* library) and Advantage 2 Synthesis kit reagents (BD Biosciences) were mixed in total volume 25 µl and run for 25 cycles in a thermal cycler using the program: 94ºC, 30s; 68ºC, 2 min. The PCR products were electrophoresised on a 1.1% agarose gel and purified with QIAquick® Gel Extraction kit (Qiagen, Sweden). Hybridisation was performed with aid of AlkPhos® Direct Labelling kit (GE Healthcare) according to the manufacturer's instructions. Signal generation and detection were done with CDP-Star (GE Healthcare).

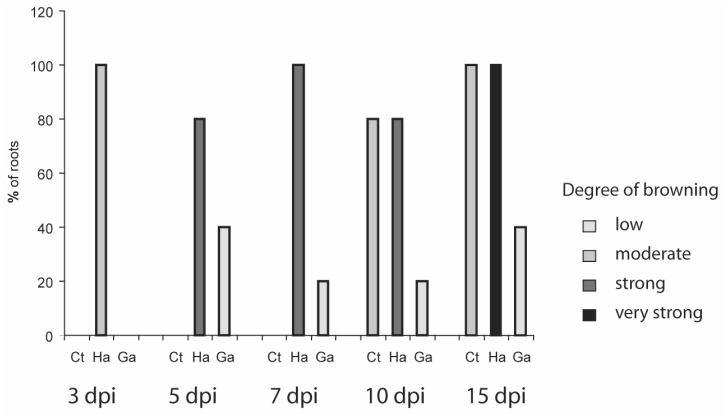

Fig. 1. The degree of necrotic browning reaction caused on *P. sylvestris* roots by *H. annosum* (Ha) or *G. abietina* (Ga) at 7 d.p.i. as compared to un-inoculated control (Ct).

Results

Pilot study on the pine response to infection with a shoot specific (*G. abietina*) and root specific (*H. annosum*) pathogen

To determine the optimal time point for sample collection macro- and microscopic observations of pine shoots and roots infected with either *G. abietina* or *H. annosum* were carried out at 1, 3, 5, 7, 10, 15 and 42 days post inoculation (d.p.i.) (Figs 1-3). It was observed that both pathogens grew equally well on the seedling roots and spore germination was documented by 1-3 d.p.i. While *H. annosum* infection resulted in necrotic reaction at 5-7 d.p.i. that became stronger at 10 d.p.i., there was either no browning or faint necrosis on the roots challenged with the shoot pathogen, *G. abietina* (Figs 1, 2). At prolonged infection (42 days) *G. abietina* challenged seedlings produced numerous short lateral roots perpendicular to the main root (Fig. 2c). At the same time the primary root of the *H. annosum* infected pines was usually dead and in the surviving seedlings formation of secondary roots was observed. The secondary roots had white tips (Fig. 2d) indicating that meristematic activity was sustained.

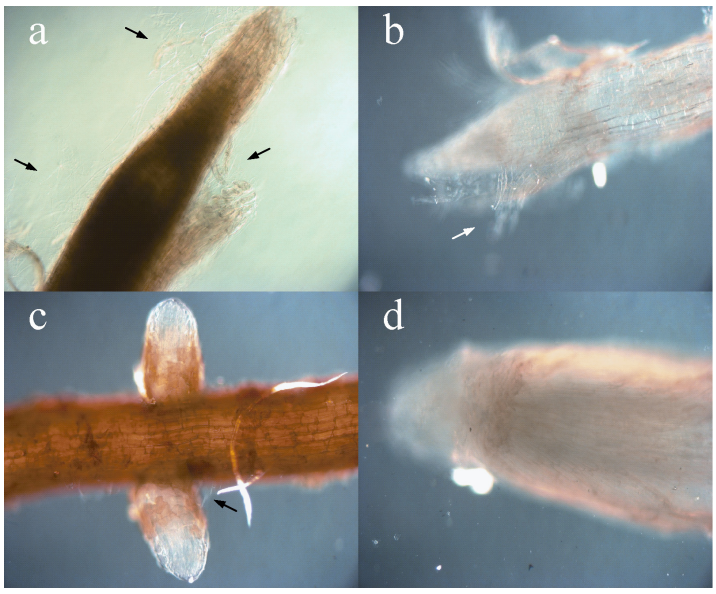

Fig. 2. Pinus sylvestris roots were inoculated with root or shoot specific pathogen: a) *H. annosum* provoked strong browning reaction at 7 d.p.i. b) After prolonged exposure to the pathogens (42 days), *G. abietina* inoculated root tips remained unaffected and c) produced numerous short lateral roots. f) At the same time, 40% of *H. annosum* infected seedlings were dead and the remaining ones formed lateral roots that avoided infection. Arrow-hyphae.

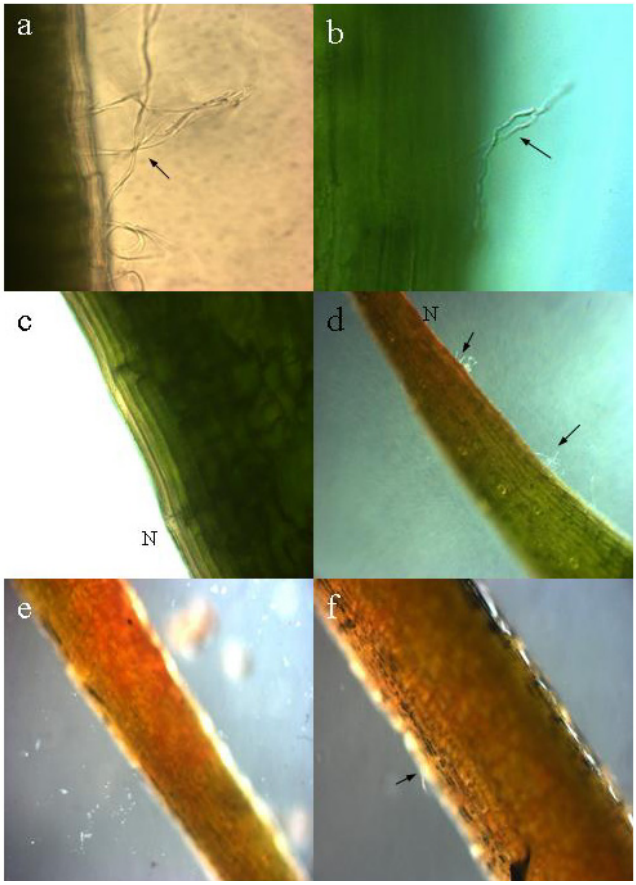

Fig. 3. Pinus sylvestris shoots were inoculated with root or shoot specific pathogen: *G. abietina* a) and *H. annosum* b) spores germinated by 5 d.p.i. At 15 d.p.i. the necrotic browning reaction (N) was weaker in needles challenged with *G. abietina* c) than *H. annosum* d). At 42 d.p.i. needles infected with both *G. abietina* e) and *H. annosum* f) were severely affected. Arrow-hyphae.

On the needles infected with either pathogen, spore germination was observed by 3-5 d.p.i. (Fig. 3a, b). Necrotic browning of the needles challenged with *G. abietina* was documented at 5 d.p.i. and until 15 d.p.i. did not increase significantly. Six weeks after inoculation with *G. abietina,* the first needles (cotyledons) remained unaffected or were light green and the secondary ones exhibited strong necrotic reaction (Fig. 3e). The root specific pathogen, *H. annosum* grew slower on the needles but at 15 d.p.i. provoked stronger browning reaction than *G. abietina* (Fig. 3c, d). Prolonged exposure to *H. annosum* led to extensive shoot necrosis (Fig. 3f). Based on this preliminary analysis, all further studies were conducted at 7 d.p.i. The development of the *G. abietina* hyphae on the root and needle surface was confirmed by PCR amplification of the pathogen specific band from surface washed seedlings (Fig. 4).

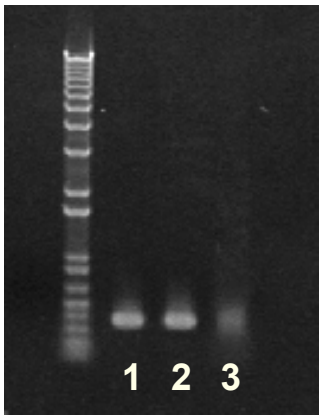

Fig. 4. G. abietina specific PCR tested presence of the pathogen in *P. sylvestris* shoots (line 1) and roots (line 2); line 3 - positive control: *G. abietina* genomic DNA.

Macroarray differential screening: pine response to challenge with organ-specific pathogens

Functional classification of the genes printed on the array is presented in Fig. 5. The most abundant functional categories were: metabolism and energy aquisition (25%), defence (13%) and transcription and translation (11%). Hypothetical proteins and genes of unknown function constituted 15% of the genes on the array.

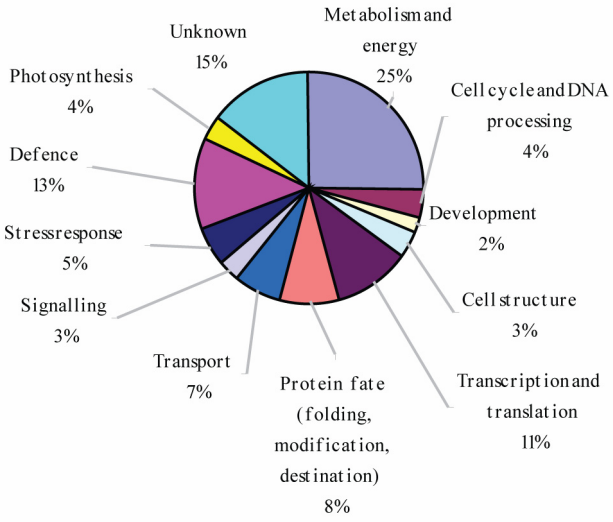

Fig. 5. Functional classification of 384 genes on the array used for the analysis of organ specific response of *P. sylvestris* inoculated with root or shoot pathogen.

Screening of the array with probes made from pine roots and shoots infected with organ-specific pathogens resulted in 92 genes significantly differentially expressed (Fig. 6a, b). Hierarchical clustering of these genes (Fig. 7) showed that the pine tissues response to challenge with *H. annosum* or *G. abietina* was more organ-specific than pathogen-specific, i.e. transcript profile of root infected with *H.*

annosum was more similar to the profile of root challenged with *G. abietina* than to shoot response to *H. annosum* (see also Fig. 6).

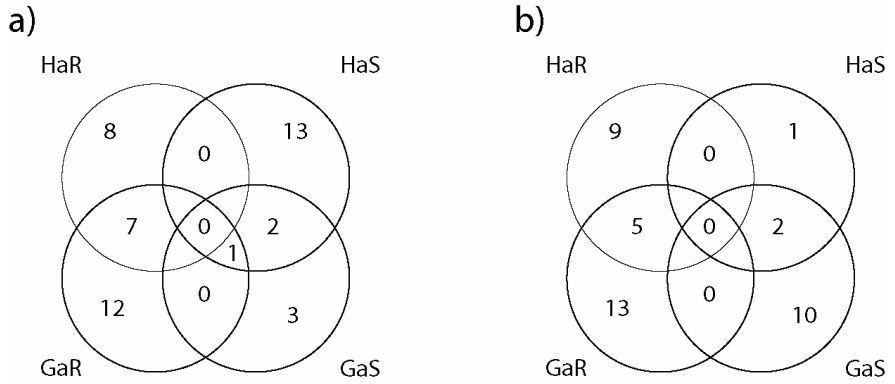

Fig. 6. Venn diagrams illustrating the number of genes a) up- or b) down-regulated by *P. sylvestris* shoots and roots inoculated with *H. annosum* or *G. abietina* at 7 d.p.i. as compared to un-inoculated control shoots and roots (HaR-*H. annosum* infected roots, GaR-*G. abietina* infected roots, HaS-*H. annosum* infected shoots, GaS-*G. abietina* infected shoots).

Pinus sylvestris root response to challenge with root- (H. annosum) or shoot-specific (G. abietina) pathogen

Pine roots infected with either root- or shoot-specific pathogen responded by differential expression of 55 genes (Fig. 6, Table 1). There were 15 and 20 genes up-regulated after inoculation with *H. annosum* and *G. abietina*, respectively; among these seven were common for both treatments. Similarly, 15 and 18 genes were down-regulated and five out of these had decreased transcript levels in both treatments. Functional classification of genes up-regulated by the roots in response to root-specific pathogen revealed that most genes had functions related to metabolism and energy acquisition (e.g. purple acid phosphatase-like, choline-phosphate-cytidylyltransferase) and defence (e.g. Avr9/Cf-9 rapidly elicited protein, harpin-induced protein) (Table 1). Few genes down-regulated as a result of *H. annosum* infection were responsible for metabolism (e.g. glyceraldehyde-3-phosphate dehydrogenase and cytochrome B5 reductase). Among Scots pine genes with increased transcript levels after *G. abietina* challenge most were functionally related to defence (e.g. cytosolic ascorbate peroxidase, NBS/LRR, putative intracellular pathogenesis related protein) and transcription and translation (e.g. auxin induced transcription factor, ribosomal protein). Inoculation of pine roots with shoot-specific pathogen, *G. abietina,* led to down-regulation of genes related to metabolism and energy acquisition (e.g. ATP synthase C-chain, ubiquinol-cytochrome c reductase-like protein) and transcription and translation (e.g. maturase K, ribosomal protein). Interestingly, three out of seven genes commonly up-regulated in response to both pathogens were defence related: antimicrobial peptide, cationic peroxidise and basic blue protein.

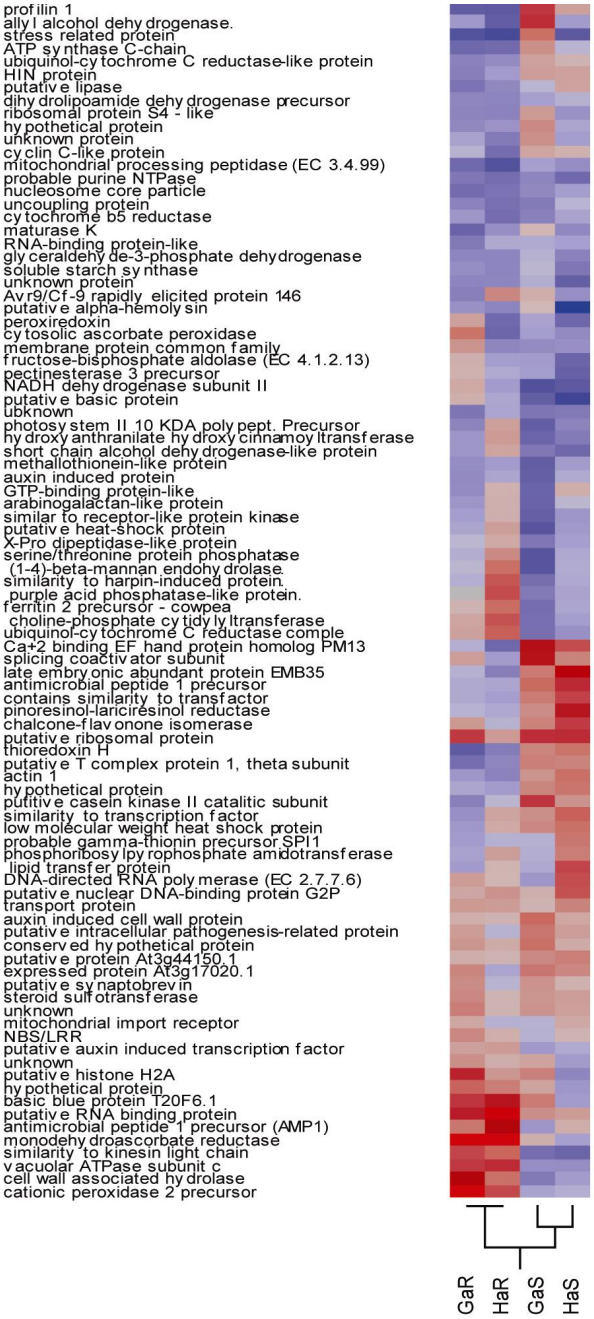

Fig. 7. Hierarchical clustering showing coordinated expression of genes differentially regulated by *P. sylvestris* shoots and roots inoculated with *H. annosum* or *G. abietina* at 7 d.p.i. as compared to un-inoculated control shoots and roots (red-blue: up- and down-regulation, HaR-*H. annosum* infected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

Pinus sylvestris shoot response to challenge with shoot- (G. abietina) or root-specific (H. annosum) pathogen

Inoculation of pine shoots with either *G. abietina* or *H. annosum* led to differential expression of 42 genes (Fig. 6, Table 1). There were six and sixteen genes up-regulated in response to *G. abietina* and *H. annosum,* respectively, and two of them were common for both pathogens. Similarly, there were twelve and three genes down-regulated and two were overlapping. Functional classification of the genes up-regulated by the shoot after infection with shoot-specific pathogen, *G. abietina,* showed that most were involved in transcription and translation (splicing coactivator subunit, containing similarity to transfactor and ribosomal protein). Genes down-regulated in response to *G. abietina* belonged mostly to functional groups of metabolism and energy acquisition (e.g. NADH dehydrogenase subunit II and (1-4)-beta-mannan endohydrolase). Inoculating the pine shoot with root-specific pathogen, *H. annosum,* resulted in up-regulation of genes with functions related to transcription and translation (e.g. DNA-directed RNA polymerase, containing similarity to transfactor) and defence (e.g. antimicrobial peptide, chalcone-flavonone isomerase). Seedlings challenged with *H. annosum* had decreased transcript levels of mostly genes responsible for metabolism (fructose-bisphosphate aldolase, NADH dehydrogenase and alcohol dehydrogenase-like protein).

Expression profile of selected genes by virtual northern blot analysis

Virtual northern blot analysis verified expression levels of a range of selected genes indicated by the macroarray to be differentially expressed. The pine shoot expression profile was validated for six genes: antimicrobial peptide, late embryogenic abundant protein, protein with similarity to transfactor, serine/threonine protein phosphatase and nuclear DNA binding protein were confirmed to be up-regulated after challenge with *H. annosum* and auxin induced cell wall protein had increased transcript level in needles infected with *G. abietina* (Fig. 8). The root expression profile was verified for 13 genes (Fig. 9). Antimicrobial peptide, monodehydroascorbate reductase and cationic peroxidase were corroborated to have elevated transcript levels in roots infected with both pathogens. *Heterobasidion annosum* infection led to up-regulation of ubiquinol-cytochrome c reductase, choline-phosphate-cytidylyltransferase and purple acid phosphatase. Similarly, inoculating pine roots with *G. abietina* resulted in up-regulation of ascorbate peroxidase, cell wall associated hydrolase, basic blue protein, putative auxin induced transcription factor and vacuolar ATPase. Two genes (intracellular pathogenesis related and vacuolar ATPase) indicated by the macroarray analysis as up-regulated in the roots infected with either *G. abietina* or *H. annosum* were shown not to be differentially expressed and two others (basic blue protein and late embryogenic abundant protein) had opposite regulation.

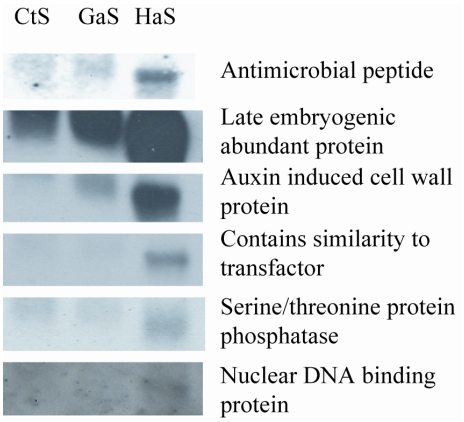

Fig. 8. Virtual northern blot verified expression level of selected genes differentially regulated by *P. sylvestris* shoots challenged with either *H. annosum* (HaS) or *G. abietina* (GaS) at 7 d.p.i. as compared to un-inoculated control (CtS).

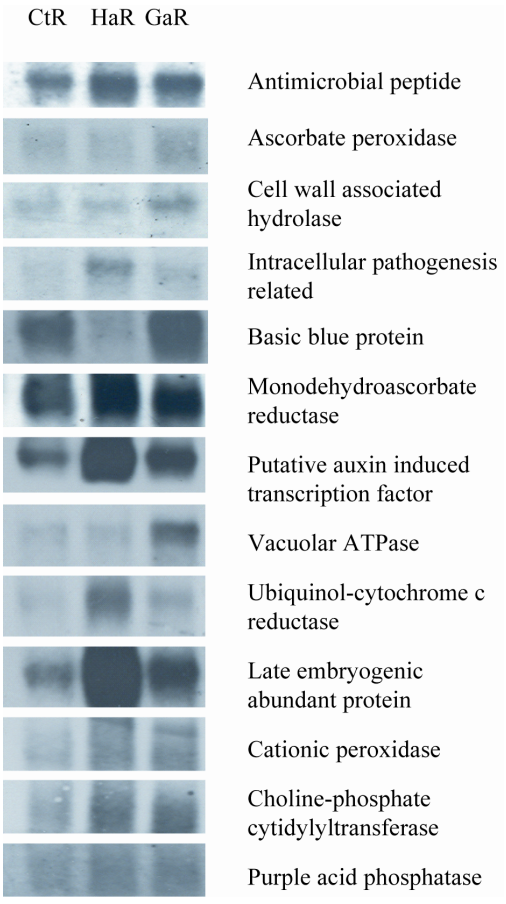

Fig. 9. Virtual northern blot verified expression level of selected genes differentially regulated by *P. sylvestris* roots challenged with either *H. annosum* (HaR) or *G. abietina* (GaR) at 7 d.p.i. as compared to un-inoculated control (CtR).

Discussion

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. *Heterobasidion annosum* 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. The *P. sylvestris* seedlings-*H. annosum* pathosystem has been used before to study pine response to the necrotrophic pathogen (Asiegbu et al. 2005b; Li & Asiegbu 2004; Adomas et al., unpublished). The process of root infection in this system is well documented (Asiegbu et al. 2005a): 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.); colonization and disintegration of the vascular region take place within 9–15 days. Similarly to *H. annosum* (Li & Asiegbu 2004), *G. abietina* is capable of killing trees of all ages (Simard, Rioux & Laflamme 2001). The fungus infects pine in the spring through stomata of developing shoots, sparsely colonizes bract tissues by late summer and during winter reaches cortical region (Patton et al. 1984). The first symptoms may not appear until the following year when brown necrotic areas develop at the base of the buds and severely affected shoots die. Bunches of light green needles may develop and have been observed in this study during prolonged exposure to *G. abietina* (EPPO, Data Sheets on Quarantine Pests http://www.eppo.org/QUARANTINE/fungi/Gremmeniella_abietina/GREMAB_ds.pdf). Moreover, the presence of the fungus at the early phase of infection was confirmed in both shoot and root (Fig.5) indicating that *G. abietina* infection was achieved in our model system.

In the present study, *H. annosum* and *G. abietina* were used to study organ-specific response to fungal infection. Pine shoots and roots were challenged with shoot- and root-specific pathogen. Macro- and microscopic observations revealed that *H. annosum* was able to cause necrosis not only on the root, the organ it infects in the nature, but also on the needles. In this way, *H. annosum* could be a second example after *M. grisea* (Sesma & Osbourn 2004) of a pathogen capable of infecting not only the organ it has been typically reported to attack.

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*, namely up-regulation of genes with functions related to metabolism and energy acquisition and defence, correlates with results obtained in a microarray profiling of pine seedlings infected with the pathogen. Major shift in primary and secondary metabolism (particularly phenylpropanoid pathway) was supplemented by production of defence-related proteins with antimicrobial properties (Adomas et al., unpublished). Although defence related genes constituted one of the main functional categories up-regulated by the shoot challenged with *H. annosum,* gene expression profile of both organs infected with the same pathogen was very different (Fig. 6, Table 1). Similar situation occurred when needles or roots were infected with shoot specific

pathogen, *G. abietina.* Interestingly, defence related genes, like peroxidases were differentially expressed in the root but not in the shoot. Increase in peroxidase activity and in lignin content together with accumulation of soluble and cell wall-bound phenolics have been described as part of conifer defence against *G. abietina* (Cvikrova et al. 2006). Peroxidases have been associated with plant defence and resistance, particularly with cross-linking phenolic compounds into papillae, production of toxic compounds and with lignin and suberin synthesis (Asiegbu, Daniel & Johansson 1994; Fossdal, Sharma & Lonneborg 2003; Takahama & Oniki 2000). Formation of ligno-suberized barrier has been shown to be the major component limiting the progression of *G. abietina* (Simard et al. 2001). But the genetic basis of pine – *G. abietina* interactions is largely unknown.

Defence-related genes, like cationic peroxidase, basic blue protein and antimicrobial peptide were commonly up-regulated by roots infected with either pathogen suggesting that they belong to broad defence mechanisms. 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). Pine antimicrobial peptide has been shown to be up-regulated also in response to challenge with non-pathogenic fungi (Adomas et al., unpublished).

Surprisingly, challenging the pine root with shoot specific pathogen that did not cause any visible symptoms led to differential expression of higher number of genes as compared to infection with root specific pathogen, *H. annosum.* Pathogens able to engage in compatible interaction leading to disease often posses mechanisms suppressing host defence (Okubara & Paulitz 2005) which may result in lower number of differentially expressed genes. Genes induced in incompatible interactions with pathogens might have role in systemic response against such attacks. An example of a gene rapidly induced in incompatible interaction and showing much lower up-regulation in compatible one is rice lipid transfer protein (LTP) (Kim et al. 2006). Members of the *LTP* gene family have been also shown to have differential expression in roots, leaves, stems and flowers (Kim et al. 2006). Interestingly, in this study LTP gene was up-regulated only in pine shoot challenged with *H. annosum,* the pathogen which naturally does not infect the aerial parts of the plant. LTPs are a family of proteins capable of moving various kinds of lipid molecules (Kader 1996), responsive to environmental stresses and suggested as signal mediator in plant defence (Blein et al. 2002).

The relative similarity of 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 et al. 2003; Jansen et al. 2006; Schafer & Yoder 1994). Only a small number of defence pathways described in leaves have so far been reported in roots (Okubara & Paulitz 2005). An example of similarity of *R* gene action in different organs was given by Poch et al. (2006) who showed that functionality of resistance gene *Hero*, which controls plant root-infecting potato cyst nematode, is preserved in leaves of tomato.

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 minerals uptake. The different tasks are performed in different surrounding: roots are anchored in soil, full of microorganisms which not always are hostile. Hence, the necessity to develop recognition mechanisms that might not be required in the leaves or needles and stems. The different requirements that must be fulfilled by the roots and aerial parts of the plant are reflected in organ specific gene expression. Global analysis of gene expression in *Arabidopsis* showed that only 40% of genes had constitutive expression in all organs and the remaining ones had usually narrow organ specificity (Obayashi et al. 2004). It is possible that only a limited set of genes and pathways are recruited in roots (Okubara & Paulitz 2005).

Technically, macroarray proved to be a useful method to elucidate pine response to challenge with organ specific pathogens. Good reproducibility of the cDNA macroarray differential screening has been shown before (Li, Osborne & Asiegbu 2006; Obayashi et al. 2004). In our study, virtual northern blot analysis essentially confirmed the results obtained with the macroarray screening. The very few exceptions could be attributed to the fact that the whole bacterial colonies that were transferred on the nylon membranes and used for the hybridisations were characterised by variable growth rate. As a result spots representing different cDNAs could have different diameter within and between the membranes, despite the efforts made to achieve uniformity in the bacteria colony size. The same reason could account for low fold changes detected in the study. In the analysis by Li at al. (2006) where plasmid DNA was blotted onto the membranes, much higher rates of up- and down-regulation in gene expression were detected. A total of 35% of the cDNAs on the macroarray originated from *P. taeda* cDNA libraries. 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.

In conclusion, by using a macroarray approach we profiled pine reaction to infection with either root (*H. annosum*) or shoot specific (*G. abietina*) pathogen. It was shown that the pine tissues response was more organ-specific than pathogen-specific, i.e. transcript profile of root infected with *H. annosum* was more similar to the profile of root challenged with *G. abietina* than to shoot response to *H. annosum.* Future efforts would focus on verifying the obtained results using field samples from older trees and monitoring not only the gene expression level but also anatomical and morphological reaction of the conifer tissues to pathogen infection.

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Tables

Table 1. Functional classification of genes differentially expressed by *P. sylvestris* roots and shoots challenged with *H. annosum* (Ha) or *G. abietina* (Ga) at 7 d.p.i. as compared to un-inoculated control roots and shoots.[a]

Gene ID[b]	Putative function	Root		Shoot	
		Ha	Ga	Ga	Ha
	Cell cycle and DNA processing				
CA305452	putative histone H2A		2,3		
CA305502	nucleosome core particle, X-ray structure of chain F		-2,0		
CA305539	cyclin C-like protein	-1,6			
NXCI_057_B04	putative nuclear DNA-binding protein G2P				1,4
ST 37 H04	HIN protein		-1,5		
	Cell structure				
CA305317	actin 1				1,3
CA305544	membrane protein common family	-1,3			
NXCI_029_H07	similarity to kinesin light chain		2,1	-1,3	-1,3
NXCI_053_H01	arabinogalactan-like protein			-1,4	
ST 24 A05	profilin 1		-2,9		
	Defence				
CA305451	basic blue protein	2,0	2,2		
CA305534	NBS/LRR		1,6		
CA305546	cytosolic ascorbate peroxidase		1,8		
CK909759	peroxiredoxin	-1,7			
CK928055	putative intracellular pathogenesis-related protein		1,4	1,3	
CK928060	antimicrobial peptide 1 precursor (AMP1)	2,1	1,8		

CK928086	Avr9/Cf-9 rapidly elicited protein 146	1,4	-1,5		
EH628377	cationic peroxidase 2 precursor	1,8	2,8		
NXCI_008_G03	similarity to harpin-induced protein.	1,7			
NXCI_062_B10	probable gamma-thionin precursor SPI1				1,3
ST 04 C10	antimicrobial peptide 1 precursor				1,5
ST 13 H03	methallothionein-like protein			-1,5	
	Development				
CA305590	pectinesterase 3 precursor				-1,3
CK909801	cell wall associated hydrolase		2,5		
	Metabolism				
CA305404	glyceraldehyde-3-phosphate dehydrogenase	-1,3	-1,4		
CA305553	hydroxyanthranilate hydroxycinnamoyltransferase			-1,4	
CA305564	short chain alcohol dehydrogenase-like protein			-1,3	-1,3
CK909763	soluble starch synthase		-1,3		
CK909796	ubiquinol-cytochrome C reductase-like protein	-1,3	-1,5		
CK928008	steroid sulfotransferase		1,6		
CK928038	X-Pro dipeptidase-like protein			-1,3	
EH628382	dihydrolipoamide dehydrogenase precursor	-1,3			
NXCI_002_B01	(1-4)-beta-mannan endohydrolase.	1,6		-1,6	
NXCI_005_G03	purple acid phosphatase-like protein.	1,8		-1,3	
NXCI_005_H04	choline-phosphate cytidylyltransferase	1,8		-1,3	
NXCI_006_F01	ubiquinol-cytochrome C reductase comple	1,7			
NXCI_017_C07	ferritin 2 precursor - cowpea	1,7			
NXCI_018_F10	pinoresinol-lariciresinol reductase				1,5
NXCI_026_G09	fructose-bisphosphate aldolase (EC 4.1.2.13)				-1,3
NXCI_027_E09	allyl alcohol dehydrogenase.	-2,1			

NXCI_038_A01	phosphoribosylpyrophosphate amidotransferase				1,3
ST 02 G06	cytochrome b5 reductase	-1,6			
ST 07 H08	chalcone-flavonone isomerase				1,4
ST 14 D02	NADH dehydrogenase subunit II			-1,6	-1,4
ST 19 F11	putative lipase		-1,8		
ST 26 D05	ATP synthase C-chain		-2,1		
ST 40 A08	thioredoxin H		-3,1		
	Photosynthesis				
ST 37 C11	photosystem II 10 kDa			-1,5	
	Protein Fate				
NXCI_057_E04	putative heat-shock protein			-1,6	
ST 24 H10	mitochondrial processing peptidase (EC 3.4.99)	-2,6			
ST 40 F04	low molecular weight heat shock protein				1,3
	Signalling				
NXCI_054_C06	GTP-binding protein-like			-1,4	
NXCI_057_E03	serine/threonine protein phosphatase			-1,6	
ST 09 C03	Ca+2 binding EF hand protein homolog PM13	-1,7			
ST 38 B04	putitive casein kinase II catalitic subunit			1,5	
	Stress response				
CA305445	monodehydroascorbate reductase	2,4	3,0		
CA305476	similar to receptor-like protein kinase			-1,4	
CK927826	late embryonic abundant protein EMB35	-1,4			1,6
ST 24 B06	stress related protein		-4,6		
	Transcription and translation				
CA305460	putative auxin induced transcription factor	1,3	1,4		
CK909850	ribosomal protein S4 - like	-1,4	-1,4		

CK927821	RNA-binding protein-like		-1,6		
CK928085	putative RNA binding protein	2,3	2,3		
CK928121	putative ribosomal protein		2,2	1,5	1,5
EH628391	contains similarity to transfactor			1,3	1,4
NXCI_049_F02	DNA-directed RNA polymerase (EC 2.7.7.6)				1,4
NXCI_054_E06	similarity to transcription factor				1,4
ST 12 G02	splicing coactivator subunit			1,5	
ST 37 H10	maturase K		-2,3		
	Transport				
CA305494	uncoupling protein	-1,6	-1,4		
CA305565	vacuolar ATPase subunit c	1,9	2,2		
CK928002	mitochondrial import receptor subunit		1,3		
CK928014	transport protein	1,3			
NXCI_054_D12	lipid transfer protein				1,4
	Unknown				
CA305321	hypothetical protein				1,3
CA305346	unknown protein	-1,5			
CA305443	hypothetical protein		2,0		
CA305461	auxin induced protein			-1,5	
CA305583	unknown protein				-1,3
CK909805	unknown			-1,3	
CK909880	hypothetical protein		-1,4		
CK909919	unknown		1,7		
CK927820	putative basic protein			-1,5	-1,6
CK927853	auxin induced cell wall protein			1,4	
CK927889	expressed protein		1,6		

CK927895	conserved hypothetical protein			1,3	
CK927959	putative alpha-hemolysin				-2,1
CK928023	putative synaptobrevin		1,6		
CK928047	putative protein At3g44150.1				1,3
CK928056	unknown		1,5		
ST 07 D02	probable purine NTPase	-1,6	-1,6		
ST 37 G12	putative T complex protein 1, theta subunit		-1,9		

[a] A gene was considered to be differentially expressed if the fold change was ≥1.3 or ≤-1.3 and p≤0.05
[b] Sequences of the ESTs are available in the GenBank or in the database at http://biodata.ccgb.umn.edu/

Supplementary material

Fig. S1. Experimental design.

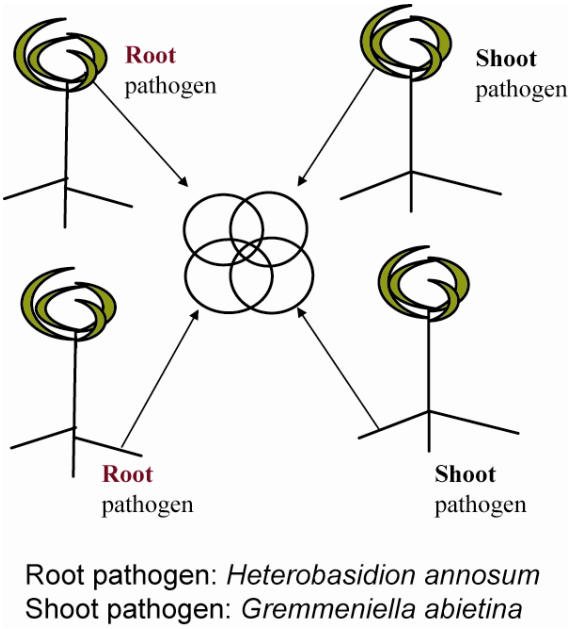

Table S1. Raw intensity values after background subtraction used for analysis of *P. sylvestris* response to infection with shoot- (*G. abietina*) or root-specific (*H. annosum*) pathogen at 7 d.p.i.

Table S1 is available upon request in electronic form.