Transcriptional Responses of *Paxillus involutus* and *Betula pendula* During Formation of Ectomycorrhizal Root Tissue

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In order to obtain information on genes specifically expressed in the ectomycorrhizal symbiosis, 3,555 expressed sequence tags (ESTs) were analyzed from a cDNA library constructed from ectomycorrhiza formed between the basidiomycete *Paxillus involutus* and birch (*Betula pendula*). cDNA libraries from saprophytically growing fungus (3,964 ESTs) and from axenic plants (2,532 ESTs) were analyzed in parallel. By clustering all the EST obtained, a nonredundant set of 2,284 unique transcripts of either fungal or plant origin were identified. The expression pattern of these genes was analyzed using cDNA microarrays. The analyses showed that the plant and fungus responded to the symbiosis by altering the expression levels of a number of enzymes involved in carbon metabolism. Several plant transcripts with sequence similarities to genes encoding enzymes in the tricarboxylic cycle and electron transport chain were down regulated as compared with the levels in free-living roots. In the fungal partner, a number of genes encoding enzymes in the lipid and secondary metabolism were down regulated in mycorrhiza as compared with the saprophytically growing mycelium. A substantial number of the ESTs analyzed displayed significant sequence similarities to proteins involved in biotic stress responses, but only a few of them showed differential expression in the mycorrhizal tissue, including plant and fungal metallothioneins and a plant defensin homologue. Several of the genes that were differentially expressed in the mycorrhizal root tissue displayed sequence similarity to genes that are known to regulate growth and development of plant roots and fungal hyphae, including transcription factors and Rho-like GTPases.

The mycorrhizal symbiosis between plants and fungi is ubiquitous, and the vast majority of plant species are dependent on this symbiosis, including many economically important forest trees and crop plants (Smith and Read 1997). The fungal

symbiont aids the growth of the plant partner by improving nutrient uptake, while the fungal partner is dependent on carbohydrates provided by the plant (Smith and Read 1997). Ectomycorrhiza (ECM) is the dominating mycorrhizal type associated with trees in temperate and boreal ecosystems. In this mycorrhizal type, the fungus colonizes the plant's fine roots, and a pseudoparenchymatous fungal mantle, the sheath, is developed surrounding the root. From this structure, fungal hyphae colonize the surrounding soil volume. Elongation of the fine root is hampered, and the fungus penetrates between the outer cells of the root, forming the so-called Hartig net. It is assumed that the nutrient exchange between the symbionts takes place in this tissue and mycorrhizas may survive for a full growth season (Dexheimer and Pargney 1991).

The molecular mechanisms governing the differentiation and development of the ectomycorrhizal structures and the shift to and maintenance of mycorrhizal metabolism are poorly understood. The mutual development of the fungus and the plant regarding the differentiation into a functional ECM requires signals that trigger the formation and the differentiation of a specific multicellular structure. These can be expected to include signals both within and between symbionts as well as signals from the environment to ensure a well-tuned regulation of gene expression at both temporal and spatial level. Although a number of symbiosis-related genes have been identified in different ECM systems (Kim et al. 1999; Laurent et al. 1999; Nehls et al. 1998, 2001; Sundaram et al. 2001; Tagu et al. 1996), the only ECM system in which the global pattern of gene expression has been analyzed in some detail is the interaction between *Eucalyptus globulus* and *Pisolithus tinctorius* (Voiblet et al. 2001).

In this paper, we present results from investigations on the global gene expression during the ectomycorrhizal symbiotic interaction between *Paxillus involutus* and birch (*Betula pendula*). The basidiomycete *P. involutus* is a common fungus in temperate ecosystems and forms ECM with a number of different hosts, including birch (Wallander and Söderström 1999). Moreover, *P. involutus* is a frequently used ECM fungus in laboratory experiments and can be easily cultivated as a saprophyte in pure culture. The development and function of ECM can be followed in several laboratory systems, including agar plates (Brun et al. 1995) and soil compartments (Ek et al. 1994). In order to characterize the global gene expression (i.e., the transcriptome) in the ECM tissue, we have conducted expressed sequence tag (EST) analysis of 10,051 cDNA clones from three different libraries. These include libraries made from ECM root tissue, saprophytically grown mycelium, and

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axenically grown plants. From the assembled EST information, we obtained a unique set of 2,284 genes of either fungal or plant origin, which was used for the construction of cDNA microarrays. Here, we describe expression profile analysis using both the EST and microarray analyses to assess the mycorrhizal stage in comparison with axenic conditions.

RESULTS

EST analysis.

The time of harvest of the birch seedlings colonized by *P. involutus* was correlated to the formation of the sheath and the Hartig net (Brun et al. 1995), as determined from microscopy on frozen sections. In parallel with the construction of a cDNA library representing the mycorrhizal tissue, libraries were constructed from axenic cultures of the plant (*B. pendula*) and saprophytically grown mycelium of the fungus. In total 10,051 high-quality ESTs were obtained from the three cDNA libraries. The sequences were assembled into 2,284 contigs that putatively represent unique transcripts (Table 1). Of the assembled sequences, 789 were represented in the *P. involutus* library,

985 in the mycorrhizal library, and 851 in the *B. pendula* library. About 44% of the contigs were singletons, i.e., they contained only one EST clone. Between 19 and 37% of the assembled sequences displayed a high degree of similarity (FASTA score was >299) to sequences in the GenBank nonredundant (nr) protein database (Table 1). The fraction of ESTs showing high-score homology was larger in the plant library than in the fungal library, which probably reflects the differences between the fractions of plant and fungal entries in the GenBank nr protein database. A large fraction (17 to 37%) of the assembled sequences showed no homology (orphans) to protein sequences in the GenBank database, and the proportion of orphans was higher in the ECM library (38%) than in the mycelium (37%) and plant (17%) libraries. Based on sequence comparisons between ESTs and information in the GenBank nr protein database, the sequences were classified into the main functional categories as used by the Munich Information Center for Protein Sequences (MIPS) (Mewes et al. 2000). At this level of resolution, only major differences between libraries were revealed, such as the relatively large difference in the number of transcripts encountered within the "cell rescue, defense, cell death

Table 1. Number of EST clones analyzed in each cDNA library^a

Library	Total EST (>99 bp)	Assembled sequences ^b		Sequence homology (FASTA/GenBank) scores (%)			
		Total	Singletons ^c	Orphans	Low (<100)	Moderate (100-299)	High (>299)
<i>Paxillus involutus</i>	3,964	789	378 (48%)	37	12	32	19
Mycorrhiza	3,555	985	499 (51%)	38	10	31	22
<i>Betula pendula</i>	2,532	851	419 (49%)	17	8	38	37
All libraries	10,051	2,284	1,018 (44%)				

^a Expressed sequence tags (EST) are distributed according to various ranges of sequence homology scores after sequence comparison with the GenBank nonredundant protein database.

^b Assembled sequences, putatively representing different transcripts, refers to the combined sets of contigs (assembled continuous sequences) and singletons after assembly by the contig assembly program (Huang 1992).

^c The percentages represent the fraction of singletons compared with the total number of assembled sequences and indicates the level of redundancy.

Fig. 1. The classification of 10,051 individual expressed sequence tags (ESTs) (>99 bp) derived from the fungal (*P. involutus*), the mycorrhizal, and the plant (*B. pendula*) cDNA libraries into main cellular functional categories. The classification is according to the Munich Information Center for Protein Sequences (Mewes et al. 2000) after putative annotation, based on sequence similarity to information in the GenBank nonredundant (nr) protein database. The distribution is plotted against the percentile of EST occurrence in each cDNA library (% EST). The "No function" category is defined as a high or moderate degree of homology (FASTA score >100) but with unclear or no defined functional role, whereas the "No homology" category is defined as weak (<100) or no homology (score <1) to sequences in the GenBank nr protein database. The "Other function" category is a collection of minor functional categories, i.e., Cellular biogenesis, Cellular communication/signal transduction, and Ionic homeostasis.

and ageing” category, corresponding to 7.0, 9.2, and 16.1% of the total number of ESTs in the fungal, mycorrhizal, and plant libraries, respectively (Fig. 1). Other functional categories showing sizeable differences between libraries were protein synthesis, with 13.8% in the fungal library versus 9.7 and 9.5% in the mycorrhizal and plant library, respectively, transcription, with 3.8% in the plant library versus 1.5 and 1.6% in the mycorrhizal and fungal library, respectively, and cellular organization, with 8.0% in the plant library versus 4.2 and 1.9% in the mycorrhizal and fungal library, respectively.

cDNA microarrays.

To allow more detailed studies on the expression of genes in the mycorrhizal tissue, we constructed a cDNA microarray from the analyzed set of EST clones. From a total of 2,284 assembled sequences or cDNAs (putatively representing different transcripts), 2,161 were successfully amplified as “single-band” polymerase chain reaction (PCR) products and spotted in quadruplicate onto microarray glass slides. Positive controls that represented several highly expressed (as revealed by EST data) fungal and plant genes were included. Heterologous PCR products for which corresponding RNAs were spiked into the RNA samples during target preparation were also used as positive controls to assess amplification and labeling of targets. Due to the fact that only milligram quantities of tissue could be harvested from the mycorrhizal system, the target RNA had to be amplified before labeling and hybridization. For this reason, we used a method of linear amplification of mRNA based on cDNA synthesis, followed by synthesis of antisense RNA (aRNA) by T7 RNA polymerase in vitro transcription. This procedure has proven to yield microarray sensitivity and precision comparable to those in protocols using mRNA directly, without altering the mRNA profiles (Baugh et al. 2001; Pabon et al. 2001; Zhao et al. 2002). To assess the accuracy of the procedure used for aRNA amplification, a mixture of heterologous mRNAs were added in different amounts to a fungal RNA preparation. The preparation was subsequently amplified by T7 in vitro transcription, and the procedure was analyzed by microarray. The measured folds of these spikes were in close agreement with the expected values (Table 2). In addition, a possible bias introduced by the labeling procedure was examined by analyzing a set of T7 in vitro-amplified heterologous spikes. The measured folds were similar to the expected values (Table 2).

Fungal and plant transcripts in the ECM tissue.

Of the 985 assembled EST clones detected in the ECM library, 644 were not clustered with sequences from either the plant or the fungal libraries and, thus, their origin is not clear.

Before the cDNA array could be used to analyze the expression of plant and fungal genes in the ECM tissue, the fungal or plant origin of these probes was investigated. In a separate microarray experiment, the putative origin of transcripts was investigated using a labeled fungal and a plant target (biological replicate 1). With the limit set at more than a twofold difference in median signal intensity, 355 of the assembled sequences could be assigned as being either of fungal or plant origin. The fungal or plant origin of mycorrhizal transcripts was also analyzed using GC content information for transcripts in the EST libraries. This analysis revealed single-humped distributions for the fungal and the plant libraries, with a mean GC content of 52 and 46% for *P. involutus* and *B. pendula*, respectively (Fig. 2, top and bottom panels). For mycorrhizal ESTs, the GC content showed a composite distribution, indicating the presence of both fungal and plant transcripts (Fig. 2, middle panel) but with unequal representations of the two. Using information on the GC content (putatively plant origin if there was $\leq 48\%$ GC and fungal origin if there was $\geq 50\%$) for the remaining 289 transcripts and in combination with sequence homology information, we assigned the putative origin of an additional 74 transcripts. In summary, from 2,284 assembled sequences, we assigned 1,048 genes as being of fungal origin and 1,021 of plant origin, whereas 215 transcripts (9%) did not meet any of the criteria set to distinguish between fungal or plant origin.

Data on the GC content were also used to calculate the proportions of plant and fungal transcripts in the ECM tissue. The maximum likelihood estimate of the proportion of plant transcripts was 31.5% with a 95% confidence interval of 24.8 to 38.4%. The confidence interval was calculated using a bootstrap procedure, in which mycorrhizal GC data was resampled 100,000 times from the *P. involutus* and *B. pendula* GC data, using the estimated probability of *B. pendula* transcripts (31.5%).

The proportion of plant and fungal transcripts in the mycorrhizal transcriptome was further examined by Northern blot analysis using 38 immobilized fungal and plant probes, respectively, and performing a hybridization using a target prepared from mycorrhizal tissue (biological replicate 1). By calculation and comparison of the combined total intensities resulting for all 38 fungal and plant probes, respectively (discussed below), the fraction of plant transcripts in the mycorrhizal transcriptome was estimated to be 29%. Similarly, by calculation and comparison of the combined total single-channel intensities for 1,048 fungal and 1,021 plant probes in a microarray experiment and using a target from mycorrhizal tissue (biological replicate 1) yielded a 30% representation of plant transcripts in the mycorrhizal transcriptome. Thus, three independent methods of analysis of fungal and plant representation in

Table 2. Evaluation of the procedures used for amplifying and labeling the target RNA for cDNA microarray analysis

Spike	Signal target (Cy3)	Control target (Cy5)	Expected fold	Observed fold (microarray)	Average signal intensity (microarray)
aRNA amplification ^a					
2	100 ^b	100 ^b	1.0	1.02	529
4	10	10	1.0	1.02	264
6	1	1	1.0	...	ND ^c
Labeling ^d					
1	500	50	10	12.9	1,885
3	250	500	0.5	0.40	1,523
5	500	250	2.0	2.05	740
7	50	500	0.1	0.099	118

^a Spikes 2, 4, and 6 (ArrayControl) were added as mRNA to a preparation of fungal RNA (total amount 4 μ g). Following one round of T7 in vitro transcription (MessageAmp) and labeling, the fold was measured using the cDNA array.

^b Measured in picograms.

^c ND = signal not detected.

^d Spikes 1, 3, 5, and 7 (ArrayControl) were amplified using one round of T7 in vitro transcription. After amplification, the concentration of aRNA was measured with RiboGreen (Molecular Probes). The aRNAs were mixed and labeled in different ratios as indicated in the table, and the fold was measured using the cDNA array.

the mycorrhizal transcriptome, i.e., using GC content distribution or total fungal and plant intensities employing either Northern blot data or microarray data, all gave similar results. Of the different approaches used, the analysis of total fungal and total plant intensities using microarray analysis may be the most convenient method and a similar assessment of the second replicate (biological replicate 2) yielded a 33% representation of plant transcripts in the mycorrhizal transcriptome. Based on these results, ratio of fungal to plant transcripts was set to roughly 7:3 and subsequently used for correction of EST counts as well as microarray expression folds (discussed below) of fungal and plant transcripts in the mycorrhizal tissue for both biological replicates (Tables 3 and 4).

Differentially regulated genes in the mycorrhizal tissue.

Microarray experiments were designed to analyze the expression of fungal and plant genes in the mycorrhizal tissue. Two biological replicates (1 and 2) were analyzed, one of them being identical to samples used for the EST library constructions (biological replicate 1). Hybridizations were per-

formed by combining a mycorrhizal target (sample labeled with Cy3) with either a fungal or a plant target (reference labeled with Cy5) representing transcripts expressed in the saprophytically grown fungus and axenically grown plants, respectively (Fig. 3A and C). To estimate the variation introduced during sample preparation, we also performed a self-self hybridization, i.e., the mycorrhizal target served as both sample (Cy3) and reference (Cy5) (Fig. 3B and D). These experiments showed high correlation between the sample and reference target (Fig. 3B and D), and only in the lower range of signal intensities were more than a twofold variations observed, which suggests that values greater than twofold indicate significant differences in expression.

In hybridization using the combined mycorrhiza/fungus target, we found, in the two biological replicates, 870 (83%) and 821 (78%), respectively, of the 1,048 fungal probes to produce signal intensities in at least one of the two channels above the twofold average background intensity on each slide. Among those and with the limit set at genes showing more than an average twofold up- or down-regulation, 93 fungal genes were found in both replicates to be differentially regulated in mycorrhizal compared with saprophytic conditions (Tables 3 and 4). Of those 93, 34 (37%) were up-regulated and 59 (63%) down-regulated (Table 3). The differentially regulated genes represented several different functional categories, but the majority (52 assembled sequences [56%]) represented genes encoding products with no clear-cut classification or were unclassified or showed no homology. Through hybridization using the combined mycorrhiza/plant target, we found in the two biological replicates 896 (88%) and 704 (69%), respectively, of the 1,021 plant probes investigated to produce signal intensities in at least one of the two channels above the twofold average background on each slide. Of these, 102 plant genes showed more than a twofold differential regulation in both biological replicates, 46 (45%) being up-regulated and 56 (55%) down-regulated. The distribution of these genes into functional categories (Table 4) showed that 57 (56%) represented genes encoding products with no clear-cut classification or were unclassified or showed no homology. This group of unknown genes forms a substantial part of all plant genes differentially regulated in mycorrhiza and is similar to the size of the fraction observed among fungal genes.

Validation of the microarray analysis.

To verify the microarray expression data, a random selection of fungal EST cDNA clones were analyzed by a Northern dot blot experiment for comparison of a fungal and mycorrhizal target (Fig. 4A). Total RNA (biological replicate 1) was amplified using the T7 in vitro transcription protocol and aRNA was finally converted to cDNA in a random labeling procedure. From the analysis of 38 fungal genes under mycorrhizal conditions, we found a correlation ($r^2 = 0.41$) between signal ratios in the microarray and Northern blot methods (Fig. 4A). Similarly, the correlation between the microarray and EST analysis was investigated. EST transcript profiling by clustering and assembly yields a frequency for each specific transcript in each library. The significance of any difference observed in EST counts between two libraries is based on the frequency for a specific transcript relative to the total sampling size, i.e., the total number of ESTs analyzed in each library (Audic and Claverie 1997). We analyzed EST counts for 1,048 fungal genes according to Audic and Claverie (1997) and found 178 genes to show significant difference in frequency ($p \leq 0.05$) between the mycorrhizal and the fungal libraries. The ratio of EST counts for these genes were then compared with the ratios between microarray signal intensities for mycorrhiza versus

Fig. 2. GC content in cDNA from *Paxillus involutus*, mycorrhiza and *Betula pendula*. From each assembled contig, one representative expressed sequence tag (EST) clone was selected (in most cases the most extended EST clone within a contig [only if <500 bp]) for analysis of GC content using the GeeCee software. The result was plotted in 0.01 increments, and the distribution is shown. The source cDNA library, together with the number of contigs analyzed, is specified in each histogram. The average GC fraction for *P. involutus* and *B. pendula* is indicated.

Table 3. Fungal genes differentially regulated in mycorrhiza^a

EST clone	GenBank ID	GenBank homologue description	Score	Biological replicate (fold)		Control (fold)
				1	2	
Cell growth, cell division and DNA synthesis						
T143B01572F	19115481	Rho2 protein (<i>Schizosaccharomyces pombe</i>)	339	-3.4	-1.6	1.1
T143B00870F	987287	Temperature sensitive supressor bem1/bud5 (<i>Schizosaccharomyces pombe</i>)	256	-2.7	-2.5	1.1
Energy						
T143A02411F	19113860	Hexokinase (<i>Schizosaccharomyces pombe</i>)	106	2.5	1.8	1.0
Metabolism						
T143A00784F	11269551	O-acetyl-L-homoserine sulphydrylase (<i>Emericella nidulans</i>)	470	4.7	1.9	1.0
T143A04701a	168922	Tyrosinase precursor (<i>Neurospora crassa</i>)	144	2.2	2.2	1.1
T143B00615F	15843160	Oxidoreductase (<i>Mycobacterium tuberculosis</i>)	272	-2.5	-1.6	1.0
T143B00264	7491600	Putative lipid metabolism protein (<i>Schizosaccharomyces pombe</i>)	329	-2.4	-1.9	1.3
T143B02978F	19075941	Putative diphthine synthase (<i>Schizosaccharomyces pombe</i>)	449	-2.1	-2.2	1.0
T143A02502a	15596060	Probable oxidoreductase (<i>Pseudomonas aeruginosa</i>)	216	-2.9	-1.8	1.1
T143B00617F	12643630	Chorismate synthase (5-enolpyruvylshikimate-3-phosphate phospholyase) (<i>Schizosaccharomyces pombe</i>)	599	-3.1	-2.9	1.1
T143B04641F	6319582	Pho88p (<i>Saccharomyces cerevisiae</i>)	413	-3.1	-2.9	1.0
T143B02594F	6321641	Phosphorylcholine transferase Pct1p (<i>Saccharomyces cerevisiae</i>)	295	-3.8	-3.6	-1.1
T143A02109F	7427848	Stearoyl-CoA desaturase (<i>Cryptococcus curvatus</i>)	406	-5.4	-4.9	1.0
Cellular organization						
T143A05011F	4008016	Hydrophobin-3 precursor (<i>Pisolithus tinctorius</i>)	305	2.6	4.1	1.2
T143B03881F	4008016	Hydrophobin-3 precursor (<i>Pisolithus tinctorius</i>)	292	2.1	2.4	1.0
T143A00886F	1729837	Tubulin alpha (<i>Schizophyllum commune</i>)	497	2.1	1.8	1.0
T143A04421F	6321542	Vacuolar H-ATPase assembly Vma21p (<i>Saccharomyces cerevisiae</i>)	114	-3.2	-2.4	-1.1
Cell rescue, defence, cell death and ageing						
T143B00573	18250469	Metallothionein (<i>Agaricus bisporus</i>)	126	1.9	2.2	1.1
T143A01393F	7493810	Rehydrin-like protein (<i>Candida albicans</i>)	512	2.4	1.7	1.0
T143B05234	13702833	Glutathione S-transferase (<i>Oryza sativa</i>)	258	-2.1	-3.0	1.0
T143B03447F	18250473	Cytochrome P450 (<i>Agaricus bisporus</i>)	170	-3.5	-3.7	-1.1
T143B01510F	416561	Glutathione S-transferase (<i>Nicotiana tabacum</i>)	122	-4.9	-3.2	-1.1
Protein destination						
T143B03709F	8393005	Bystin (<i>Mus musculus</i>)	220	-2.0	-2.3	1.0
T143B00344	13928974	Ribophorin II (<i>Rattus norvegicus</i>)	216	-2.7	-2.4	-1.2
Protein synthesis						
T143A04021F	5757449	L41 ribosomal protein (<i>Coprinopsis cinerea</i>)	280	4.6	2.1	1.1
T143B04049F	12329977	Ribosomal protein L13A (<i>Xanthophyllomyces dendrorhous</i>)	842	2.4	2.8	1.1
T143B00315	1173072	Acidic ribosomal protein P2 (<i>Bos taurus</i>)	353	3.0	1.8	1.1
T143A04854F	1813337	Ribosomal protein S11 homologue (<i>Schizosaccharomyces pombe</i>)	500	2.0	2.1	1.1
T143A04915F	15825503	Ribosomal protein L11 (<i>Branchiostoma belcheri</i>)	714	2.4	1.5	1.0
T143A03073F	19113857	40s ribosomal protein S9 (<i>Schizosaccharomyces pombe</i>)	594	1.7	2.3	1.0
T143B03743F	6324085	Translation initiation factor 3 Sui1p (<i>Saccharomyces cerevisiae</i>)	350	-2.4	-1.7	1.1
T143B05380F	6322711	15.5 kDa mitochondrial ribosomal protein YmL31 (<i>Saccharomyces cerevisiae</i>)	154	-2.3	-2.0	1.1
Transport facilitation						
T143A02176F	19112801	MSF transporter (<i>Schizosaccharomyces pombe</i>)	262	-4.1	-3.3	1.4
Cellular transport and transport mechanisms						
T143A00221	19075934	Putative coatomer zeta subunit (<i>Schizosaccharomyces pombe</i>)	437	8.0	23.7	1.1
T143A01761F	15229865	Putative syntaxin (<i>Arabidopsis thaliana</i>)	205	2.8	3.1	1.0
T143B04983F	19114254	Mitochondrial inner membrane translocase subunit tim17 homologue (<i>Schizosaccharomyces pombe</i>)	467	-2.8	-1.5	1.0
T143B05214F	17558550	RAS-related protein (<i>Caenorhabditis elegans</i>)	537	-2.4	-2.3	-1.4
T143B00471	19075520	Putative component of COPII-coated vesicles possibly involved in cargo sorting (<i>Schizosaccharomyces pombe</i>)	309	-2.7	-2.7	-1.2
T143B00262	19114749	Mitochondrial carrier protein (<i>Schizosaccharomyces pombe</i>)	152	-3.8	-1.8	1.1
T143B03605F	461475	Adenine nucleotide translocator (<i>Rattus norvegicus</i>)	109	-3.4	-2.4	1.0
T143B04084F	7493804	Probable mitochondrial carrier protein (<i>Candida albicans</i>)	317	-4.7	-1.8	-1.1
T143B00732F	113466	ADP/ATP carrier protein (<i>Neurospora crassa</i>)	139	-4.1	-2.8	-1.1
No clear-cut classification or unclassified genes						
A total of 36 genes, 12 up- and 24 down-regulated (range 16.3- to -7.1-fold)						
Orphans (transcripts with no homology)						
A total of 16 genes, 6 up- and 10 down-regulated (range 41.6- to -3.5-fold)						

^a Genes showing significant (≥ 2 -fold) differential regulation in mycorrhiza compared with axenic conditions as analyzed by microarrays (a total of 93 genes, 34 were up- and 59 were down-regulated). The control value refers to folds assessed in a self-self hybridization experiment, i.e., mycorrhiza versus mycorrhiza, and deviation from unity indicates technical variance.

Table 4. Plant genes differentially regulated in mycorrhiza^a

EST clone	GenBank ID	GenBank homologue description	Score	Biological replicate (fold)		Control (fold)
				1	2	
Cell growth, cell division and DNA synthesis						
T143C00420	15231065	High mobility group protein 2-like (<i>Arabidopsis thaliana</i>)	436	2.7	3.2	1.1
T143A04305F	15228312	AP2 domain transcription factor RAP2.3 (<i>Arabidopsis thaliana</i>)	359	-1.7	-2.4	-1.1
T143C03043F	15238782	NAM (no apical meristem)-like protein (<i>Arabidopsis thaliana</i>)	277	-2.9	-3.1	-1.1
T143C02304F	6996315	ABI3-interacting protein 2 (AIP2) (<i>Arabidopsis thaliana</i>)	510	-2.2	-4.6	-1.2
Energy						
T143C00263	15237260	succinyl-CoA synthetase, alpha subunit (<i>Arabidopsis thaliana</i>)	542	-1.8	-2.4	-1.1
T143A00972F	15218058	Putative ubiquinol-cytochrome-c reductase (<i>Arabidopsis thaliana</i>)	382	-2.3	-2.3	1.2
T143C02075F	15239430	Ubiquinol-cytochrome-c reductase-like protein (<i>Arabidopsis thaliana</i>)	548	-1.6	-3.2	-1.2
T143A00364	8954298	Chlorophyll-a/b-binding protein CP24 precursor (<i>Vigna radiata</i>)	402	-2.6	-3.3	1.3
T143C01246F	115766	Chlorophyll-binding protein (<i>Petunia</i> sp.)	200	-3.5	-2.9	...
Metabolism						
T143C00430	20137323	Acyl carrier protein (<i>Casuarina glauca</i>)	513	2.2	2.1	-1.1
T143C02092F	9797761	Similarity to Oryza sativa PIR7A protein (<i>Arabidopsis thaliana</i>)	365	-2.8	-3.4	1.0
T143C03291F	3914600	Ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (<i>Marchantia paleacea</i>)	586	-4.5	-4.0	1.2
T143C02657F	15228666	Putative adenylsulphate kinase (<i>Arabidopsis thaliana</i>)	399	-2.2	-6.5	1.4
T143A01252F	15227060	Putative tropinone reductase (<i>Arabidopsis thaliana</i>)	497	-6.6	-6.1	-1.2
T143C02214F	5030906	Beta-glucosidase (<i>Polygonum tinctorium</i>)	472	-8.9	-8.3	1.0
Cell rescue, defence, cell death and aging						
T143A02009F	1171035	Metallothionein-like protein type 2 (<i>Actinidia deliciosa</i>)	323	66.6	47.8	1.0
T143C00549	14581677	Hsp70 interacting protein/thioredoxin chimera (<i>Vitis labrusca</i>)	299	2.4	4.3	1.0
T143C00473	8272394	Metallothionein-like protein (<i>Pyrus pyrifolia</i>)	290	3.8	2.4	1.0
T143C02821F	266579	Metallothionein (<i>Ricinus communis</i>)	395	2.2	2.7	1.0
T143C01959F	11385461	Glutathione S-transferase GST 23 (<i>Glycine max</i>)	623	-2.1	-2.6	1.2
T143C02593F	17646156	Phospholipid hydroperoxide glutathione peroxidase (<i>Momordica charantia</i>)	150	-3.7	-2.3	-1.1
T143A04401F	15228488	Glutathione S-transferase (<i>Arabidopsis thaliana</i>)	307	-4.2	-3.1	1.0
T143C01218F	10444372	Kunitz trypsin inhibitor protein (<i>Phaseolus coccineus</i>)	294	-5.3	-4.3	1.3
Protein destination						
T143A04213F	15237785	Putative 26S proteasome, non-ATPase regulatory subunit (<i>Arabidopsis thaliana</i>)	337	3.7	5.8	1.0
T143C00484	464981	Ubiquitin carrier protein (<i>Lycopersicon esculentum</i>)	756	4.4	2.2	1.0
T143A04804F	20152203	Ubiquitin-conjugating enzyme (<i>Oryza sativa</i>)	615	2.4	1.6	...
T143C02832F	15237785	Putative 26S proteasome non-ATPase regulatory subunit (<i>Arabidopsis thaliana</i>)	376	-1.8	-2.5	-1.2
T143C02252F	18416821	grpE-like protein (<i>Arabidopsis thaliana</i>)	202	-2.2	-3.9	-1.2
Protein synthesis						
T143A00980F	13811954	40S ribosomal protein S29A (<i>Guillardia theta</i>)	248	133.2	165.6	1.0
T143C00558	20346622	Ribosomal protein L41 (<i>Mus musculus</i>)	137	8.0	8.1	1.0
T143C02273F	2282584	Elongation factor 1-alpha (<i>Zea mays</i>)	896	5.0	2.1	1.0
T143A02906F	3334346	SUI1 homologue (<i>Salix bakko</i>)	495	4.4	2.2	1.1
T143A04286F	13957631	Putative translation initiation factor (<i>Oryza sativa</i>)	126	-2.0	-2.0	1.0
T143C00754	1173234	Ribosomal protein S25 (<i>Lycopersicon esculentum</i>)	471	-1.9	-2.4	-1.4
T143C03282F	3334320	Ribosome-associated protein p40 (<i>Glycine max</i>)	848	-2.2	-2.1	-1.2
T143C01755F	18253059	Ribosomal protein S29 (<i>Spodoptera frugiperda</i>)	232	-4.7	-4.6	-1.1
Transcription						
T143C00512	18403023	Putative spliceosome-associated protein (<i>Arabidopsis thaliana</i>)	367	3.3	4.6	-1.1
T143C00751	7439984	Glycine-rich protein (<i>Oryza sativa</i>)	300	4.6	2.8	1.1
T143C00740	1173351	DNA binding protein s1Fa (<i>Spinacia oleracea</i>)	293	-2.2	-1.8	-1.1
T143A04661F	15222432	Putative transcription factor scarecrow-like 14 (<i>Arabidopsis thaliana</i>)	450	-1.7	-2.7	1.2
Transport facilitation						
T143A04641F	8699604	Putative aquaporin TIP3 (<i>Vitis berlandieri</i> x <i>Vitis rupestris</i>)	734	-3.3	-2.2	1.0
Cellular organization						
T143C03004F	17352496	H3 histone (<i>Drosophila melanogaster</i>)	193	1.7	2.3	1.1
T143A02240F	15237024	Histone H2A-like protein (<i>Arabidopsis thaliana</i>)	285	-2.0	-2.3	2.1
T143C00882	15242942	Outer membrane lipoprotein-like (<i>Arabidopsis thaliana</i>)	351	-3.0	-3.3	1.1
Cellular transport and transport mechanisms						
T143C01496F	728882	Putative ADP-ribosylation factor 3 (<i>Arabidopsis thaliana</i>)	428	-4.1	-2.7	1.2
No clear-cut classification or unclassified genes						
A total of 45 genes, 24 up- and 21 down-regulated (fold range 95.3 to -11.7)						
Orphans (transcripts with no homology)						
A total of 12 genes, 6 up- and 6 down-regulated (fold range 9.7 to -6.0)						

^a Genes showing significant (≥ 2 -fold) differential regulation in mycorrhiza compared with axenic conditions as analyzed by microarrays (a total of 102 genes, 46 were up- and 56 were down-regulated). The control value refers to folds assessed in a self-self hybridization experiment, i.e., mycorrhiza versus mycorrhiza, and deviation from unity indicates technical variance.

fungus. This comparison, in contrast to comparison of microarray and Northern dot blot data, showed no obvious correlation between ratios of EST counts and ratios between microarray signal intensities (Fig. 4B). However, applying Spearman's rank test for correlation (as implemented in the R-language), we could not reject the null hypothesis of "no correlation" on an error level of 5% (R software).

DISCUSSION

The cDNA microarray contained probes representing 1,048 *P. involutus* genes and 1,021 *B. pendula* genes, which obviously embody only a minor part of all genes present in the two organisms. The fraction of *P. involutus* genes that is represented on the array can be estimated to correspond to approximately 14% of the total number of genes in the fungal genome, assuming a gene content of 7,700 (Le Quéré et al. 2002). However, due to the fact that a substantial fraction of the DNA probes were obtained from EST sequencing in cDNA libraries constructed from RNA extracted from ECM tissue, it could be expected that the set of probes represent an enriched portion of symbiosis-related genes.

Plant and fungal development.

Approximately 10% of the probed plant and fungal genes were at least twofold down- or up-regulated in the ECM tissue, as compared with the levels found in the free-living fungus or plant. Several of the genes that were differentially expressed in the ECM tissue displayed sequence similarity to genes that are known to be involved in plant and fungal development (Tables 3 and 4). Among the down-regulated plant genes was a transcript (T143C03043F, average fold -3.0) with sequence similarity to a no-apical-meristem (NAM) protein. In *Arabidopsis*, the NAM family of proteins is large and includes members that have roles in determining the positions of meristem and primordia (Sablowski and Meyerowitz 1998; Souer et al. 1996). Two other contigs were identified containing birch EST with the NAM domain, but were not, however, differentially regulated in the ECM tissue. Through sequence similarity searches among the EST, we also identified a number of transcription factors that are known to be involved in plant development. An AIP2 homologue (T143C02304F, average fold -3.4) was down-regulated in the ECM tissue. In *Arabidopsis*, AIP2 interacts with ABI3, which is a major regulator of embryogenesis and germination programs (Kurup et

Fig. 3. Microarray analysis designed to assess differential regulation among 1,048 *Paxillus involutus* fungal genes and 1,021 *Betula pendula* plant genes in mycorrhiza compared with free-living conditions (biological replicate 1). Scatter plots are shown of background-corrected median signal intensities at wavelengths of 635 and 532 nm for **A**, mycorrhiza (Cy3) versus fungus (Cy5) (only fungal genes shown), **B**, mycorrhiza (Cy3) versus mycorrhiza (Cy5) (fungal genes only), **C**, mycorrhiza (Cy3) versus plant (Cy5) (only plant genes shown), and **D**, mycorrhiza (Cy3) versus mycorrhiza (Cy5) (plant genes only). B and D show plant and fungal subsets, respectively, of data from the same experiment. Signal intensities were normalized such that the total median ratio (635:532 nm) equals 1.0. The three diagonal lines indicate ratios (635:532nm) equal to 0.5, 1.0, and 2.0.

al. 2000). Another EST (T143A04305F, average fold -2.0) that was down-regulated in the ECM tissue showed sequence similarity to AP2-domain-containing proteins. This is a large family of DNA-binding proteins in *Arabidopsis* that is known to be involved in controlling the development of flowers and seeds but possibly also other tissues, including roots (Okamuro et al. 1997). The most highly up-regulated plant transcript in the ECM tissue showed a high level of homology to the 40S ribosomal protein S29 (T143A00980F, average fold $+149.4$). The sequence similarity between the deduced peptide sequence of the birch EST and the ribosomal S29-like protein of *Arabidopsis* is 92%. The function and expression pattern of the S29-like protein in *Arabidopsis* is not known, but the rat and the human homologues have been shown to be differentially regulated during growth and differentiation in e.g., cancer cells (Coppock et al. 2000). Another plant transcript (T143C00558, average fold $+8.1$) that was significantly up-regulated during ECM formation showed a high degree of homology with the basic 60S ribosomal protein L41. The deduced polypeptide of the EST sequence exhibited 100% identity with an L41 ribosomal protein in *Pisum sativum* (PsRbL41), which has been shown to be expressed in dividing cells of the root caps (Woo and Hawes 1997).

The intercellular penetration and formation of the Hartig net induces a profound change in the morphology of the fungal hyphae, including an increased branching (Brun et al. 1995; Smith and Read 1997). The factor or factors regulating this shift in morphology are not known, but it can be expected that they are based on signal-mediated changes in the actin network and associated proteins (Timonen and Peterson 2002). In eukaryotic cells, including *Saccharomyces cerevisiae*, the evolutionary conserved Rho-like GTPases act in the signal transduction pathways that link plasma membrane receptors to the organization of the actin cytoskeleton (Hall and Nobes 2000). Based on sequence similarity, we identified a fungal Rho2 homologue (T143B01572F, average fold -2.5) that was down-regulated in the ECM tissue. The EST clone corresponds to an 81-amino acid (aa) polypeptide that has a sequence identity of 82% to the *Ustilago maydis* Rho2 protein (Weinzierl et al. 2002). Apart from the Rho2 homologue, we identified another fungal EST with sequence similarity to a different family of Rho GTPases, a *Schizophyllum commune* cdc42 homologue (T143B03506F, average fold $+1.4$) but at comparable expression levels in both ECM and axenic fungal tissue. A cdc42 homologue (*SbCDC42*) was recently characterized in the ECM fungus *Suillus bovinus*, and the gene was highly and similarly expressed in both vegetative hyphae and ectomycorrhizal root tissue (Gorfer et al. 2001).

Voiblet and associates (2001) analyzed the patterns of genes expressed during the early stage of ectomycorrhizal interaction between *Pisolithus tinctorius* and *Eucalyptus globulus*. Among the genes being up-regulated were several fungal cell wall proteins, including hydrophobins. Hydrophobins are small hydrophobic proteins that are involved in a number of morphogenetic activities in fungi, including the formation of aerial hyphae, sporulation, fruit body development, and infection structure formation and play essential roles in cell-to-cell communication and adhesion (Kershaw and Talbot 1998; Wessels 1997). We identified five different *P. involutus* EST contigs displaying significant sequence similarities to fungal hydrophobins, including those from *Pisolithus tinctorius* and *Tricholoma terreum* (Mankel et al. 2002; Tagu et al. 1996). Two of these putative hydrophobins were up-regulated in the ECM tissue of *P. involutus* and birch (T143A05011F and T143B03881F, average folds $+3.4$ and $+2.3$, respectively) (Table 3).

Carbon metabolism.

The microarray analysis indicated a shift in the plant and fungal carbon metabolism in the ECM tissue. Several identified plant transcripts with sequence similarities to genes encoding enzymes in the tricarboxylic (TCA) cycle were significantly down-regulated, including succinyl-CoA ligase (EC 6.2.1.4) (T143C00263, average fold -2.1) and dihydrolipoamide succinyltransferase E2 subunit (EC 2.3.1.61) (T143C03020F, average fold -2.5). Other homologues to components of the TCA cycle, such as phosphoenol pyruvate carboxykinase (EC 4.1.1.49) (T143C03133F, average fold -1.7), dihydrolipoamide dehydrogenase (EC 1.8.1.4) (T143C01035F, average fold -1.5), and malate dehydrogenase (EC 1.1.1.37) (T143A04242F, average fold -1.2) also indicated a general down-regulation but not the same extent. Furthermore, plant ESTs with sequence similarities to genes encoding enzymes in the cytochrome bc1 complex (EC 1.10.2.2) of the electron transport chain were down-regulated in the ECM tissue, including a 7.8-kDa subunit (T143A00972F, average fold -2.3), a 14-kDa subunit (T143C02075F, average fold -2.4), and an 8-kDa subunit (T143C02520F, average fold -2.1). The above data indicate that the carbon metabolism of the plant tissue responded to the formation of mycorrhiza by slowing down the oxidative degradation of glucose and by lowering the rate of respiration. Previous biochemical studies have shown that the activity of the TCA enzyme fumarase was lower in mycorrhizal tissues than in noncolonized roots (Namysl et al. 1989). The sucrose transported to the ECM tissue is thought to be

Fig. 4. Correlations between microarray data and independently assessed expression levels for fungal genes in mycorrhiza versus fungus. **A**, Correlation between median signal intensity ratios (Cy3) for a selection of 38 fungal genes in a microarray and a Northern dot blot experiment. A correlation factor (r^2) of 0.41 was calculated. **B**, Correlation between expressed sequence tag (EST) count ratios for 178 fungal genes showing significant EST redundancy (Audic and Claverie 1997) and median signal intensity from microarray analysis of mycorrhiza versus fungus.

hydrolyzed by plant invertases to monosaccharides including glucose, which are taken up by the fungal cells (Nehls and Hampp 2000). It is most likely that the assimilated glucose functions as a signal affecting the expression of a number of fungal genes among others involved in the utilization of alternative carbon sources (Gancedo 1992). In *S. cerevisiae*, the *HXX2* gene, encoding a hexokinase (Hxk2), plays a pivotal role in the glucose signaling system (Moreno and Herrero 2002). Among up-regulated fungal genes in the ECM tissue, we found a putative hexokinase homologue (T14302411F, average fold +2.1). The deduced polypeptide contains a hexokinase domain (pfam 00349.6), and the N-terminal region (95-aa residues) displays a moderate sequence similarity (identity 23 to 26%) to various fungal hexokinases, including the *hxx-1* gene product from the white truffle *Tuber borchii* (Agostini et al. 2001). An indication of glucose repression is the observation that several fungal transcripts with putative roles in the lipid metabolism were down-regulated in the ECM tissue compared with those in the saprophytically growing mycelium. Among them was a homologue (T143B00615F, average fold–2.1) to the *Fox2* gene in *S. cerevisiae* encoding a peroxisomal multifunctional β -oxidation protein (Hiltunen et al. 1992). A *Fox2* homologue has previously been characterized in the endomycorrhizal fungus *Glomus mosseae*, which is down-regulated during interactions with rhizobacteria (Requena et al. 1999). Another down-regulated gene (T143B00264, average fold –2.1) in the *P. involutus*-birch ECM tissue with a role in lipid metabolism displayed sequence similarity to the *S. cerevisiae* *ISC1* gene encoding an inositol phosphosphingolipid phospholipase (sphingomyelinase) (32% identity over 154-aa residues) (Sawai et al. 2000). Two fungal transcripts that were also down-regulated showed sequence similarities to genes involved in the synthesis of membrane lipids, including a *PCT1* homologue (T143B02594F, average fold –3.7) encoding a putative cholinophosphate cytidyltransferase and an

OLE1 homologue (T143A02109F, average fold –5.1) encoding a putative fatty-acid desaturase (Stukey et al. 1990). In addition, a *Paxillus* homologue (T143B00617F, average fold –3.0) to the chorismate synthase (5-enolpyruvylshikimate-3-phosphate phospholyase) (EC 4.2.3.5) was down-regulated in the ECM tissue. This enzyme catalyzes the last step in the biosynthesis of chorismate in the shikimate pathway. Chorismate is a common precursor in the biosynthesis of several compounds, including aromatic amino acids, folate coenzymes, ubiquinone and secondary metabolites (Dosselaere and Vanderleyden 2001). Other enzymes involved in secondary metabolism are extracellular hydrolytic and oxidative saprotrophic enzyme systems, and a recent screening study demonstrated that genes for lignin peroxidase and manganese peroxidase (key enzymes in lignin degradation) are present in a broad taxonomic range of ECM fungi (Chen et al. 2001). We did not identify any fungal EST with sequence similarities to either lignin or manganese peroxidase, but a laccase homologue (T143B01984F, average fold –2.0) was detected down-regulated in mycorrhizal tissue as compared with saprophytically growing fungus. In addition to lignin and manganese peroxidases, laccase is also thought to contribute to lignin degradation, and it has been suggested that laccases produced by ECM fungi may be involved in a number of processes related to the functioning of the symbiosis, involving depolymerization and polymerization reactions of the plant cell wall (Burke and Cairney 2002). Glucose repression of laccase production has been observed in the white-rot basidiomycete *Phanerochaete chrysosporium* (Srinivasan et al. 1995), and considering the assumed carbon situation in the fungal ECM symbiont, laccase transcription may here be governed by a similar kind of regulation.

Cellular transport.

The most highly up-regulated fungal transcript (T143A00221, average fold +15.9) displayed a significant sequence similarity

Fig. 5. Structural comparison of various plant defensins. **A**, A multiple alignment of 13 plant defensins, including the *Betula pendula* polypeptide deduced from the expressed sequence tag information of clone T143C01218F. The consensus sequence shows major agreement between all sequences, which includes eight conserved cysteine residues. **B**, A phylogenetic tree for the same set of 13 different plant defensins, constructed using the neighbor-joining method (Saitou and Nei 1987).

to the zeta subunit of the coatomer complex including the *RET3* protein of *S. cerevisiae* (gi: 6325247). Coatomer is the major building block of the coat of COPI-coated vesicles. COPI-coated vesicles have been shown to be involved in several different transport steps within eukaryotic cells, including the anterograde transport of secretorial proteins from the endoplasmic reticulum (ER) to the Golgi body and between successive cisternae of the Golgi stack, in retrograde transport from the Golgi back to the ER, and in endosomal trafficking (Mallabiabarrena and Malhotra 1995). Through sequence similarities, we identified two other components of the vesicular transport system of *P. involutus* that, in contrast to the COPI subunit, were down-regulated in the ECM tissue. These transcripts include a *S. cerevisiae EMP24* (gi: 1177628) homologue (T143B00471, average fold -2.7) that encodes a subunit of the COPII-coated vesicles and a homologue (T143B05214F, average fold -2.3) to the *S. cerevisiae YPT1* gene (gi: 19112997) encoding a small GTPase. The COPII-coated vesicles selectively transport molecules from the ER to the Golgi and have a much more specialized function than the COPI-coated vesicles (Mallabiabarrena and Malhotra 1995). *YPT1* belongs to the rab family of ras-like GTPases, which are key components in the control of vesicular transport. In total, 11 rab GTPases have been identified in *S. cerevisiae* that differ in localization and function in vesicle transport. The *YPT1* GTPase is required for the docking of COPII-coated vesicles to the *cis*-Golgi membranes (Lazar et al. 1997). Apart from the *YPT1* homologue, we identified several other rab GTPases among the EST of *P. involutus*, including putative homologues to *YPT7*, *YPT31/YPT32*, and *YPT53*. None of the corresponding transcripts was significantly regulated during ECM formation.

Defense and stress response.

There are several reports indicating that mycorrhizal fungi can induce stress responses in plants similar to the responses induced by pathogenic fungi (Garcia-Garrido and Ocampo 2002; Lamb and Dixon 1997). The responses include the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radicals (OH^-), the induction of ROS detoxifying enzymes such as superoxide dismutase (SOD), catalase, and L-ascorbate peroxidase, and the synthesis of pathogenesis-related proteins such as chitinases and phenylpropanoid biosynthesis enzymes like phenylalanine ammonia-lyase (PAL). Additionally, H_2O_2 induces expression of polyubiquitin and glutathione S-transferase (GST), which detoxify oxidation products. Despite the fact that a substantial number of the analyzed EST displayed significant sequence similarities to proteins known to be involved in various stress responses, only a few of them showed differential regulation in the ECM tissue. For example, a fungal transcript (T143A01267F, fold +2.1) identical to a previously characterized *P. involutus* SOD cDNA (Jacob et al. 2001) was significantly up-regulated in the ECM tissue as compared with the free-living mycelium, whereas a plant *Populus tremuloides* SOD homologue (T143A01474F, average fold -1.1) was not differentially regulated as compared with axenic plant tissue. A previous study showed a transient increase in PAL activity, as measured by an enzymatic assay, and transient expression of the wound-inducible plant *Bet v1-SC1* gene when *B. pendula* was colonized by *P. involutus* (Feugey et al. 1999). In our study, a fungal PAL homologue (T143C02049F, average fold +1.1) was not differentially expressed in the ECM tissue, whereas significant up-regulation of a plant *Prunus avium Bet v1* homologue (T143A01633F, average fold +48.9) was detected. Another form of this allergen, the plant *Prunus avium Bet v1-SC3* homologue (T143C02841F,

average fold +1.3), was expressed at similar levels in the ECM tissue and nonsymbiotic roots. A significant increase in plant GST (EgHypar) transcription has been observed during the early development of a mycorrhizal association between *Pisolithus tinctorius* and *Eucalyptus globulus* (Nehls et al. 1998). On microarray, we assessed eight different plant GST homologues and observed a complex pattern of regulation (average fold range -3.7 to +1.9), some unaffected and some genes differentially regulated compared with the levels present in the axenically grown roots. Also, fungal GST homologues, 10 in total, showed a complex pattern of regulation (average fold range -2.4 to +1.8) as compared with axenic conditions. Neither plant polyubiquitin (T143C00277, average fold +1.0; T143C01692F, average fold -1.1) nor fungal polyubiquitin homologues (T143B01795F, average fold +1.0) were differentially expressed in the ECM tissue. Another family of proteins with a proposed function in various stress responses are metallothioneins (MT) (Kim et al. 2001; Palmiter 1998). We identified several plant MT genes that were up-regulated in ECM tissue (Table 4), and one of them, an *Actinidia deliciosa* MT homologue (T143A02009F, average fold +57.2), was highly up-regulated. The fungal partner also shows MT expression under mycorrhizal conditions, and we found an *Agaricus bisporus* MT homologue to be up-regulated (T143B00573, average fold +2.1), whereas another *Agaricus bisporus* MT homologue (T143B00624F, average fold +1.2) remained unregulated. Plant defensins are small highly basic cysteine-rich peptides. They are ubiquitous throughout the plant kingdom and inhibit the growth of a broad range of fungi, including various plant pathogens, and involve the binding of the defensin protein to specific fungal membrane targets (Thomma et al. 2002). A *Phaseolus coccineus* defensin (syn. Kunitz trypsin inhibitor protein) homologue (Table 4) (T143C01218F, average fold -4.8) shows a significant down-regulation in mycorrhizal tissue compared with uncolonized plant tissue. This result is intriguing and suggests a specific down-regulation in a plant antifungal defense system as a result of the mycorrhizal interaction. Plant defensins show a clear but limited sequence conservation restricted to the eight structurally important cysteines, and a multiple sequence alignment of various plant defensins shows the *B. pendula* variant to significantly match the overall structure, which also includes a signal peptide for extracellular secretion (Fig. 5A). Furthermore, it seems phylogenetically related to one of two *A. thaliana* defensin gene families (Thomma et al. 2002) (Fig. 5B).

Conclusion and perspective.

We have generated a large EST database containing sequence information and functional annotation of genes being expressed in the mycorrhizal fungus *P. involutus* and its host *B. pendula*. The EST library generated from the mycorrhizal root tissue contained a mixture of plant and fungal transcripts. A substantial part of these ESTs displayed no homology to protein sequences in the GenBank nr protein database. The plant or fungal origin for the majority of these orphans was determined using a combination of methods including clustering of EST sequences, analysis of GC content, and hybridizations.

Based on the EST data, a cDNA microarray was constructed, containing probes of 1,048 *P. involutus* genes and 1,021 *B. pendula* genes. Using this array, it was possible to demonstrate that the formation of the mycorrhiza root tissue affects the expression of a significant number of genes in both the fungal and plant partner, including genes with putative functions in developmental, metabolic, and defense responses.

MATERIALS AND METHODS

Biological material, media, and culture conditions.

Paxillus involutus (Batsch: Fr.) Fr. (ATCC 200175) was maintained on MMN agar medium with an increased amount of glucose (2.5 g/liter) (Brun et al. 1995). Dry seeds of *Betula pendula* (Skuleskogen, SkogForsk, Sweden) were stored at 8°C until used. A system for the formation of ectomycorrhiza between *B. pendula* and *P. involutus* has been described previously (Brun et al. 1995) and was used here but with reduced amounts of carbon (0.25 g/liter). The colonization and development of mycorrhiza was monitored microscopically, and the material was harvested at the time of the formation of the Hartig net (approximately 25 days after synthesis). Axenic cultures of *P. involutus* and *B. pendula* were grown under identical conditions.

cDNA libraries.

At harvesting, sample material from fungi, plant (discarding the green part of the seedling), and mycorrhiza (discarding the green part of the seedling and extraradical mycelium) was snap-frozen in liquid nitrogen and was ground in a precooled mortar with a pestle. Total RNA was isolated from 100 mg of the sample, using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and a guanidine hydrochloride lysis buffer, according to the manufacturer's instructions. Total RNA was finally eluted in 60 µl H₂O and was stored at -20°C until use. Three directional and full-length cDNA libraries were constructed from total RNA, using the PCR-based SMART cDNA library construction kit (BD Clontech, Palo Alto, CA, U.S.A.) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) for the first-strand cDNA synthesis. After ligation of cDNA to λTriplEx2 arms (BD Clontech) and lambda phage packaging (GigaPack III gold extract; Stratagene, La Jolla, CA, U.S.A.), the primary titers for the unamplified libraries were determined to be 4.9×10^6 PFU for mycorrhiza, 3.4×10^6 PFU for *P. involutus*, and 2.9×10^6 PFU for *B. pendula*. For amplification of the libraries, the phage suspensions were used to transduce *Escherichia coli* XL1-Blue, which resulted in titers of more than 1.0×10^{10} PFU/ml. The λTriplEx2 clones were mass-excised into pTriplEx2 plasmid clones by transduction into the Cre-recombinase-expressing strain *E. coli* BM25.8, according to the manufacturer's instructions (BD Clontech). The following protocol was used for mass excision. For each library and after the preparation of an *E. coli* BM25.8 overnight culture, 1.5 ml of cell suspension was transferred into 15 ml of fresh medium Luria Bertani (LB) broth (10 mM MgSO₄, 0.2% maltose) and was incubated at 31°C, 150 rpm until optical density at 600 nm equaled 0.7. MgCl₂ was added to a final concentration of 10 mM, and a total of 1.5×10^7 PFU of the amplified library stock was added. The culture was incubated at 31°C, 225 rpm for 1 h, and the cell suspension was then plated (1 ml/plate) on 150-mm LB agar petri dishes containing 50 µg/ml carbenicillin (Sigma-Aldrich, St. Louis). Plates were incubated overnight at 37°C, after which each petri dish received 12 ml of LB broth, and cells were collected. A total of 10 plates per library were processed, and after centrifugation, cells were resuspended in 10 ml of LB broth and an equal volume of freezing medium (60% [vol/vol] SOB [Sambrook et al. 1989], 40% [vol/vol] glycerol). Cells were aliquoted into Eppendorf tubes and were stored frozen at -80°C. The procedure yielded approximately 10^6 CFU/ml. The average transcript length in the cDNA libraries was estimated from the analysis of 96 individual clones from each of the three libraries. PCR amplification followed by gel electrophoresis showed that more than 90% of the randomly collected clones contained inserts. The average size of the inserts was approximately 850, 930, and

920 bp in the mycorrhiza, the *P. involutus*, and the *B. pendula* cDNA libraries, respectively. The size of the transcripts ranged from 300 to 2,400 bp in all three libraries.

EST analysis.

Plasmid clones were randomly collected from each of the three libraries, were plated on LB/amp agar, and were transferred to 150 µl of 0.1× TE buffer in 96-well microtiter plates. The cells were immediately lysed in a microwave oven and then stored frozen at -20°C until use. The bulk of the cDNA sequencing was automated, with bacterial lysate as the starting material for PCR amplification of the plasmid insert and with final 5' DNA sequencing on purified PCR products. Reactions were prepared on a Biomek 2000 (Beckman Coulter, Fullerton, CA, U.S.A.) in 96-well microtiter format. PCR amplifications were performed using pTriplEx2-specific universal primers P104 (5'-GGGAAGCGCGCCATTGTGTT-3') and P105 (5'-AGTGAGCTCGAATTGCGGCC-3') and standard PCR protocols, followed by assessment of the size and quality of the products by gel electrophoresis. Partial nucleotide sequences of the cDNA inserts were determined using the dideoxy chain-termination method (Sanger et al. 1977) employing the DYEnamic ET dye terminator kit (Amersham Biosciences, Uppsala, Sweden) and a biotinylated 5'-sequencing primer (P104). The sequencing products were purified on a magnetic workstation (Magnatrix 1200, Magnetic Biosolutions, Stockholm, Sweden), using M280-Streptavidin paramagnetic beads (Dyna, Oslo, Norway), and then loaded on a MegaBACE 1000 sequencer (Amersham Biosciences). Base-calling of DNA sequencer traces was conducted using the PHRED program with the quality level set to 20 (Ewing et al. 1998). After vector trimming and translation into all three forward frames, sequence comparisons against the GenBank nr protein database were performed using the FASTA program (Brenner et al. 1998; Pearson 1994). EST from all three stages were assembled using the contig assembly program (CAP) (Huang 1992). CAP parameters were optimized for the EST sequence data and the following values were set: OVERLEN = 20; PERCENT = 0.85; CUTOFF = 40; DELTA = 8.5; OPEN = 0; and POS5 = 20. Based on sequence homology, the ESTs were assigned functional roles and EC numbers (when applicable). Functional roles were classified into categories according to catalogues used for the *S. cerevisiae* genome, provided by MIPS (Mewes et al. 2000). All EST information was stored in a relational database (using MySQL) on a Linux (Red Hat 6.2) platform, and the data were processed using the PHOREST tool (Ahren et al. 2003).

DNA sequence information from the fungal and the plant EST libraries was analyzed to elucidate differences in GC content between *P. involutus* and birch. Each library was analyzed by selecting one EST representative from a number of contigs, with the limit set to a sequence length of more than 500 bp and with no trimming for 5' or 3' untranslated regions, using the GeeCee software. Due to the fact that the GC content of both *P. involutus* and *B. pendula* transcripts was significantly different from normal distributions (Lilliefors test, $P < 0.01$, in both cases), the proportion of plant transcripts in the mycorrhizal EST was calculated using a maximum likelihood method. In the estimation procedure, we used the empirical distributions of *P. involutus* and *B. pendula* GC content (Fig. 2). Due to this, we had to exclude one mycorrhizal sample (GC content = 0.31), since there was no such observation in either the plant or fungal library.

Construction of cDNA arrays.

A single EST cDNA clone was selected to represent each assembled sequence (putatively unique transcript). For an

assembled sequence represented by multiple EST, the rule followed was to select the clone with the most extensive DNA sequencing read length. These clones were collected from the master EST library stock bacterial lysate plates, and aliquots were transferred into new 96-well plates. Plasmid inserts were amplified by PCR, using 5 µl of bacterial lysate in 100-µl volume reactions, and each clone was amplified in two independent PCR reactions. Amplification was performed in an ABI 9700 thermocycler (Applied Biosystems, Foster City, CA, U.S.A.), and each reaction contained 10 µl of 10× PCR buffer (100 mM Tris-HCl, pH 8.0, 500 mM KCl), 3.0 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM each of primers P104 and P105, and 2.0 U Taq polymerase (AmpliTaQ Gold, Applied Biosystems). The amplification conditions were 95°C for 7 min, followed by 31 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2.5 min, followed by one cycle at 72°C for 7 min. Each PCR product (5 µl) was analyzed on a 1.2% agarose gel. In the case of weak or multiple bands, the clone was replaced by another clone of the same contig, if available. The two replicates of each PCR reaction were pooled and amplicons were purified and concentrated on 96-well multiscreen PCR plates (Millipore, Bedford, MA, U.S.A.), according to the manufacturer's instructions. After filtering, the PCR products were resuspended in 60 µl of H₂O and were transferred to new 96-well plates. From these PCR backup plates, 30 µl were transferred into 384-well microplates, and an equal volume of dimethyl sulphoxide (Sigma-Aldrich) was added. Plates were sealed and stored at -20°C until printing. From a uniset of 2,284 assembled sequences, 2,161 clones were successfully processed. cDNA PCR products were printed on GAPSII-coated slides (Corning Glass, Corning, NY, U.S.A.), using a 16-pin configured MicroGrid II array printer (BioRobotics, Cambridge) controlled by the MicroGrid TAS Application Suite (ver. 2.2.0.6). The general design of the microarrays was two identical tool arrays, each containing 16 blocks (sub-arrays) and with each clone replicated twice within each block, which provided on-chip quadruplicates for each clone. Additionally, various positive and negative control probes were spotted as multiple replicates in various positions within each block, such as positive homologous controls, i.e., *P. involutus* and *B. pendula* genes as well as negative heterologous controls such as human probes. Also included in the blocks were eight different heterologous and commercial PCR probes (ArrayControl; Ambion, Austin, TX, U.S.A.) for which complementary RNAs were spiked into the amplification and labeling process of targets. Each control probe was present in up to 64-per-chip replicates. DNA was cross-linked to the slides by baking at 80°C for 2 h in glass jars, followed by UV cross-linking at 90 mJ/cm². Spot and printing quality were assessed by staining with POPO3 dye (Molecular Probes, Eugene, OR, U.S.A.), and subsequent scanning showed no spots missing or joined on the slides.

cDNA microarray analysis.

Microarray analysis was performed on two independent biological replicates (biological replicates 1 and 2) grown under identical conditions. Total RNA was extracted as described above from mycorrhizal root tips (developed after 25 days of synthesis) and from axenically grown mycelium and plant roots of *P. involutus* and *B. pendula*. The RNA preparation for biological replicate 1 was identical to that used for construction of the fungal, plant, and mycorrhizal EST libraries. Antisense RNA (aRNA) was synthesized by T7 polymerase in vitro transcription, using one or two rounds of amplification (MessageAmp; Ambion). The aRNA was analyzed by gel electrophoresis, and the titer was determined spectrophotometrically. For dual color labeling (Cy3 and Cy5)

of targets the CyScribe post-labeling kit was used (Amersham Biosciences). A total of 1 µg of aRNA for each target was used, including exogenous spikes according to Table 2 (Array Control). After cDNA synthesis and labeling, targets were finally purified using the QIAquick PCR purification kit (Qiagen). After purification the Cy3- and Cy5-labeled targets were combined in an amber Eppendorf tube, and after evaporation, labeled cDNA was dissolved in 6 µl of H₂O. After denaturation at 95°C for 2 min and a short period of incubation on ice, 1.5 µl of dT₈₀ (1 mg/ml) was added, followed by incubation at 75°C for 45 min. cDNA was mixed with an equal volume of hybridization buffer (Amersham Biosciences), and 15 µl of 100% formamide was added. The hybridization mixture was pipetted onto the slide and was sealed under a LifterSlip cover slip (Erie Scientific Company, Portsmouth, NH, U.S.A.). Slides were placed in humidified CMT hybridization chambers (Corning) and were incubated in a hybridization oven at 42°C overnight. After hybridization the slides were rinsed with a series of 5-min washes in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% sodium dodecyl sulfate (SDS) at 55 °C; 0.1× SSC, 0.2% SDS at 55 °C; 0.1× SSC, 0.2% SDS at room temperature; and 0.1× SSC at room temperature. Finally, the slides were rinsed in water and were dried by a short centrifugation. The slides were scanned using an Axon 4000A scanner, and images were analyzed using GenePix Pro software (ver. 3.0.6.89) (Axon Instruments, Union City, CA, U.S.A.). For spotted probes to be included in the normalization and analysis, each spot had to satisfy three quality criteria: i) the spot signal intensity had to be within the dynamic range of the photomultiplier tube (the photomultiplier gain was determined and adjusted by the user aiming at equal distribution of signal intensities for both channels on each slide); ii) each spot had to be of high quality as decided by manual ocular inspection of each slide; and iii) the signal intensity in each spot had to be significantly greater than the local background (namely, in one of the two channels scanned, the signal intensity had to be greater than the twofold average background signal intensity for each slide). Raw median signal and background pixel intensities were further processed using GeneSpring software (ver. 5.1 build 1096) (Silicon Genetics, Redwood City, CA, U.S.A.). For normalization, a Lowess curve was fit to the log-intensity versus log-ratio plot. A portion of the data (20%) was used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control value was lower than 10, then 10 was used instead. In the experiments performed to analyze fungal and plant gene expression in mycorrhizal root tissues, the signal intensities were also corrected for the 7:3 representation of fungal and plant transcripts in the mycorrhizal transcriptome by multiplying fold values for fungal and plant genes with a factor of 1.43 and 3.33, respectively.

Northern dot blot analyses.

A selection of 38 fungal and 38 plant cDNA clones were PCR amplified, using plasmid and universal primers (P104 and P105). Products were purified and 400 ng of each amplicon were denatured in 400 µl of 10× SSC, 0.2 M NaOH by incubation for 10 min at room temperature. The denatured DNA was split and then blotted on two separate Hybond-N+ membranes (Amersham Biosciences). In order to normalize the hybridization intensities between the two membranes, a serial dilution of heterologous DNA (in seven dilutions ranging from 1 to 200 pg) was treated in the same way as the sample PCR products and was spotted in parallel onto the membranes. The probes were prepared from 20 ng of either fungal or mycorrhizal cDNA (biological replicate 1), were synthesized

from aRNA after amplification from total RNA (MessageAmp, Ambion), and were spiked with 5 ng of heterologous DNA before [α - ^{32} P]dCTP labeling using a random-primer labeling protocol (RediPrime, Amersham Biosciences). The probes were added to the membranes, and hybridization was performed at 68°C overnight, after which the membranes were washed. The conditions for hybridization and washing were those recommended by the manufacturer (Amersham Biosciences). Membranes were imaged onto phosphor screens and were analyzed using a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). From the spike signal intensities, a standard curve was produced for each membrane and was used to normalize sample signal intensities between membranes. After correction for the 7:3 representation of fungal and plant transcripts in the mycorrhizal transcriptome (see section above), signal intensity ratios were calculated for the mycorrhiza versus fungus comparison.

Estimation of the proportions of fungal and plant transcripts in the mycorrhizal transcriptome.

Besides estimations of the fungal and plant representation in mycorrhizal transcriptome from the GC distribution among EST contigs, the total signal intensities for fungal and plant probes were investigated by both Northern and microarray analyses. From the Northern analysis of mycorrhizal tissue, the total signal intensities for 38 fungal genes ($[F_i]$) and for 38 plant genes ($[P_i]$), respectively, were calculated. To then estimate the fraction of plant transcripts in the mycorrhizal transcriptome, the formula $[P_i]/([P_i]+[F_i])$ was used. Similarly, from microarray analysis, the total median intensity in the mycorrhizal channel for 1,048 fungal and 1,021 plant probes were calculated and used for estimation of plant representation in the mycorrhizal transcriptome.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Codoncode Corporation software solutions for DNA sequencing PHRED software: www.codoncode.com
- The European Molecular Biology Open Software Suite GeeCee software: www.emboss.org
- Munich Information Center for Protein Sequences (MIPS): mips.gsf.de
- MySQL open source database: www.mysql.com
- National Center for Biotechnology Information (NCBI) GenBank database: www.ncbi.nlm.nih.gov
- R Project for statistical computing software: www.r-project.org/
- Significance of digital gene expression profiles (Audi and Claverie 1997): igs-server.cnrs-mrs.fr/~audic/significance.html