Identification and analysis of differentially expressed cDNAs during nonself-competitive interaction between *Phlebiopsis gigantea* and *Heterobasidion parviporum*

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Abstract

The molecular factors regulating interspecific interaction between the saprotrophic biocontrol fungus *Phlebiopsis gigantea* and the conifer pathogen *Heterobasidion parviporum* were investigated. We constructed cDNA libraries and used expressed sequence tag analysis for the identification and characterization of genes expressed during the self and nonself-hyphal interaction. cDNA clones from either the pathogen or biocontrol agent were arrayed on nylon membrane filters and differentially screened with cDNA probes made from mycelia forming the barrage zone during nonself-interactions, mycelia growing outside the barrage zones or monocultures. BlastX analysis of the differentially expressed clones led to the identification of genes with diverse functions, including those with potential as virulence factors, such as hydrophobins. Because of the high sequence conservation ($r^2 = 0.81$) between *P. gigantea* and *H. parviporum*, a selected number of genes from either fungus were used to monitor the expression profile under varying interaction conditions by virtual northern blot. The results are discussed with respect to the potential role of the induced genes during the nonself-competitive interaction for space and nutrients between *P. gigantea* and *H. parviporum*.

Introduction

The taxa within *Heterobasidion* species complex are the main cause of root and butt rot in coniferous forests in the northern hemisphere (Asiegbu *et al.*, 2005). In Europe, the species complex consists of three species, previously known as intersterility groups P, S and F, which differ in their host preferences. *Heterobasidion annosum sensu stricto* (Fr.) Bref. (P-type) mainly attacks pine trees, *Heterobasidion parviporum* Niemelä & Korhonen (S-type) primarily infects spruce and *Heterobasidion abietinum* Niemelä & Korhonen (F-type) attacks mainly fir species (Niemelä & Korhonen, 1998). In North America, S- and P-types have not been given new scientific names. The fungi infect fresh stump surfaces by means of aerial basidiospores released mainly during the summer period (Stenlid, 1985).

Protection measures against *Heterobasidion* spp. have been focused on preventing germination and growth of the fungi after basidiospore deposition. Over the last 50 years, a large number of commercially produced compounds have been tested as stump protectants. Of these, urea and borates have been shown to be the most effective against *Heterobasidion* spp. and are used commercially (Pratt & Lloyd, 1996; Johansson *et al.*, 2002). In spite of their effectiveness, there have been increased objections to the use of commercially produced compounds, for both practical and environmental reasons (Westlund & Nohrstedt, 2000). Biocontrol has been proposed as an alternative to the chemical control and a number of fungal species (*Phlebiopsis gigantea*, *Bjerkandera adusta*, *Fomitopsis pinicola*, *Resinicium bicolor*, *Hypholoma* spp., *Trichoderma* spp., *Scytalidium* spp.) have been tested on the stump as competitors and antagonists against *Heterobasidion* spp. (Holdenrieder & Grieg, 1998). Among these, only *P. gigantea* (Fr.) Jül is currently used and has shown very good results (Holdenrieder & Grieg, 1998).

Phlebiopsis gigantea is common on conifer stumps in boreal and temperate forests. As a primary colonizer of conifer logs, it gradually causes a significant decay on such material. Rishbeth (1952) in Great Britain was the first to discover that *P. gigantea* was able to replace *Heterobasidion* spp. on conifer (pine) stumps and proposed the use of the fungus as a biocontrol agent. Korhonen (1993) isolated a heterokaryotic strain of *P. gigantea*, which is now commercially produced and marketed in Scandinavia, as a

biocontrol product (Rotstop®, manufactured by Verdera Oy, Espoo, Finland) for control of *Heterobasidion* spp. in both spruce and pine stumps. *Phlebiopsis gigantea* is also commercially available in Poland and Great Britain as PG IBL® (Biofood s.c., Walcz, Poland) and PG Suspension® (Forest Research, Surrey, UK), respectively.

However, almost nothing is known about the physiological and molecular basis for the interspecific interaction between *P. gigantea* and *Heterobasidion* spp. Several modes of action of biocontrol agents are documented in the literature and these include antibiosis (Raaijmakers *et al*., 2002), production of lytic enzymes (Viterbo *et al*., 2002), parasitism (Benhamou & Chet, 1997), induced resistance (Kosaka *et al*., 2001) and competition for space and nutrients (Poppe *et al*., 2003). At present, there is no evidence of either antibiotics or toxins being secreted by *P. gigantea* (Holdenrieder & Grieg, 1998). Paired growth of *P. gigantea* and *Heterobasidion* spp. on the same agar medium results in confluence and overgrowth by *P. gigantea* without formation of a growth inhibition zone (Kallio, 1971). Ikediugwu *et al*. (1970) and Ikediugwu (1976) found that *P. gigantea* hyphae changed the structure of adjacent *H. annosum* hyphae: penetration, granulation and vacuolation of the cytoplasm and loss of opacity were observed. A similar observation was made by Behrendt & Blanchette (2001) in trials of biocontrol of blue stain fungi in pulpwood by *P. gigantea*. This phenomenon, called hyphal interference, usually a consequence of exploitation of resources or parasitism, is not uncommon in interspecific antagonistic fungal interactions (Cooke & Rayner, 1984; Rayner & Boddy, 1988; Boddy, 2000) but its precise mechanism has not been elucidated (Whipps, 2001). Such interspecific antagonistic interactions are one of the major factors determining fungal community development and functioning and are known to have a potential in biocontrol (Boddy, 2000). Generally, antagonistic microbial agents used in biocontrol do not give any guarantee for sustainable efficacy and longevity; an appreciation of the underlying mechanism for such nonself-interaction provides a good starting point for understanding and using them more intelligently and effectively.

In the present study, a genomic approach was applied to identify genes differentially expressed during the interaction of *P. gigantea* and *H. parviporum*. Genomics enables analysis of the expression level of numerous genes in a single experiment (for a review, see Yoder & Turgeon, 2001; Colebatch *et al*., 2002) and has been applied recently for studying fungus–plant interactions involving pathogens like *Magnaporthe grisea* (Lee & Dean, 1993), *Colletotrichum lagenarium* (Inagaki *et al*., 2000) and *H. annosum* (Karlsson *et al*., 2003; Abu *et al*., 2004). We report the use of cDNA macroarrays for differential screening and identification of genes which are expressed during nonself-competitive interaction between *P. gigantea* and *H. parviporum.*

Materials and methods

Fungal species and maintenance of cultures

Heterobasidion parviporum (FS6: S-type) and *Phlebiopsis gigantea* were obtained from K. Korhonen (Finnish Forest Research Institute, Finland) and maintained on Hagem agar medium (Stenlid, 1985) at 18 ± 1 °C.

Pathogen–biocontrol agent interaction study: experimental model and sampling

An experimental model (Figs 1 and 2a) consisting of dual cultures grown at 18 ± 1 °C on Hagem agar was used to examine pathogen–biocontrol agent interaction. Prior to inoculation of the fungal species, the agar surface was overlaid with a cellophane membrane to facilitate harvesting of the mycelia. The cellophane membrane was cut to the size of a 90 mm diameter Petri dish, washed with distilled water and autoclaved for 20 min. Agar plugs of actively growing cultures of either *P. gigantea* or *H. parviporum* were inoculated as paired cultures on agar plates at a distance of 30 mm from each other. Total RNA was extracted following the procedures described by Chang *et al.* (1993) from the barrage zones, mycelia outside the barrage zones or pure cultures during the self- or nonself-interactions (Fig. 1) at different periods ranging from 5 to 28 days. To avoid collecting contaminating hyphae from either fungus, no sample was taken outside the barrage zone at 28 days postinoculation (dpi) due to increased potential for overgrowth (see Fig. 2e, f). The cDNA was synthesized from the same amount of RNA (1 μg) using SMART™ PCR cDNA synthesis kit (Clontech, Franklin Lakes, NJ).

Construction and storage of *Phlebiopsis gigantea* (GIGA) and *Heterobasidion parviporum* (HAGS) cDNA libraries

Phlebiopsis gigantea was grown in liquid Hagem medium at 18 ± 1 °C in static conditions. After 14 days the total RNA was extracted (Chang *et al*., 1993). The cDNA library was constructed using the Creator™ SMART™ cDNA Library Construction kit (Clontech). The library (GIGA) consisted of 2636 clones. The clones were stored in 96-well plates at –80 °C. The cDNA library for *H. parviporum*-germinated spores (HAGS) was similarly constructed and the recombinant cDNA clones (3072) stored in 384-well microtitre plates, as previously described (Abu *et al*., 2004).

Macroarray construction of *Phlebiopsis gigantea* (GIGA) and *Heterobasidion parviporum* (HAGS) cDNA libraries and the subset of 94 selected HAGS cDNA clones

A total of 716 clones randomly selected from the GIGA cDNA library were manually transferred onto Hybond® N^+

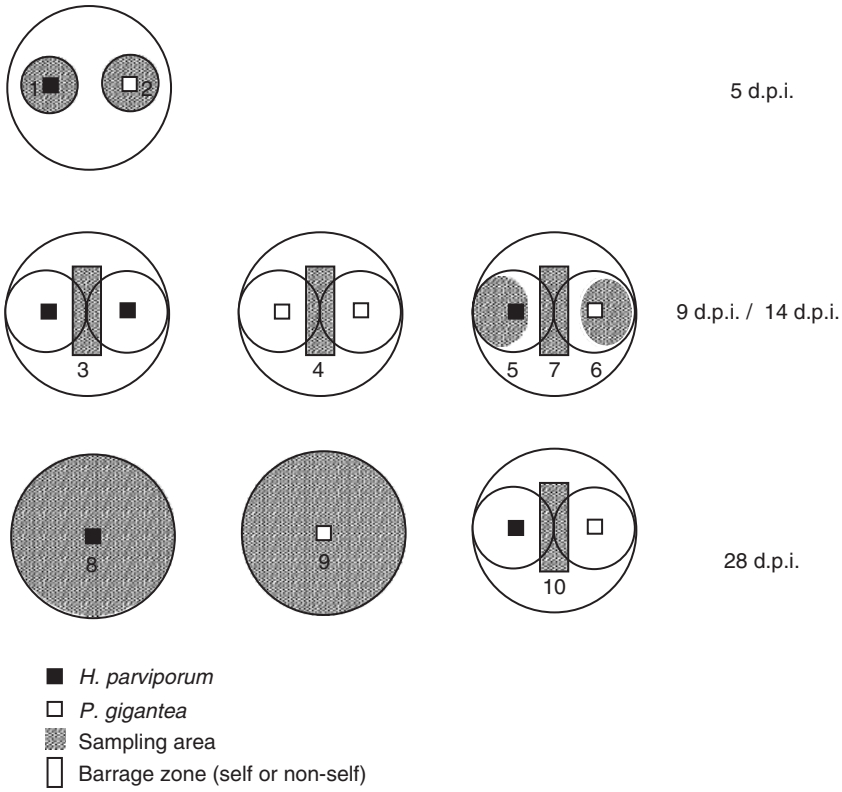

Fig. 1. Schematic illustration of the experimental design for sample collection used either for macroarray differential screening or for virtual northern blot: *Heterobasidion parviporum* (■), *Phlebiopsis gigantea* (□), sampling area (▩), contact (self-) or barrage zone (non-self-interaction) (□). Descriptions of numbers: 1, precontact interacting *H. parviporum* (Hp) hyphae [5 dpi (days postinoculation)]; 2, precontact interacting *P. gigantea* (Pg) hyphae (5 dpi); 3, hyphae contact zone for self-interaction of *H. parviporum* (Hp–Hp) (9 dpi); 4, hyphae contact zone for self-interaction of *P. gigantea* (Pg–Pg) (9 dpi); 5, *H. parviporum* domain outside the barrage zone during non-self-interaction with *P. gigantea* (9 or 14 dpi); 6, *P. gigantea* domain outside the barrage zone during nonself-interaction with *H. parviporum* (9 or 14 dpi), 7, barrage zone during nonself-interaction of *P. gigantea* and *H. parviporum* at either 9 or 14 dpi; 8, monoculture of *H. parviporum* (28 dpi), 9, monoculture of *P. gigantea* (28 dpi); 10, barrage zone for non-self-interaction of *P. gigantea* and *H. parviporum* (28 dpi).

nylon membranes (Amersham Pharmacia Biotech, Bucks, UK) with the aid of a 96-well replicator. After 18 h of incubation on Luria Broth–chloramphenicol medium, the membranes were placed for 7 min on Whatman paper presoaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl). They were then transferred to another Whatman paper saturated with neutralizing solution (1.5 M NaCl, Tris-HCl) for 7 min and air dried for 2 h. The membranes were later transferred to a Whatman filter paper saturated with alkali fixation buffer (0.4 M NaOH) for 20 min and then to another Whatman filter paper saturated with 5 × SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7) for 7 min. After overnight drying, the membranes were stored at room temperature until used. A total of 3072 clones from the *H. parviporum* cDNA library (HAGS) were replicated onto Hybond N^+ membranes (Amersham Pharmacia Biotech) using a Q-bot automated workstation (Genetix, Boston, MA) (Abu *et al.*, 2004). A subset of 94 clones selected from the HAGS cDNA library, mostly of known identity (see supplementary Table S1a, b), was also transferred manually onto Hybond N^+ membranes.

Pilot study

A pilot study was conducted to investigate the interaction of *P. gigantea* and *H. parviporum* in varying environmental conditions and time-dependent changes in the interaction pattern. The dual cultures and monocultures of *P. gigantea* were grown on different agar media: Hagem or Norkrans (1963) containing sawdust as sole carbon source. The mycelial structures in the barrage zone were collected 14 and 28 dpi (Fig. 1). Total RNA was extracted and cDNA synthesized as described above (*Macroarray construction*) and was used for differential screening of a subset of 140 *P. gigantea* cDNA clones. The labelling and hybridization were done using AlkPhos® Direct Labelling kit (Amersham Pharmacia Biotech). Signal generation and detection were done with CDP-Star (Amersham Pharmacia Biotech). The interaction of *H. parviporum* and *P. gigantea* on a sterile spruce wood disc was also studied (Fig. 2a–f).

Differential screening of macroarrays of *Phlebiopsis gigantea* (GIGA) and *Heterobasidion parviporum* (HAGS) cDNA libraries and the subset of 94 selected HAGS cDNA clones

The biological samples (see Fig. 1) designated as 5, 6 and 7 at 14 dpi as well as 8, 9 and 10 at 28 dpi were used for differential screening of macroarrays as described above. The arrays containing 716 *P. gigantea* (GIGA) cDNA library clones were screened with probes made from monoculture or interacting *P. gigantea* as well as barrage zones. The arrays

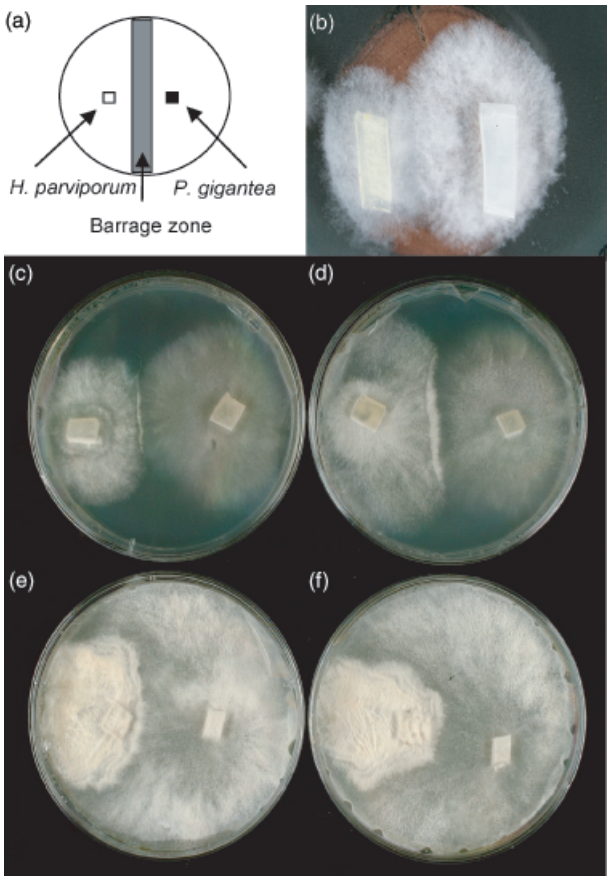

Fig. 2. (a) Experimental model designed to investigate the interaction between *Heterobasidion parviporum* and *Phlebiopsis gigantea* consisting of dual cultures grown at 18 ± 1 °C; the interaction pattern of *P. gigantea* (right) and *H. parviporum* (left) grown in dual cultures (b) on spruce disk (c) and on Hagem at 18 °C: the initial contact between both species occurring at 5–7 dpi (d), formation of barrage zone at 9–14 dpi (e), overgrowth of H. parviporum by *P. gigantea* at 30–40 dpi (f).

containing 3072 *H. parviporum* genes (HAGS) (Abu *et al.*, 2004) were used to identify the pathogen genes using probes made from pure or interacting *H. parviporum* mycelia and barrage zones. There were three biological replications for each array screening. To assay for the extent and degree of sequence conservation between *P. gigantea* and *H. parviporum* genes, labelled cDNA probes made from RNA extracted from mycelia of either fungus pre-grown on Hagem liquid medium were used to screen a macroarray containing a subset of 94 selected cDNA clones from the *H. parviporum* cDNA library. There were three biological and two technical replicates in this analysis.

Virtual northern blot analysis

To examine the expression pattern of a number of selected genes, virtual northern blot was done according to the manufacturer's instructions (Clontech) and earlier published methods (Franz *et al.*, 1999; Hammerle *et al.*, 2003). The genes from the GIGA (fructose bisphosphate aldolase – CV632240, glutamine synthetase – CV632209, cyclophilin – CV632234, hydrophobin 1 – CV632212, hydrophobin 2 – CV632371, hydrophobin 3 – CV632331, glyceraldehyde-3-phosphate dehydrogenase – CV632224) and HAGS library (phosphoglucomutase – CK817377, arginase – CK92772, cytochrome P450 monooxygenase – BU672561, transcript antisense to ribosomal RNA – CK817421) and *H. annosum* hydrophobin (08C04) (kindly provided by Magnus Karlsson, Uppsala University, Sweden) were selected because of their functional relevance, abundance in the cDNA library or for significant expression level during nonself-interaction. Equal amounts of total RNA (1 μg) extracted from varying interaction conditions (see Fig. 1) were used for SMART™ PCR cDNA synthesis. The cDNA (0.5 μg) was separated on an agarose gel, denatured and subsequently blotted onto nylon membrane. For preparation of the DNA probes, GIGA or HAGS plasmids of interest were extracted with QIAprep® Spin Miniprep kit (Qiagen). The inserts were amplified using the following PCR reaction conditions: 50 ng of plasmid DNA, M13 forward and reverse primers and Advantage 2 Synthesis kit reagents (BD Biosciences, Bedford, MA) were mixed and run for 25–30 cycles in a thermal cycler using the program: 94 °C, 30 s; 68 °C, 2 min. The PCR products were electrophoresized on a 1.1% agarose gel and purified with QIAquick® Gel Extraction kit (Qiagen, Courtaboeuf, France). Hybridization was performed with aid of AlkPhos® Direct Labelling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Signal generation and detection were done with CDP-Star (Amersham Pharmacia Biotech). Virtual northern blot analysis was done on all samples taken at 5, 9 and 28 days (Fig. 1).

Sequencing of cDNA clones from the GIGA library and sequence analyses

A total of 191 clones from the *P. gigantea* (GIGA) cDNA library were randomly selected for the sequencing. For plasmid DNA isolation, clones were grown overnight at 37 °C in a 96-well plate, each well containing 2 mL of Terrific broth medium (Zhu *et al.*, 1997) and 35 μg mL^{-1} chloramphenicol (Sigma, St Louis, MO). The sequencing of plasmid DNA was done with DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (Art. Nr. US81090) and performed on the MegaBACE 1000 sequencing machine (Amersham Biosciences). For sequencing reactions, plasmids, primers and sequence kit reagents were mixed according to the manufacturer's instructions and run for 40 cycles in a thermal cycler using the following program: 95 °C, 20 s; 50 °C, 15 s; 60 °C, 90 s. The PCR

products were precipitated with ethanol and ammonium acetate, dried and dissolved in a MegaBACE loading solution and injected into the MegaBACE 1000 for sequencing. After deleting vector and contaminating sequence, the obtained cDNA sequences were compared with GenBank database sequences using BlastX. Phylogenetic analysis was done with the CLUSTALW Method (DNASTAR, MegAlign™ expert sequence analysis software, version 5.05) using data obtained from this study and from GenBank.

Data collection and statistical analysis

Signal intensity data were evaluated with Quantity One program, version 4.4.0 (Bio-Rad; www.biorad.com). To remove systematic sources of variation, a normalization procedure mimicking print-tip normalization (commonly used when normalizing microarrays) was used. The assumption behind print-tip normalization is that systematic variations in the expression data of a particular gene are correlated with the gene being printed within a certain print-tip group at a certain location on the microarray (Yang *et al.*, 2001). Here, 716 GIGA cDNA clones were printed on eight macroarrays and there were three biological replications for each array screening. Each of the total 48 macroarrays was regarded as a 'print-tip group' and thus individually normalized, using *z*-score normalization, $Zn = [\log2(Xn+1) - \mu]/\sigma$ (van Zyl *et al.*, 2002). The normalized expression values were concatenated to form a gene expression matrix containing normalized expression measurements for all clones and replicates as well as both differential screenings. A parametric empirical Bayes approach (Lönnstedt & Speed, 2002) was used to identify differentially expressed genes. The normalized data for 94 clones used to ascertain the extent and degree of sequence conservation between *P. gigantea* and *H. parviporum* were also statistically analyzed using the Bayes approach (Lönnstedt & Speed, 2002). For each replicate, the background, corrected intensity values and estimates of gene expression for each gene were adjusted so that the lowest intensity for any gene was one. To remove systematic sources of variation, quantile-normalization was used as outlined in Bolstad *et al.* (2003). Technical replicates were treated with a pooled correlation method (Wettenhall & Smyth, 2004; Smyth *et al.*, 2005). The *P*-value was fixed at 0.01. Because of the pattern in which the 3072 HAGS cDNA clones were printed on the high-density nylon filter by the Q-bot, it was not possible to quantify signal intensity on those membranes; as a result, no statistical analysis was applied to the data.

GIGA sequences deposition

A total of 191 sequences were submitted to the GenBank with the following accession numbers CV632203 – CV632393. The additional expressed sequence tags (ESTs) from the HAGS cDNA library used for virtual northern analysis (see above) have also been deposited to the GenBank. Similarly, *P. gigantea* hydrophobin genes (*Pgh 1* and *Pgh 2*) have been deposited in the GenBank with accession numbers AY822597 and AY822598, respectively.

Results

Interaction pattern

The interaction between *Heterobasidion parviporum* and *Phlebiopsis gigantea* in dual cultures was characterized by development of a barrage zone consisting of dense mycelia formed between both fungi (Fig. 2b–f). At 18 °C initial contact between growing mycelia occurred 5–7 dpi. The barrage zone was formed 9–14 dpi. The deadlock situation where neither of the fungi was capable of further growth usually switched to overgrowth of *H. parviporum* by *P. gigantea* 30–40 dpi. The timing and occurrence of the overgrowth depended on the temperature and nutrients regime. At 28 °C overgrowth occurred within 7 days after contact. Moreover, *P. gigantea* overgrew *H. parviporum* regardless of the initial inoculum size. When the study was repeated on a spruce wood disc, the natural substrate for both fungi, a similar interaction pattern (deadlock and overgrowth) was observed on the disc surface (Fig. 2b).

Construction and sequencing of the *Phlebiopsis gigantea* cDNA library (GIGA)

The *P. gigantea* cDNA library (GIGA) consisted of 2636 clones with 0.5–1.0 kb average insert size. Random sequencing and BlastX analysis of 191 sequences identified (threshhold for a sequence match set to $<e^{-5}$) genes coding for proteins belonging to the following functional categories: protein synthesis (7%), defence/virulence (5%), metabolism (7%), transport (2%), hypothetical (7%) and unknown (72%). Representative sets of ESTs belonging to diverse functional groups are presented in Table 1. Contig assembly of the 191 sequenced ESTs showed that 141 were singletons, and 47 grouped into 15 contigs (multicopy sequences). The contig with the highest number of sequences (nine) had similarity to mitochondrial protein of unknown function (transcript antisense). The next most prevalent group of ESTs (six) clustered in a contig with homology to fungal hydrophobins. Other contigs aligned closely with genes coding for glyceraldehyde-3-phosphate dehydrogenase as well as with those of unknown function. Analysis of the *H. parviporum* cDNA library (HAGS) has been described earlier (Abu *et al.*, 2004).

Pilot study on the interaction between *Heterobasidion parviporum* and *Phlebiopsis gigantea*

The pilot study using RNA extracted from *P. gigantea* monocultures and the barrage zone of dual cultures grown on either Hagem or sawdust containing agar medium revealed that about 60% of genes highly expressed in the barrage zone were overlapping in both media. The initial results obtained after screening 140 *P. gigantea* cDNA clones (GIGA) also showed that 52% of the highly expressed genes were overlapping in the barrage zones of mycelia harvested at either 14 or 28 dpi. Consequently, the self- and nonself-interaction studies were conducted for periods ranging from 5–28 days on Hagem agar medium.

Table 1. Representative expressed sequence tags (ESTs) from the *Phlebiopsis gigantea* cDNA library (GIGA) and GenBank accession numbers matching to homologues described in this paper

EST	GenBank accession number
Metabolism	
Glutamine synthetase	CV632209
Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	CV632224
Fructose-bisphosphate aldolase	CV632240
Sterol reductase	CV632300
Endo-galacturonase	CV632316
Mitochondrial protein, Tar1p (transcript antisense)	CV632287
Protein synthesis	
60S ribosomal protein L17	CV632214
Ribosomal protein	CV632393
Ubiquitin	CV632334
Defence/virulence	
Hydrophobin 1	CV632212
Hydrophobin 2	CV632371
Hydrophobin 3	CV632331
Heat shock protein 90	CV632379
Cyclophilin	CV632234
Transport	
V type ATPase (mitochondrial)	CV632327
Member of the ATP-binding transporters	CV632376

Macroarray differential screening: pathogen–biocontrol agent interaction

To identify genes expressed by each fungus during nonself-interactions, cDNA probes made from *P. gigantea* and *H. parviporum* RNA extracted either from the barrage zone, outside the barrage zone or pure cultures (see Fig. 1) were used to screen the GIGA and HAGS cDNA libraries, respectively. The normalized signal intensity values from appropriate membranes of the GIGA cDNA library were compared. The range of normalized expression values varied from 6 to 12 and had a Gaussian (normal) distribution. A total of 234 genes with strong expression were recorded at 28 dpi; of these, 57 and 49 genes were uniquely associated with pure culture of *P. gigantea* and barrage zones, respectively, and 128 genes were common to both conditions. Further, 15 ESTs with strong expression were identified when this study was repeated 14 dpi with RNA extracted from mycelia located outside the barrage zone (see Fig. 1). BlastX analysis of the genes from the GIGA cDNA library (Table 2a) with increased level expression in the barrage zone led to the identification of hydrophobin 1, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, endo-galacturonase, ATP binding transporter, pyruvate

Table 2a. Subset of genes of known identity from the *Phlebiopsis gigantea* cDNA library (GIGA) with differential expression in the barrage zone at 14 and 28 dpi

Origin/regulation	Accession number	Homology	*e* value
↓ Pg	AY822598	Hydrophobin 2 (*Tricholoma terreum*)	6e – 18
↓ Pg	CV632331	Hydrophobin 3 (*Flammulina velutipes*)	6e – 09
↓ Pg	CV632286	Mitochondrial protein, Tar1p (transcript antisense) (*Saccharomyces cerevisiae*)	9e – 18
↓ Pg	CV632339	Peptide *N*-myristoyl transferase (*Aspergillus fumigatus*)	2e – 05
↓ Pg	CV632379	Heat-shock protein 90 (*Cryptococcus bacillisporus*)	1e – 13
↓ Pg	CV632376	ATP-binding cassette (ABC) transporter (*Cryptococcus neoformans*)	8e – 20
↑ Pg*	AY822597	Hydrophobin 1 (*Tricholoma terreum*)	2e – 13
↑ Pg*	CV632226	Glyceraldehyde-3-phosphate dehydrogenase (*Phanerochaete chrysosporium*)	2e – 77
↑ Pg	CV632218	60S ribosomal protein (*Cryptococcus neoformans*)	1e – 38
↑ Pg	CV632234	Cyclophilin (*Pleurotus* sp.)	8e – 05
↑ Pg	CV632209	Glutamine synthetase (*Suillus bovinus*)	7e – 09
↑ Pg	CV632316	Endopolygalacturonase (*Chondrostereum purpureum*)	2e – 12

↓ Gene down-regulated at 28 days in the barrage zone (and up-regulated in the monoculture) (see Fig. 1).
↑ Gene up-regulated at 28 days in the barrage zone (and down-regulated in the monoculture) (see Fig. 1).
*Gene up-regulated at 14 days in the barrage zone (and down-regulated outside the barrage zone).

Table 2b. Subset of genes of known identity from the *Heterobasidion parviporum* cDNA library (HAGS) with positive hybridization to probes made from mycelium growing outside (14 dpi) and within (14 and 28 dpi) the barrage zone

Origin/library	Accession number	Homology	*e* value
Hp*	CK927627	Predicted protein (*Magnaporthe grisea*)	2e – 21
Hp†	CK927573	Succinyl-CoA synthetase (*Neocallimastix patriciarum*)	7e – 11
Hp†	CK927599	Ubiquitin (*Aspergillus fumigatus*)	2e – 29
Hp†,‡	CK817461	Similar to rRNA intron-encoded homing endonuclease (*Pan troglodytes*)	1e – 14
Hp*,†,‡	CK817421	Mitochondrial protein, Tar1p (transcript antisense) (*Saccharomyces cerevisiae*)	1e – 16
Hp*,†,‡	CK859650	CDH-1 (*Gallus gallus*)	2e – 16
Hp*,†,‡	CK927667	Unknown (*Saccharomyces cerevisiae*)	11e – 09

*Sample collected from region outside the barrage zone at 14 dpi (see Fig. 1).
†Sample collected from region within the barrage zone at 14 dpi (see Fig. 1).
‡Sample collected from region within the barrage zone at 28 dpi (see Fig. 1).

decarboxylase, cyclophilin, heat shock protein, 60S ribosomal protein and ubiquitin.

The result of the differential screening of the HAGS cDNA library with RNA extracted either from the barrage zone or pure *H. parviporum* cultures 28 dpi indicated that of 3072 clones on the nylon membrane there were 62 positive hybridizing clones. Of these, 33 clones were expressed by pure culture of *H. parviporum*, 29 in the barrage zone of *P. gigantea* and *H. parviporum*, and 16 ESTs were common. When this study was repeated using RNA extracted from outside and within the barrage zone at 14 dpi, respectively 44 and 52 positive hybridizing clones were identified. Twenty-one of these clones were common to mycelia growing outside and within the barrage zone. BlastX analysis of the sequenced clones (Table 2b) led to the identification of transcript antisense to ribosomal RNA, CDH-I (gallus), an unknown *Saccharomyces cerevisiae* protein, hypothetical protein, an un-named protein product, rRNA intron encoded homing endonuclease, succinyl CoA synthetase, ubiquitin and several other genes of unknown function.

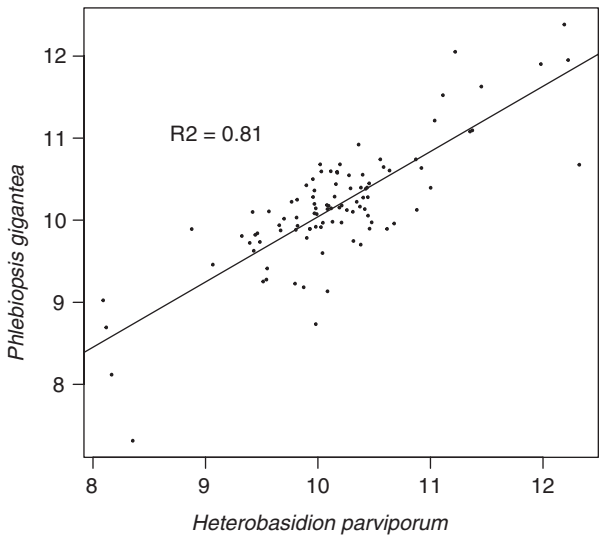

Fig. 3. The linear regression of the estimated gene expression in mycelia of *Heterobasidion parviporum* and *Phlebiopsis gigantea*. The gene expression value for every gene in *H. parviporum* has been plotted against the expression value for the same gene in *P. gigantea* Each point represents the mean of six randomized replicates of the expression of each of the 94 genes of known identity in the *H. parviporum* cDNA array estimated by hybridization of the array with labelled cDNA from pure cultures of either *P. gigantea* or *H. parviporum*.

Pairwise comparison of gene expression by *Phlebiopsis gigantea* and *Heterobasidion parviporum*

To evaluate the possibility that the genes identified within the barrage zone may have come from either fungus, we tested the level of sequence conservation between *P. gigantea* and *H. parviporum* using linear regression analysis of the normalized data. The expression values for the selected 94 HAGS genes hybridized with cDNA probes from each species (see supplementary Table S1a, b) were plotted against each other. Pearson's product-moment correlation rho was measured for *P. gigantea* vs. *H. parviporum* and the result was found to be high ($r^2 = 0.81$) (Fig. 3). The results further confirmed the potential for cross hybridization among related genes from either fungus. However, because of this high sequence conservation ($r^2 = 0.81$), genes from either fungus were used to study gene expression in the other by the virtual northern blot analysis.

Expression profile of selected genes by virtual northern blot analysis

Virtual northern blot analysis (Fig. 4a, b) verified expression levels of a range of selected genes, notably those with functions related to carbohydrate metabolism (fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglucomutase), amino acid and nitrogen metabolism (glutamine synthetase, arginase), defence and virulence (cyclophilin, hydrophobin 1, 2 and 3), stress and toxin detoxification (cytochrome P450 monooxygenase) as well as genes related to other cellular functions (transcript antisense to ribosomal RNA).

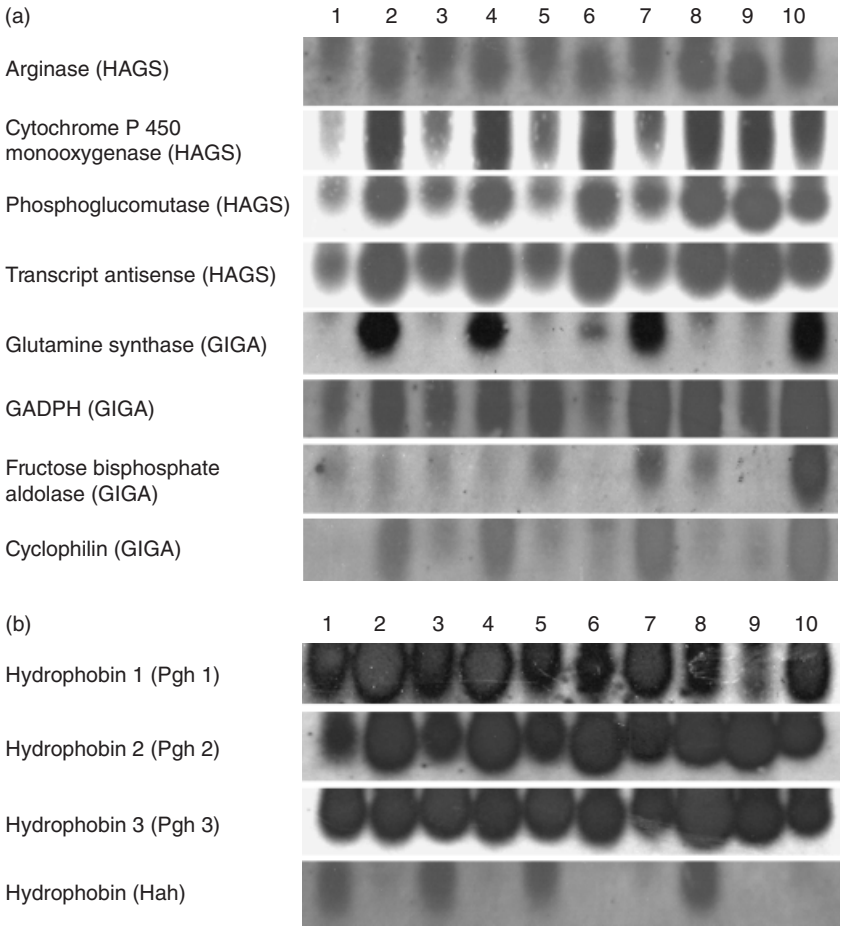

Fig. 4. Virtual northern blot verified expression level of (a) selected genes isolated from the *Heterobasidion parviporum* (HAGS) or *Phlebiopsis gigantea* (GIGA) cDNA library in self and non-self interactions of *P. gigantea* (Pg) and *H. parviporum* (Hp); For description of numbers (lanes 1–10), see the legend for Fig. 1. Numbers 5–7 refer to 9 dpi. (b) Virtual northern blot verified expression level of hydrophobins from *P. gigantea* (Pgh 1, Pgh 2 and Pgh 3) and *H. annosum* (Hah) in self and nonself-interactions of *P. gigantea* (Pg) and *H. parviporum* (H. parviporum); For description of numbers (lanes 1–10), see the legend to Fig. 1. Numbers 5–7 refer to 9 dpi.

The expression levels for fructose bisphosphate aldolase representing carbohydrate metabolism (Fig. 4a) were comparatively low in both fungi at the early stages of growth as well as during self–self-interaction. An increased expression of the gene was recorded in the barrage zones formed during nonself-interaction with stronger expression at 28 than at 9 dpi. Glyceraldehyde-3-phosphate dehydrogenase was highly expressed in both fungi and was up-regulated in the barrage zones at both 9 and 28 dpi. By contrast, high expression of the gene encoding phosphoglucomutase (Fig. 4a) was documented over time in both fungi with notable down-regulation at early (9 dpi) and late (28 dpi) stages of barrage zone formation during nonself-interaction.

Glutamine synthetase, representing genes with functions related to nitrogen and amino acid metabolism, was expressed mostly by *P. gigantea*, with increased levels observed during nonself-interaction in the barrage zones. The expression level of the gene encoding arginase was stronger at 28 dpi than at 5–9 dpi. However, the gene was slightly down-regulated in the barrage zone at 9 and 28 dpi (Fig. 4a).

Analysis of the expression profile of the genes encoding proteins related to defence and virulence revealed that cyclophilin was expressed primarily by *P. gigantea* and was up-regulated in the barrage zones. High transcript levels were recorded for cytochrome P450 monooxygenase (involved in stress tolerance and toxin detoxification) in *P. gigantea,* with observed down-regulation at both early and late stages of barrage zones formed during nonself-interactions. The expression and regulatory pattern for transcript antisense to ribosomal RNA was similar to cytochrome P450 monooxygenase (Fig. 4a), but with higher expression by *H. parviporum*. Finally, a much stronger expression level for arginase, cytochrome P450 monooxygenase, phosphoglucomutase, fructose bisphosphate aldolase, cyclophilin and glyceraldehyde-3-phosphate dehydrogenase was observed in the barrage zone at 28 dpi than at 9 dpi (Fig. 4a).

Comparison of gene expression during hyphal contact in self and nonself-interactions

Higher transcript levels of fructose bisphosphate aldolase, cyclophilin, and glyceraldehyde-3-phosphate dehydrogenase were observed in the barrage zone during nonself-inter-

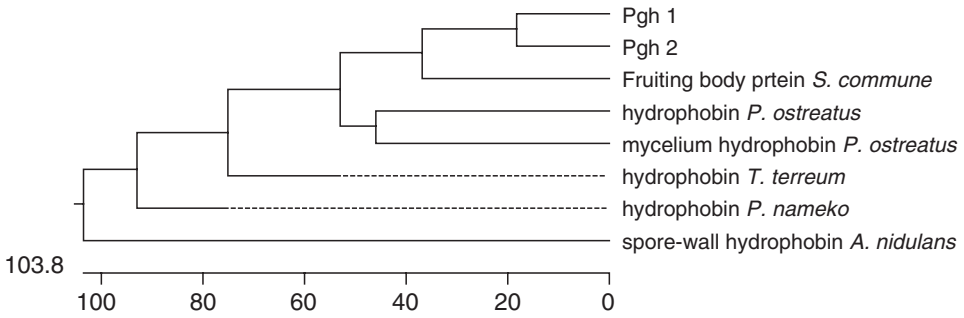

Fig. 5. A dendrogram representation of the sequence similarities between the different fungal hydrophobins. The dendrogram was produced with CLUSTALW method using data obtained from this study (Pgh 1 and Pgh 2) and from GenBank: spore-wall hydrophobin of *Aspergillus nidulans* (GenBank accession number EAA58809.1); fruit body protein of *Schizophyllum commune* (P16934); hydrophobins of *Pleurotus ostreatus* (CAB41405.1 and CAA74987.1); *Pholiota nameko* (BAB84546.1); and *Tricholoma terreum* (AAL05426.1).

action than at hyphal contact zone of self–self-interaction. On the other hand, stronger expression of cytochrome P450 mono-oxygenase, phosphoglucomutase and transcript antisense was recorded during hyphal contact in self–self-interaction of *P. gigantea* than in barrage zones of nonself-competitive interaction.

Hydrophobin gene analysis

A particular gene encoding hydrophobin, earlier identified by other authors to have a functional role as a fungal virulence factor and found in this study to be differentially expressed, was further analyzed. Bioinformatic analysis indicated that eight of the 191 sequenced ESTs had homology to fungal hydrophobins. Contig analysis showed that six of the eight hydrophobin ESTs clustered together (hydrophobin 1), while the other two were separate (hydrophobin 2 and 3). Macroarray analysis indicated that hydrophobin 1 was up-regulated in the interaction zone, whereas hydrophobin 2 and 3 were down-regulated. The expression profile of these genes was further analyzed using virtual northern blot (Fig. 4b). The result showed a high expression level for hydrophobin 1 in both fungi at the early time points and low transcript levels at late time points but strong up-regulation within the barrage zones. Hydrophobin 2 was strongly expressed by *P. gigantea* and an increased transcript level was recorded for *H. parviporum* at 28 dpi. Hydrophobin 2 was down-regulated in barrage zones at 9 and 28 dpi as observed in macroarray differential screening. With hydrophobin 3, no major differences in expression level between *H. parviporum* and *P. gigantea* were documented at early time points. At a later time point (28 dpi), stronger expression was observed in *H. parviporum*. The gene was down-regulated within barrage zones at 9 and 28 dpi. In contrast to *P. gigantea* hydrophobin, the hydrophobin that originated from *H. annosum* showed strong expressions only with *H. parviporum,* very low expressions with *P. gigantea* and down-regulation in the barrage zones (Fig. 4b).

The sequence of hydrophobin 1 (*Pgh 1*, AY822597) was analyzed and found to contain an open reading frame encoding a polypeptide of 108 amino acids with seven conserved cysteine residues. The gene had 5′ untranslated region 50 bp long and 3′ untranslated region 156 bp long. Hydrophobin 2 (*Pgh 2*, AY822598) coded for a 153-amino acid polypeptide containing seven conserved cysteine residues. Hydrophobin 3 (Pgh 3) contained only partial sequence and was not included in further analysis. Conserved cysteine residues are characteristic for fungal hydrophobins. Phrap clustering of sequences from hydrophobin 1 (Pgh1), 2 (Pgh2) and *H. annosum* hydrophobin (Hah) showed they have 36–64% nucleotide sequence identity to each other, Pgh1–Pgh2 shared 64% identity and Pgh1–Hah and Pgh2–Hah shared 43 and 36% nucleotide identity, respectively.

A dendrogram was produced on the aligned amino acid sequences of the *P. gigantea* hydrophobins 1 and 2 and hydrophobins from an ascomycete and a few basidiomycetes (Fig. 5). The dendrogram confirmed that hydrophobin 1 and 2 are closely related. On the amino acid level, Pgh1–Pgh2 shared 70% identity. Spore-wall hydrophobin from an ascomycete *Aspergillus nidulans* (GeneBank accession number EAA58809.1) was distantly related to *P. gigantea* hydrophobins. Hydrophobin from fruiting body of *Schizophyllum commune* (P16934) was closely related to hydrophobin 1 and 2.

Discussion

In this study, our aim has been not only to describe the interaction patterns but also enhance our knowledge of microbial interaction during competition for space and nutrients. The *Phlebiopsis gigantea* cDNA library used for assessing gene expression during interaction with *Heterobasidion parviporum* was constructed from mycelia grown on rich complex medium rather than on wood, the natural substrate for the fungus. However, a critical distinction is

whether genes expressed during hyphal–hyphal interaction are the same as genes used during growth on rich medium. In contrast to the *Trichorderma* biocontrol system, where the mechanism of action is due to lytic enzyme secretion (Viterbo *et al*., 2002), in the *P. gigantea* system, the basis is not antibiosis, parasitism or toxin production but rather competitive advantage in nutrient acquisition (Holdenrieder & Grieg, 1998). In fresh woody stumps, two types of nutrients have been identified: soluble sugars (Asiegbu, 2000) and lignified cellulosic constituents (Cowling & Kirk, 1976). The primary interaction between *P. gigantea* and *H. parviporum* occurs on the surface of stumps where soluble sugars are abundant and lignoceullose biodegradation is usually accomplished during secondary metabolism in fungi (ten Have & Teunissen, 2001). Therefore, we reasoned that genes and proteins important in primary metabolism could be major determinants of the outcome of *P. gigantea–H. parviporum* interaction, particularly during initial substrate colonization. Thus, it is equally useful to identify genes vital for efficient substrate utilization that occurs when fungi are competing for space and nutrients (Poppe *et al*., 2003) during growth on fresh stump surface or on artificial rich media.

A number of studies have analyzed interactions between organisms using artificial media to elucidate the basis for biological control and the dynamics of fungal competition (Carruthers & Rayner, 1979; Magan & Lacey, 1984a, b). These studies have usually used paired cultures of fungi in Petri dishes of agar medium. However, Dowding (1978) expressed doubts about extrapolating results obtained from surface culture to natural situations. In turn, Magan & Lacey (1984a, b) suggested that such strategies were the best available for analyzing the interaction between fungi. Strong correlations have been stated by some authors between the combative ability of fungi in dual cultures and their ecological roles (Henningson, 1967; Rayner, 1978; Carruthers & Rayner, 1979; Boddy & Rayner, 1983; Rayner & Webber, 1984; Woods *et al*., 2005). In other systems, however, contrasting results have been obtained between tests carried out on high sugar media and woody substrates (Lundborg & Unestam, 1980; Nicolotti & Varese, 1996; Highley, 1997). Surface agar-based culture systems using high sugar media to test competitive interactions between fungi may, in certain instances, give interaction results comparable to those obtained under field conditions (Woods *et al*., 2005). In our study, the dominance of *P. gigantea* over *H. parviporum* on wood was also replicated on the artificial Hagem medium (see Fig. 2). In addition, the high number of overlapping highly expressed genes detected in a pilot study during growth on Hagem or sawdust containing medium suggests that useful information could be obtained using Hagem in this *in vitro* study.

Interspecific fungal interactions have so far been characterized with respect to activity of extracellular enzymes, particularly laccase (Iakovlev & Stenlid, 2000; Boddy, 2000), and use of mRNA differential display for identification of genes involved in the interaction (Iakovlev *et al*., 2004). To our knowledge, this is the first study using a macroarray genomic tool to investigate nonself-competitive–fungal interaction relating to biocontrol of forest pathogens by *P. gigantea*. Genomics can provide information about previously uncharacterized genes and it is considered a novel approach for detecting unique and potentially important genes. In this study, we differentially screened a cDNA library from *H. parviporum* (HAGS) (Abu *et al*., 2004) and *P. gigantea* (GIGA) containing 3072 and 716 (from a total of 2636) clones, respectively. The BlastX analyses of 191 randomly sequenced EST clones from the *P. gigantea* cDNA library (GIGA) revealed that a large number of sequences shared homology with genes of fungal origin currently available in the NCBI database. But a significant number (72%) had no match with genes of known function. The lack of information for such a high number of genes may be due to the fact that a vast number of fungal genes have yet to be cloned, sequenced and fully identified. The differential screening of the cDNA libraries led to identification of several genes of diverse functions (see Table 2a, b). Several genes encoding hypothetical proteins or genes of unknown function or no homology to any gene in NCBI database were also noted. The presence of genes encoding hypothetical proteins or with no known function should not necessarily reduce the value of such datasets.

As indicated earlier, the interaction between *H. parviporum* and *P. gigantea* is known to be a deadlock or overgrowth, rather than antibiosis or parasitism commonly observed with other biocontrol agents such as *Trichoderma* spp. (Viterbo *et al*., 2002). In the *Trichoderma* spp. biocontrol system, secretion of chitinase, a major enzyme involved in breakdown of hyphal cell walls, has been implicated in the success of the biocontrol process (Haran *et al*., 1996; Viterbo *et al*., 2001). On the other hand, in the *P. gigantea–H. parviporum* interaction, preferential expression of genes encoding a diverse range of proteins, including those important for efficient substrate utilization and nutrient acquisition (fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, endo-galacturonase, glutamine synthetase), were documented. Fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase are enzymes involved in glycolysis and gluconeogenesis. Endo-galacturonases are known to be involved in breakdown of pectic compounds, and glutamine synthetase is a key enzyme in nitrogen metabolism. The results of the virtual northern blot analysis suggest that the increased transcript level of fructose bisphosphate aldolase in the barrage zone could be due to strong expression by *H. parviporum*, whereas high levels of glutamine synthetase and glyceraldehyde-3-phosphate dehydrogenase most likely originated

from *P. gigantea* mycelia. It is, however, difficult to explain whether the expression of these genes is specific for the interaction or perhaps a response to stress or nutrient starvation.

Two other interesting genes encoding cyclophilin and hydrophobin 1 were up-regulated in the barrage zone but their role in the interaction remains unclear. Cyclophilin is a highly conserved protein that regulates a variety of cellular processes, including the response to environmental stresses, cell cycle control, the regulation of calcium signalling and the control of transcriptional repression. It is also a virulence determinant in rice blast fungus *M. grisea* (Viaud *et al.*, 2002). Cyclophilin has also been identified in another biological control agent – *Trichoderma harzianum* (Grinyer *et al.*, 2004). Hydrophobins are small secreted multifunctional proteins that have been detected in ascomycetes and basidiomycetes (for review see Wessels, 1997; Whiteford & Spanu, 2002). They possess ability to self-assemble at hydrophilic–hydrophobic interfaces into highly amphipathic films. This property is important in formation of aerial structures and attachment of hyphae to hydrophobic surfaces. Hydrophobins have also been implicated in cell-wall assembly and in pathogenic interactions where the monomers could act as toxins and elicitors (Tucker & Talbot, 2001). Changes in hyphal hydrophobicity might lead to sealing-off of the hyphal boundaries at the interaction interface and formation of barrage zone (Rayner *et al.*, 1994). The differences in the homology among the different hydrophobins studied probably explain the reasons for the varying regulatory patterns observed with the virtual northern analysis. It is also possible that the hydrophobin gene which originated from *H. annosum* that was down-regulated in the interaction zone is of a separate gene family. The results of the virtual northern blot further suggest that the up-regulation of hydrophobin 1 and cyclophilin in the barrage zone is most likely due to increased expression by the interacting *P. gigantea* mycelia.

Four other genes (phosphoglucomutase, arginase, cytochrome P450 monooxygenase, transcript antisense to ribosomal RNA) from *H. parviporum* were found to be strongly expressed by *P. gigantea*. Phosphoglucomutase functionally interconverts the 1- and 6-phosphate isomers of α-D-glucose. This reaction is also important in glycogen biosynthesis, trehalose anabolism, sucrose biosynthesis and galactose metabolism. Arginase is a manganese-containing polypeptide that catalyzes the conversion of L-arginine to ornithine and urea. Ornithine is a central part of the urea cycle, which allows for the disposal of excess nitrogen. Urea is considered a much less toxic compound than ammonia; therefore organisms, which cannot easily and quickly remove ammonia usually have to convert it to some other substance, like urea or uric acid. Several authors studied the changes in mRNA levels of genes involved in amino acid biosynthesis and observed that most of these genes are expressed at significantly higher levels during periods of isotropic growth or in response to nutritional shifts (Sachs & Yanofsky, 1991; dEnfert, 1997). Cytochrome P450 monooxygenase, on the other hand, has been implicated in pathogenesis, antifungal resistance, biosynthesis of secondary metabolites and toxin detoxification (van den Brink *et al.*, 1998). Transcript antisense mRNAs (mitochondrial protein, Tar1p) have been shown to regulate mRNA transcription, processing, translation and DNA replication (Coelho *et al.*, 2002). Overexpression of the mitochondrial protein (Tar1p) has been found to increase the efficiency of oxidative phosphorylation and may act indirectly by influencing the DNA stability of mitochondria (Coelho *et al.*, 2002). The four genes described above were slightly down-regulated in the barrage zone, and stronger expressions were documented within *P. gigantea* mycelia located outside the barrage zone. This suggests that the signals emanating from barrage zone formation could lead to increased expression of some key genes in other regions of the hyphae during nonself-combative interaction. Consequently, the ability of *P. gigantea* efficiently to produce many of these functionally important enzymes necessary for nutrient acquisition and other metabolic processes will presumably confer some competitive advantage during colonization of available niches on a suitable substrate. Future studies will seek to use gene knockout to establish the precise role of each of these transcripts in nonself-competitive interactions.

Technically, the EST analysis, macroarray screening and virtual northern blot are robust approaches for identification of genes which are differentially expressed in nonself-fungal interactions. To address the issue of the potential cross hybridization resulting from high sequence conservation between *P. gigantea* and *H. parviporum*, RNA samples were obtained not only from the barrage zone (containing hyphae of the two fungi) but also from mycelia of the respective fungi located outside the barrage zone. Together with RNA samples obtained from different time points under varying self- and nonself-interaction conditions, we were able to ascertain the potential origin of a number of major up-regulated genes identified within the barrage zones. Due to the high sequence conservation, we were also able to use genes isolated from one strain for gene expression study of the other. Interestingly, some genes isolated from *H. parviporum* were found to be strongly up-regulated by *P. gigantea* during the nonself-interactions and *vice versa*. The data on pairwise comparison support the conclusion that arrays from one species could give useful information from hybridization with labelled cDNA from related species. This obviously will reduce the need for mass cDNA sequencing projects and allow laboratories to share resources and exploit opportunities available for using functional genomics tools to study global gene expression in related cell types.

This study is the first of its kind to highlight the molecular basis of this poorly understood ecological process which regulates interspecific interaction between *P. gigantea* and a major forest pathogen (*H. parviporum*). It is hoped that identification of genes crucial for nonself-competitive interactions will lead to a better understanding of interactions occurring between microorganisms in the natural environment. The knowledge to be acquired will be of immense value for improving *P. gigantea* efficiency as a factor in the control of *Heterobasidion parviporum*-caused root rot, either by arming the biocontrol agent or by weakening the pathogen via enriching applied pesticides in appropriate regulatory compounds.

Supplementary material

The following supplementary material is available for this article online.

Table S1. Genes on the array used to determine the extent of sequence conservation between *Heterobasidion parviporum* and *Phlebiopsis gigantea*. Raw signal intensity values for (a) *H. parviporum* and (b) *P. gigantea* expression level.

The material is available as part of the online from http://www.blackwell-synergy.com

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