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Published in:
Molecular Ecology

DOI:
10.1111/j.1365-294X.2004.02369.x

2004

Link to publication

Citation for published version (APA):
Le Quéré, A., Schutzendubel, A., Rajashekar, B., Canbäck, B., Hedh, J., Erland, S., Johansson, T., & Tunlid, A. (2004). Divergence in gene expression related to variation in host specificity of an ectomycorrhizal fungus. *Molecular Ecology*, *13*(12), 3809-3819. https://doi.org/10.1111/j.1365-294X.2004.02369.x

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Molecular Ecology (2004) **13**, 3809–3819 doi: 10.1111/j.1365-294X.2004.02369.x

Divergence in gene expression related to variation in host specificity of an ectomycorrhizal fungus

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Abstract

Ectomycorrhizae are formed by mutualistic interactions between fungi and the roots of woody plants. During symbiosis the two organisms exchange carbon and nutrients in a specific tissue that is formed at the contact between a compatible fungus and plant. There is considerable variation in the degree of host specificity among species and strains of ectomycorrhizal fungi. In this study, we have for the first time shown that this variation is associated with quantitative differences in gene expression, and with divergence in nucleotide sequences of symbiosis-regulated genes. Gene expression and sequence evolution were compared in different strains of the ectomycorrhizal fungus *Paxillus involutus*; the strains included Nau, which is not compatible with birch and poplar, and the two compatible strains Maj and ATCC200175. On a genomic level, Nau and Maj were very similar. The sequence identity was 98.9% in the 16 loci analysed, and only three out of 1075 genes analysed by microarray-based hybridizations had signals indicating differences in gene copy numbers. In contrast, 66 out of the 1075 genes were differentially expressed in Maj compared to Nau after contact with birch roots. Thirty-seven of these symbiosis-regulated genes were also differentially expressed in the ATCC strain. Comparative analysis of DNA sequences of the symbiosis-regulated genes in different strains showed that two of them have evolved at an enhanced rate in Nau. The sequence divergence can be explained by a decreased selection pressure, which in turn is determined by lower functional constraints on these proteins in Nau as compared to the compatible strains.

Keywords: comparative genomics, ectomycorrhiza, gene expression, host specificity, microarray

Received 22 June 2004; revision received 9 September 2004; accepted 9 September 2004

Introduction

It has been estimated that at least 6000 species of fungi, primarily basidiomycetes with some ascomycetes and zygomycetes, form mutualistic relationships with woody plants (Malloch *et al*. 1980). These ecologically very important associations are called ectomycorrhizae and are the dominant mycorrhizal type associated with trees in temperate and boreal ecosystems (Smith & Read 1997). In ectomycorrhizae, the fungal partner obtains photosynthetic sugars from the host plant while, in return, the plant receives mineral nutrients from the fungus. The exchange of nutrients occurs in a specific symbiotic tissue that is formed between the fungal hyphae and the host roots. This tissue consists of a mantle, which develops from the fungal hyphae surrounding the root, and a Hartig net, which is formed by the hyphae penetrating between the outer cells of the root (Smith & Read 1997).

Analyses of DNA-based phylogenies have shown that the ancestors of the ectomycorrhizal homobasidiomycetes were free-living and that mycorrhizal symbionts have evolved repeatedly from saprophytic precursors (Hibbett *et al*. 2001). Many of these basidiomycetes form symbioses with several host species. Furthermore, it is known that fungi can differ markedly in their ability to form ectomycorrhizae and to promote the growth of the host plant (Smith & Read 1997). Screenings, mainly based on records

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of sporocarp–plant associations, have shown that ectomycorrhizal fungi include both generalist and specialist species (Trappe 1962). Studies have also demonstrated that the magnitude of intraspecific differences in host preferences can be as large as the between-species differences (Smith & Read 1997; Cairney 1999). This variation is associated with differences in the ability to form the mantle and Hartig net. The molecular basis for the differential infectivity is not clear, but it has been suggested to reside in the capacity to produce hormones, extracellular enzymes and not yet identified recognition factors (Cairney 1999). Furthermore, it has been shown that the patterns of polypeptides produced by strains of the ectomycorrhizal fungus *Pisolithus tinctorius* are correlated with the infectivity of the strain (Burgess *et al*. 1995).

The genomic mechanisms that could account for the variation in host preferences between species or strains of ectomycorrhizal fungi are, however, not known. Generally such phenotypic differences could be the result of variations in gene content, of quantitative differences in gene expression, or of structural differences in gene products (King & Wilson 1975; Wray *et al*. 2003). DNA microarray technology has opened up new possibilities of comparing transcript abundance between closely related species and/or strains, and of identifying genes that are associated with morphological and physiological divergence (Ferea *et al*. 1999; Jin *et al*. 2001; Enard *et al*. 2002). In addition, DNA microarray-based comparative genomic hybridization can be used to assess genome rearrangements like amplification or deletion at single gene resolution, which might play an important role in adaptive evolution (Hughes *et al*. 2000; Dunham *et al*. 2002).

We have used a cDNA microarray to examine the differences in gene expression between incompatible and compatible strains of the ectomycorrhizal fungus *Paxillus involutus* (Basidiomycetes; Boletales). This fungus is widespread in the northern hemisphere and forms ectomycorrhizae with many species of coniferous and deciduous trees (Wallander & Söderström 1999). Strains of *P. involutus* are known to differ in their ability to form ectomycorrhizal associations with various hosts (Laiho 1970; Gafur *et al*. 2004). In this study we included a strain Nau that was originally isolated from oak in France (Marschner *et al*. 1999). It was recently shown that Nau, in contrast to several other strains of *P. involutus*, does not infect poplar *in vitro* (Gafur *et al*. 2004). For the comparative analysis, we identified the closest evolutionary relative of Nau to be the poplar-compatible strain Maj. In addition, we included a third strain ATCC 200175 (henceforward called ATCC), in which gene regulation during the formation of ectomycorrhizae on birch (*Betula pendula*) has previously been studied (Johansson *et al*. 2004). The cDNA array contained cDNA probes putatively representing 1075 *P. involutus* genes. The probes were obtained from a nonredundant set of expressed sequence tags (ESTs) produced either from cDNA libraries of the ectomycorrhizal tissue formed between the ATCC strain and birch mycelium or from saprophytically grown mycelia of the ATCC strain (Johansson *et al*. 2004). 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 7700 (Le Quéré *et al*. 2002).

Materials and methods

Biological material, media and culture conditions

The strains of *Paxillus* (Table 1) were maintained on Modified Melin-Norkrans (MMN) agar medium (Brun *et al*. 1995). An axenic system for the formation of ectomycorrhizae between *Betula pendula* (Skuleskogen, SkogForsk, Sweden) and *Paxillus involutus* was used with reduced amounts of carbon (0.25 g glucose/L) (Brun *et al*. 1995). The association was grown under controlled conditions of 22 °C day/15 °C night temperatures, a day/night period of 15 h/9 h, and 60% humidity. The material was harvested after 14 days. After fixation the fungal–root tissues were examined under a light microscope (Axioplan, Carl Zeiss) (Gafur *et al*. 2004).

cDNA microarray, labelling and hybridization analysis

Microarrays had previously been constructed (Johansson *et al*. 2004; Le Quéré & Wright *et al*. manuscript in preparation) using a nonredundant set of 2159 ESTs, either of fungal (1075 ESTs) or plant (1074 ESTs) origin. The plant ESTs, and 10 ESTs with uncertain origin, were excluded from this investigation. The mycorrhizal tissue (Maj and ATCC) or root tips with attached hyphae (Nau) was harvested and total RNA was isolated as described (Johansson *et al*. 2004). Antisense RNA (aRNA) was synthesized by T7 polymerase *in vitro* transcription using one round of amplification (MessageAmp, Ambion (Europe) Ltd). The aRNA was analysed by gel electrophoresis and the titre was determined spectrophotometrically. The aRNA targets were labelled (Cy3 or Cy5) and the microarray slides were hybridized and scanned as previously described (Le Quéré, Wright *et al*. manuscript in preparation). The experiments were designed as two-sample comparisons (ATCC and Maj; ATCC and Nau; Maj and Nau) using three independent biological replicates, including technical and dye-swapped control experiments.

Statistical analysis

Data images were manually inspected and low-quality spots were excluded from further analyses. Reporters

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Table 1 Strains of *Paxillus* used for ITS phylogeny

Order	Species and strain name	Location	Habitat	Woody plants within a 10-m radius	Compatibility in laboratory	Accession number
Paxillus involutus						
1	Pi11	Germany, Bavaria		*Picea*		AF167698†
2	Pi2	Germany, Bavaria		*Fagus*, *Quercus*		AF167699†
3	Pi3	Germany, Bavaria		*Salix*		AF167695†
4	PiM3	Germany, Bavaria		*Fagus*		AF167700†
5	Pi9H	Germany, Bavaria		*Picea*		AF167696†
6	SE03-09–2808	Sweden, Småland	Pinus heath forest	*Pinus*, *Picea*, *Betula*		AY585920*
7	Pi10H	Germany, Bavaria		*Betula*		AF167697†
8	Pi01SE	Sweden, Viken	Pine sand forest	*Pinus*		AY585918*
9	Pi08BE	Belgium, Maatheide			*Pinus* (+), *Populus* (+), *Picea* (+)	AY585919*
10	ATCC200175	Scotland		*Betula*	*Betula* (+), *Picea* (+), *Pinus* (+), *Fagus* (+)	AY585913*
11	SE03-10–0903	Sweden, Torna Hällestad	Fagus forest	*Sorbus*, *Fagus*, *Betula*, *Quercus*		AY585916*
12	PAO03-09–1401	Sweden, Hässleholm	Mixed forest	*Pinus*		AY585914*
13	SE03-10–0501	Sweden, Kjugekull	Mixed tree stand on pasture	*Fagus* (*Fraxinus*), *Betula*, *Quercus*, *Corylus*		AY585921*
14	HW03-09–2501	Sweden, Torna Hällestad	Road side outside spruce hedge	*Betula*, *Picea*		AY585922*
15	SE03-07–1610	Sweden, Lund	Small grove on public lawn	*Populus* (*Cornus*), *Tilia*, *Acer*, *Fagus*, *Quercus*, *Sorbus*		AY585912*
16	Pi12	Germany, Bavaria		*Corylus*		AF167691†
17	PiM4	Germany, Bavaria		*Betula*		AF167693†
18	PiM1	Germany, Bavaria		*Quercus*		AF167692†
19	PiM2	Germany, Bavaria		*Populus*		AF167694†
20	SE03-07–1622	Sweden, Lund	Small grove on public lawn	*Populus* (*Cornus*), *Tilia*, *Acer*, *Fagus*, *Quercus*, *Sorbus*		AY585911*
21	Pi1	Germany, Bavaria		*Tilia*		AF167690†
22	SE03-07–1001	Sweden, Lund		*Populus* (*Cornus*), *Tilia*, *Acer*, *Fagus*, *Quercus*, *Sorbus*		AY585910*
23	Nau	France		*Quercus*	*Betula* (–), *Populus* (–)	AY585915*
24	Maj	France		*Populus*	*Populus* (+), *Picea* (+)	AY585917*
Paxillus vernalis						
25	Pv2	Canada				AF167689†
Paxillus filamentosus						
26	304	Germany, Bavaria		*Alnus*		AF167687†
27	133	Germany, Bavaria		*Alnus*		AF167688†
Melanogaster broomeianus						
28	Mbr1			*Tilia*		AF098383‡

Data taken from: *this study, †(Jarosch & Bresinsky 1999), and ‡NCBI.

related to *B. pendula* (Le Quéré, Wright *et al*. manuscript in preparation) were excluded and only data for reporters representing fungal and control genes were extracted. For those spots remaining, the raw fluorescence intensities for each channel on each slide were collected. For each channel, the mean background fluorescence was calculated. After local background correction for each spot, the reporters yielding intensities less than twice the background intensity were excluded and the fluorescence for the remaining reporters was multiplied to give a common channel mean of 5000 fluorescence units for each slide. As a result, data for 1022 reporters remained in the data set. The statistical approach, the mixed model analyses of variance (ANOVA) (Wolfinger *et al*. 2001), served two purposes: first, normalization of the data to remove systemic biases that may have affected all genes simultaneously, such as differences in the amount of RNA that was labelled for a particular replicate of a treatment; and second, assessment of the contribution of biological and experimental sources of error to the variation in the expression of each individual gene. This procedure used differences in normalized expression levels, rather than ratios, as the unit of analysis of expression differences. We subjected the corrected $\log_2$-transformed measures (y_{gij}) for the gene g ($g = 1, \ldots, 1022$), which included scores for 50 573 fungal spot measures, to a normalization model of the form: $y_{gij} = \mu + A_i + D_j + (A \times D)_{ij} + \varepsilon_{gij}$, where μ is the sample mean, A_i is the effect of the i^{th} array ($i = 1$–11), D_j is the effect of the j^{th} dye (Cy3 or Cy5), $(A \times D)_{ij}$ is the array–dye interaction (channel effect) and ε_{gij} is the stochastic residual. We then subjected the residuals from this model, which can regarded as a crude indicator of the relative expression level (and are referred to in the text as 'normalized expression levels'), to 1015 gene-specific models of the form: $r_{ijl} = \mu + A_i + S_l + D_j + \varepsilon_{ijl}$, where S_l is the l^{th} strain [ATCC, Maj, or Nau; two degrees of freedom (d.f)]. In the gene models, which were fitted using PROC MIXED in SAS (SAS/STAT Software Version 8, SAS Institute Inc.), the A_i variable controls for spot effects and is random (10 d.f., leaving 8 d.f. for the residual error). The microarray data are available at the EBI-EMBL ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) (accession number E-MEXP-179).

The overall distance between the expression profiles of two strains was calculated using the hybridization values of the transcriptome profiles as described by Enard *et al*. (2002). Briefly, the distance between the samples was calculated by summing the $\log_2$ values of the hybridization–signal ratios for genes that were expressed in all comparisons. The resulting distance matrix was used to build neighbour-joining trees (Saitou & Nei 1987) using the PHYLIP program (Felsenstein 1993). In a similar way, the genomic distances between the strains were calculated using data from comparative genomic hybridizations. In this experiment, DNA from the different strains was cohybridized (ATCC and Maj; ATCC and Nau; Maj and Nau) to cDNA microarrays that were identical to those used for the expression studies (Le Quéré, Rajashekar *et al*. manuscript in preparation).

DNA sequencing

For complete DNA sequencing of the cDNAs representing candidate genes, plasmid clones were selected from a collection of EST clones constructed in the pTriplEx vector (BD Clontech) and maintained as bacterial lysates (Johansson *et al*. 2004). The bacterial lysate was used for retransformation into *Escherichia coli* DH5α and transformants were verified by standard procedures. Plasmids were prepared and used as starting material for DNA sequencing using the dideoxy chain-termination method, employing the BigDye Terminator Kit (Applied Biosystems) and either using the pTriplEx2-specific universal forward primer P104 (5′-GGGAAGCGCGCCATTGTGTT-3′), the reverse primer T23V (5′-T_{23}V-3′, V = A, G or C), or template-specific primers. The polymerase chain reaction (PCR) products were purified by isopropanol precipitation and finally loaded onto an ABI3100 DNA sequencer (Applied Biosystems). In addition, genomic regions of 17 different loci were PCR amplified and sequenced from different *Paxillus* strains using template specific primers (Table 2). Sequence information was validated and assembled using the SEQUENCHER 3.0 software (Gene Codes Corporation).

Phylogenetic analysis

To examine the phylogenetic relationship between the *Paxillus* strains (Table 1), the internal transcript spacer region (ITS) of the rDNA was amplified by PCR and sequenced in both directions using the primer pair ITS1–ITS4 (White *et al*. 1990). To construct phylogenetic trees of the *cipC*, *cchA* and *rabA* genes, we retrieved homologous genes from GenBank (Benson *et al*. 2004), the COGEME EST database (Soanes *et al*. 2002) and from the genome database of *Phanerochaete chrysosporium* (http://www.jgi.doe.gov) using BLAST (Altschul *et al*. 1990) searches with the ATCC strain as query sequence. The sequences were then aligned using CLUSTAL-W (Thompson *et al*. 1994). Regions with ambiguous sites were removed manually from the amino acid or nucleotide sequence alignments by SEAVIEW (Galtier *et al*. 1996). Phylogenetic trees of nucleotide sequence alignments for the ITS region, *cipC* and *cchA* genes were calculated by the maximum likelihood method using PAUP (Swofford 1998). A bootstrap of 100 replicates was generated in all three cases. The software MODELTEST (Posada & Crandall 1998) was used to evaluate the appropriate models and parameter values used in the likelihood analysis. An unrooted phylogenetic tree of the *rabA* gene was constructed using PHYLO_WIN software (Galtier *et al*. 1996). Protein

Table 2 Genes of *Paxillus* used for estimating per cent identity* and d_n/d_s analysis†

Gene	GenBank homologue	Strains analysed	Alignment length	Regulation in ECM	Accession number
actA	Actin 1 (gi:14194451) [*Schizophyllum commune*]	6‡	482	+ ‡‡	AY585949,6027–31§§
β-tubA	beta tubulin 2 (gi:23477228) [*Suillus bovinus*]	6‡	316		AY585948,6022–26§§
calA	Calmodulin (gi:18150814) [*Paxillus involutus*]	5§	432	+ ‡‡	AY586017–21§§, AAD17455***
cipC1	CipC protein (gi:20429042) [*Emericella nidulans*]	5§	256	+ ‡‡¶¶	AY586008–12¶¶, CAC87272***
cipC2	CipC protein (gi:20429042) [*Emericella nidulans*]	6‡	234	+ ‡‡¶¶	AY585947,6003–7¶¶
cchA	copper chaperone (gi:25956321) [*Trametes versicolor*]	4¶	156	+ ¶¶	AY586013–16¶¶, AAN75572***
ppiA	putative cyclophilin (gi:16943775) [*Pleurotus ostreatus*]	6‡	444	+ ‡‡	AY585941,60–64 §§
gpiA	Glc-6-P isomerase (gi:40713647) [*Agaricus bisporus*]	6‡	1483		AY585946,5998–6002 §§
lecA	lectin (gi:18478668) [*Xerocomus chrysenteron*]	5§	354	+ ‡‡¶¶	AY585973–77¶¶, AAL73235***
gstA	glutathione S-transferase (gi:1353751) [*Naegleria fowleri*]	5§	509	+ ‡‡¶¶	AY585993–97¶¶, XP_391216***
hetC1	het-c2 protein (gi:2133321) [*Podospora anserina*]	6‡	508	+ ‡‡	AY585945,88–92§§
hxtA	hexose transporter (gi:6715095) [*Aspergillus parasiticus*]	5§	1532	+ ‡‡	AY585978–82§§, XP_391358***
hspA	small heat shock protein (gi:21950718) [*Laccaria bicolor*]	6‡	453		AY585944,83–87§§
micA	mitochondrial carrier protein (gi:19075302) [*Schizosaccharomyces pombe*]	4**	770	+ ‡‡	AY585943,70–72§§
ndkA	nucleoside diphosphate kinase (gi:32405858) [*Neurospora crassa*]	6‡	427	+ ‡‡	AY585942,65–69§§
ptrA	inorganic phosphate transporter (gi:586531) [*Saccharomyces cerevisiae*]	6‡	473	+ ††	AY585940,55–59§§
rabA	small GTPase (gi:7108528) [*Mus musculus*]	5§	485	+ ¶¶	AY585950–4¶¶, EAK82432***

ECM, ectomycorrhiza.
*All genes (except *micA*) from ATCC, Maj and Nau strains were used for per cent identity estimation.
†β-*tubA*, *gpiA*, *hspA* and *micA* were not used for d_n/d_s analysis.
‡Isolates ATCC200175, Pi01SE, Pi08BE, Maj, Nau and Pf01DE.
§Isolates ATCC200175, Pi01SE, Pi08BE, Maj and Nau.
¶Isolates ATCC200175, Pi08BE, Maj and Nau.
**Isolates ATCC200175, Pi01SE, Pi08BE and Pf01DE.
††Taken from Johansson *et al*. 2004.
‡‡Taken from Le Quéré, Wright *et al*. (manuscript in preparation).
§§Taken from Le Quéré, Rajashekar *et al*. (manuscript in preparation).
¶¶This study.
***Used as outgroup in d_n/d_s analysis.

alignments were made using CLUSTAL-W (Thompson *et al*. 1994) and a neighbour-joining method (Saitou & Nei 1987) with 500 bootstrap replications was used to develop the phylogeny.

Calculation of substitution rates

Nucleotide sequence information for 13 *Paxillus* coding regions (Table 2) was translated and the protein alignments for each gene were made by CLUSTAL-W (Thompson *et al*. 1994). Then the protein alignment was used as a template to align the corresponding coding nucleotide sequences by TRANALIGN of EMBOSS (Rice *et al*. 2000). The rates of nonsynonymous (d_n) and synonymous (d_s) nucleotide substitutions per site were estimated using the CRANN software (Creevey & McInerney 2003). The presumed species tree used in the input to CRANN was based on the ITS phylogeny.

Results

Phylogeny

To identify the closest evolutionary relative to Nau, an ITS phylogenetic tree using a number of strains and isolates of *Paxillus* was constructed (Fig. 1). The strain Maj that has been shown to be compatible with poplar (Gafur *et al*. 2004) was recognized as the closest relative to Nau. The Maj and Nau strains formed a clade with high bootstrap support (value 89) within the so-called 'park' group of *Paxillus involutus* strains (Fries 1985; Jarosch & Bresinsky 1999). The ATCC strain was found in another clade with high bootstrap support (value 83). This clade contained strains collected in various coniferous or mixed forests. This comprises the 'forest' group of *P. involutus* (Fries 1985; Jarosch & Bresinsky 1999).

The close genetic relationship between Maj and Nau was also evident from the DNA sequences of 16 loci (Table 2),

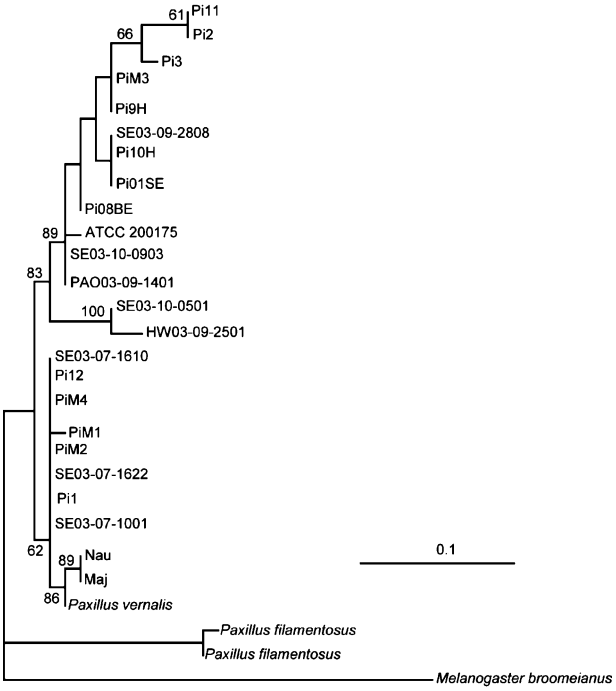

Fig. 1 Phylogenetic relationships between strains of *Paxillus involutus*. The maximum likelihood tree for the ITS region is shown. Values at nodes represent the bootstrap support value in per cent for 100 replicates. Strains are listed in Table 1.

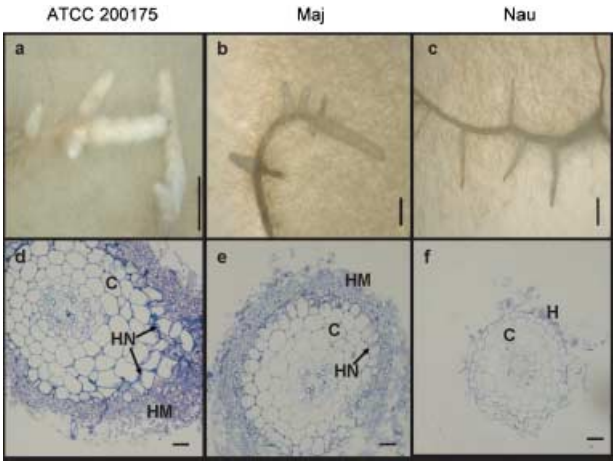

Fig. 2 Interactions between *Paxillus involutus* and birch (*Betula pendula*). The birch seedlings were grown for 14 days on cellophane-covered agar plates together with three different strains of *P. involutus*: (a, d) ATCC; (b, e) Maj; (c, f) Nau. Upper panel shows micrographs taken under a binocular microscope with tenfold magnification (bar = 1 mm). Lower panel shows cross-sections embedded as described in the Materials and methods. Micrographs of cross-sections were taken under a microscope (bar = 30 μm). Note that both the ATCC and the Maj strain, but not Nau, have colonized the plant root by forming a pseudoparenchymatous mantle (HM). From this structure the fungus has penetrated between the outer epidermal cells of the root forming the Hartig net (HN) [(C) cortical cells; (H) fungal hyphae].

which showed a 98.9% identity between Maj and Nau. The identities between ATCC and Maj, and ATCC and Nau in these loci were 95.6 and 95.2%, respectively.

Phenotypic differences

After 2 weeks of interaction, the ATCC and Maj strains of *P. involutus* colonized the roots of birch seedlings and developed a typical ectomycorrhizal tissue, as visualized by the hampered elongation and swelling of the fine roots, and the presence of a pseudoparenchymatous mantle. In addition, the hyphae of these strains had penetrated between the outer epidermal cells of the root and formed the Hartig net where nutrients and carbohydrates are exchanged between the two symbiotic partners (Fig. 2a,b,d,e) (Brun *et al*. 1995). In contrast, the Nau strain did not develop such a mycorrhizal interface (Fig. 2c,f). Thus the differences in compatibility between Maj and Nau on birch were similar to those observed on poplar (Gafur *et al*. 2004). It is not known whether the examined isolate of Nau has completely lost its ability to form ectomycorrhiza with all potential hosts including oak.

Differences in gene expression

The patterns of gene expression for the ATCC, Maj and Nau strains after 14 days of contact with the birch were compared using cDNA microarrays. In total, 926 (86%), 747 (70%), and 747 (70%) of the 1075 fungal genes spotted on the cDNA arrays produced signal intensities above the twofold average background threshold in the hybridizations comparing ATCC and Maj, ATCC and Nau and Maj and Nau, respectively. The relative rates of evolutionary change in the transcriptomes of the three strains were estimated by summing the $\log_2$ values of the ratios of the hybridization signals for genes that were commonly expressed in all comparisons (Fig. 3). These analyses showed that the transcriptome distance between ATCC and Maj was slightly shorter than the distance between ATCC and Nau.

Following statistical analyses, we identified 66 genes that differed significantly in expression levels between the closely related compatible Maj and the incompatible Nau strain (Table 3). A subset of these genes [in total 37 genes, 36 being up-regulated and one down-regulated (EST clone CD273016)] was also differentially expressed in the compatible ATCC strain as compared to Nau. We considered that these commonly regulated genes in Maj and ATCC were associated with the colonization and development of the symbiotic ectomycorrhizal tissue. A visual presentation of the expression patterns of the 37 symbiosis-related genes is presented in Fig. 4 (left panel). Notably, 20 of them

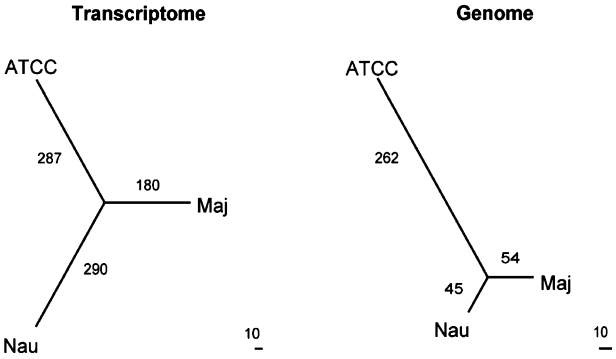

Fig. 3 Distance trees representing the relative extent of changes in transcriptome and genome between the ATCC, Maj and Nau strains of *Paxillus involutus* as analysed by cDNA microarrays. The distance between the samples was calculated by summing the $\log_2$ values of the hybridization–signal ratios for genes that were expressed in all pairwise comparisons (in total 744 genes).

Table 3 Significant differences in gene expression between three strains of *Paxillus involutus* during interaction with birch seedlings (*Betula pendula*) as estimated by the mixed model ANOVA*

Comparisons	Up	Down	Total
ATCC and Maj	24	77	101
Maj and Nau	63	3	66
ATCC and Nau	66	38	104

*The number of genes with $P < 0.05$ (Bonferroni corrected). Analyses were made on 1075 fungal genes and three different strains (ATCC, Maj and Nau).

have previously been reported to be regulated in the ectomycorrhizal root tissue formed between the ATCC strain and birch (Johansson *et al*. 2004) (Le Quéré, Wright *et al*. manuscript in preparation).

Rapidly evolving genes

To assess the possibility that the sequences of regulated genes have evolved at different rates in the compatible and incompatible strains, we calculated the d_n and d_s values for six regulated genes (*cipC1*, *cipC2*, *cchA*, *lecA*, *gstA* and *rabA*). In addition, we included seven genes in the analysis that had been found to be regulated in the interaction between the ATCC strain and birch (Fig. 5). Out of a total of 126 pairwise comparisons, 58 showed a d_n/d_s ratio below 0.6, which is indicative of purifying selection associated with functional constraints. In 61 comparisons the frequencies of d_n, d_s, or both had a zero value. In the remaining seven comparisons the d_n/d_s ratio exceeded one. These were only found in two genes (*cchA* and *cipC1*). Interestingly, all the observations were between Nau and different compatible strains. However, the differences between the

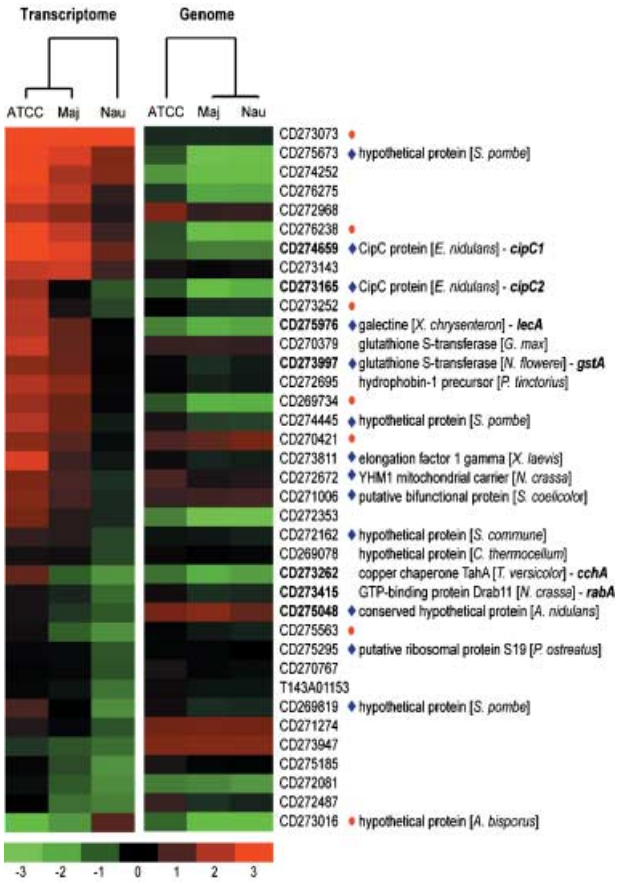

Fig. 4 Visual representation of strain variation at the transcriptome and genome levels for genes found to be related to mycorrhizal efficiency in *Paxillus involutus*. The left panel (Transcriptome) shows $\log_2$-transformed relative expression levels (residuals) from the mixed model ANOVA for 37 genes (out of 1075) that were identified as commonly regulated in both the compatible strains, ATCC and Maj, but not in the incompatible Nau strain. Genes were hierarchically clustered using a matrix of correlation coefficients (CLUSTER 3.0) (Eisen *et al*. 1998). Higher and lower expression levels than the calculated mean are indicated by red and green, respectively. The next panel (Genome) shows data from comparative genomic hybridization (array-CGH) of the ATCC, Maj and Nau strains. The raw hybridization intensities were normalized, $\log_2$-transformed and centred according to Le Quéré, Rajashekar *et al*. (manuscript in preparation). The genes were placed in order in accordance with the hierarchical clustering of the expression data. Genes are identified by EST clone names and the GenBank description for putative homologues. The red circles (18) and the blue diamonds (Le Quéré, Wright *et al*. manuscript in preparation) mark genes that have previously been found to be regulated in the interaction between the ATCC strain and birch. The identities of each of the clones in boldface have been confirmed by resequencing. Genes examined for sequence divergence (*cipC1*, *cipC2*, *cchA*, *lecA*, *gstA* and *rabA*) are marked (cf. Table 2 and Fig. 5).

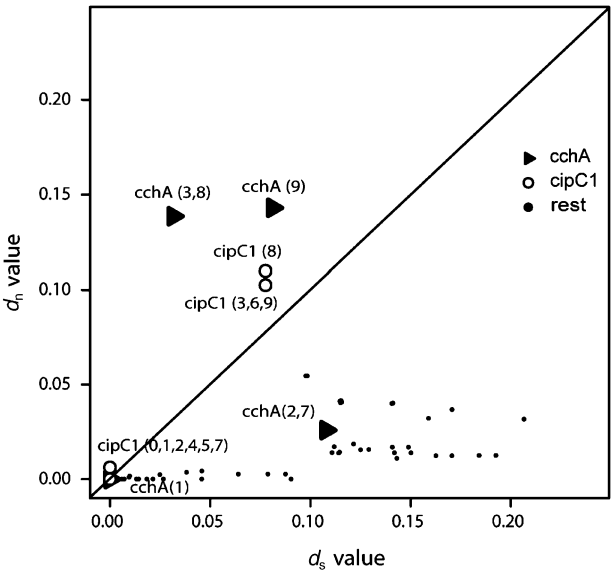

Fig. 5 Comparison of rates of nonsynonymous (d_n) and synonymous (d_s) nucleotide substitutions per site. Each point represents a pairwise comparison made between genes [*cipC1*, *cchA*, or rest (*actA*, β-tubA, *calA*, *ppiA*, *gpiA*, *lecA*, *gstA*, *hetC1*, *hxtA*, *hspA*, *micA*, *ndkA*, *ptrA*, *rabA*)] from five different strains of *Paxillus involutus* (ATCC, Pi01SE, Pi08BE, Maj and Nau) and one strain of *P. filamentosus* (Pf01De). The highlighted gene names in the scatter plot refer to the following comparisons: (0) ATCC vs. Pi01SE; (1) ATCC vs. Pi08BE; (2) ATCC vs. Maj; (3) ATCC vs. Nau; (4) Pi01SE vs. Pi08BE; (5) Pi01SE vs. Maj; (6) Pi01SE vs. Nau; (7) Pi08BE vs. Maj; (8) Pi08BE vs. Nau; (9) Maj vs. Nau. The diagonal line shows the neutral expectation when d_n is equal to d_s. See Table 2 for additional details.

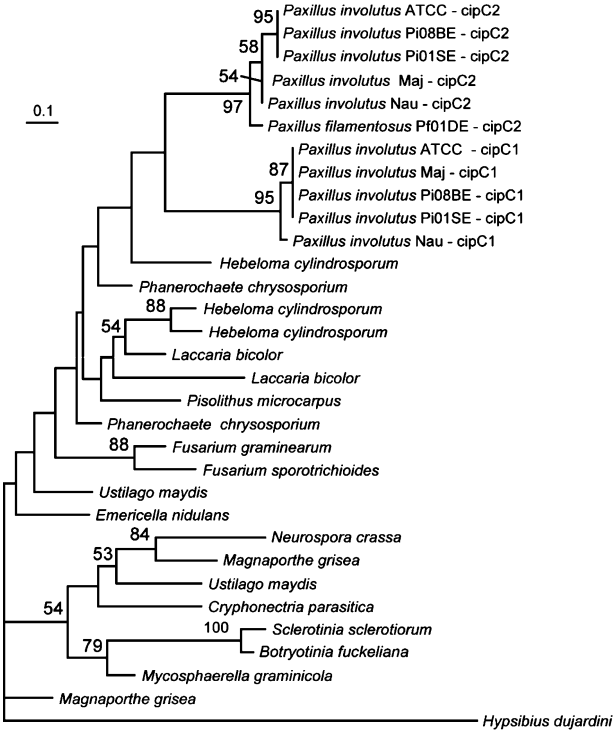

Fig. 6 Phylogeny of cipC proteins. The maximum likelihood tree for cipC proteins based on nucleotide sequence information is shown. Values at nodes represent the bootstrap support value in per cent for 100 replicates. Accession numbers of the protein/nucleotide sequences from top to bottom are: AY586003, AY586005, AY586004, AY586006, AY586007, AY585947, AY586008, AY586011, AY586010, AY586009, AY586012, BU963955, Scaffold_4 (490068–490202,490254–490334, http://www.jgi.doe.gov), BU963954, BU963951, CB010355, CB011958, CB010733, Scaffold_50 (61264–61419,61464–61526, http://www.jgi.doe.gov), EAA71391, FsCon[0257] (COGEME database), UmCon[1708] (COGEME database), CAC87272, XP_323537, EAA52181, UmCon[0073] (COGEME database), CpCon[0039] (COGEME database), CD645941, BfCon[0008] (COGEME database), mg[0591] (COGEME database), BM863150 and CK326079.

d_n and d_s values were not statistically significant using the *Z*-test.

In *P. involutus*, we identified two *cipC* genes, one encoding the CipC1 protein (108 amino acids) and one the CipC2 protein (107 amino acids) with a sequence identity of 47%. A phylogenetic analysis of fungal *cipC* genes shows that the *cipC1* and *cipC2* genes of different strains of *P. involutus* were separated in two well-resolved clades with bootstrap support values of 95 and 97, respectively (Fig. 6). The duplication of the ancestral *cipC* gene has occurred rather recently, after the divergence of *Paxillus* from its ancestor within the bolete clade, as inferred from the phylogeny of ribosomal DNA sequences (Hibbett *et al*. 2001). Most importantly, the analysis shows that the *cipC1* sequence from Nau, diverged before the corresponding sequences from the compatible strains. The most probable explanation for this topology is the observed rate increase in the Nau strain. In contrast, the *cipC2* portion of the tree shows the same topology as the ITS tree (Fig. 1) where Nau and Maj are the most closely related strains. Based on the alignment of the CipC1 protein sequences (83 amino acids), we identified 15 substitutions between Nau and the closely related compatible strain Maj. The d_n/d_s ratio was estimated at 1.32 for the corresponding nucleotide sequences. When comparing Maj with a more distant strain like the compatible ATCC, there were no amino acid substitutions whatsoever. Furthermore, alignment of intron sequences of the *cipC1* gene revealed nine substitutions between Nau and Maj, and none between Maj and ATCC.

The other gene that showed a d_n/d_s ratio above one (1.76 when comparing Nau and Maj), was the *cchA* gene. Also in this case, the Nau gene has evolved rapidly resulting in 11 amino acid substitutions between Nau and Maj, but only three between the more distantly related Maj and ATCC. The phylogenetic tree showed a well-supported *P. involutus* clade (bootstrap support value of 95) with Nau and Maj diverging before the other strains (Fig. 7).

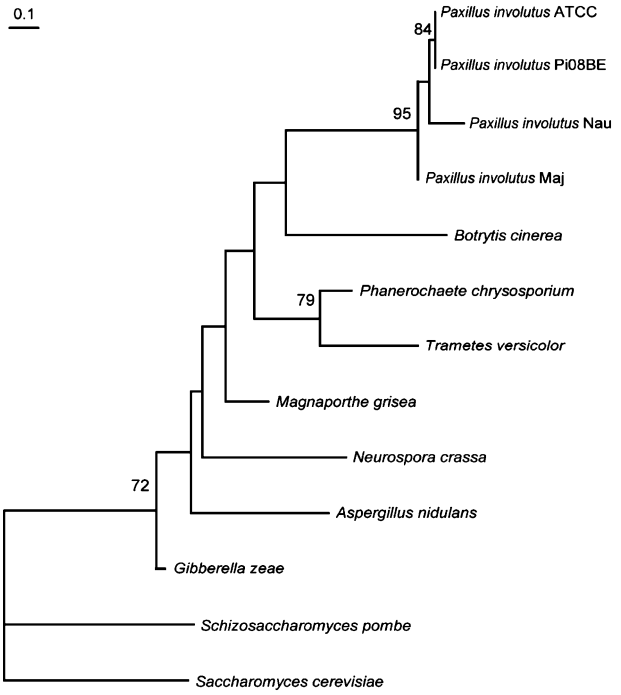

Fig. 7 Phylogeny of *cchA* genes. Fungal *cchA* genes homologous to the *Paxillus involutus* ATCC strain were downloaded from public databases. A maximum likelihood tree from nucleotide sequence alignment was generated using PAUP. Values at nodes represent bootstrap support in per cent of 100 replicates. Accession numbers of the protein/nucleotide sequences from top to bottom are: AY586013, AY586014, AY586016, AY586015, AL114957, Scaffold_41 | *P. chrysosporium* (69102–69158 | 69232–69327, http://www.jgi.doe.gov), AAN75572, EAA54422, CAD70315, EAA65220, EAA74307, NP_595443 and NP_014140.

Discussion

On the genomic level, there are several compatible mechanisms that can account for the intraspecific variations of host specificity in ectomycorrhizal fungi, including quantitative differences in gene expression, alterations in gene content, and structural differences in gene products. By comparing closely related strains of the fungus *Paxillus involutus*, we have demonstrated in this study that variation in host specificity was associated with changes in the patterns of plant-induced gene expression. Approximately 3.4% (37 out of 1075 genes represented on the cDNA array) of the genes were differentially regulated in the two compatible strains compared to the incompatible strain during the interaction with the roots of a putative host plant (birch). The variation in expression profiles between the compatible and incompatible strains was not reflected in the genomic distances as analysed by comparative genomic hybridizations (Fig. 3). Analyses of the genomic array data showed that Maj and Nau were very similar and were equally distant from the ATCC strain. A statistical analysis using the mixed model ANOVA showed that only 0.3% (three out of 1075) of the genes had hybridization signals indicating differences in gene copy numbers between Maj and Nau. These three genes were not found to be among the symbiosis-regulated genes. A similar analysis showed that approximately 200 out of 1075 genes had significantly different signal intensities when comparing ATCC with either Maj or Nau. Ten of these variable genes were found among the symbiosis-regulated genes (Fig. 4).

Among the symbiosis-regulated genes was a homologue (*lecA*) to a family of saline-soluble, low molecular weight fungal lectins (carbohydrate-binding proteins) (Balogh *et al*. 2003). The function of these proteins is unclear but it has been proposed that they are involved in development, storage of nutrients and defence reactions (Balogh *et al*. 2003). Two glutathione S-transferase (GST) homologues were also up-regulated. One of them (*gstA*) displayed a high degree of homology to a GST from the human pathogen *Naegleria fowleri* (identity 49%). GSTs are recognized as detoxification enzymes and are involved in the removal of reactive oxygen species such as H_2O_2 (Sheehan *et al*. 2001). H_2O_2 is an important component of the stress response in plants following their interaction with pathogens, and its production stimulates the expression of a number of cell-protecting enzymes including GSTs (Lamb & Dixon 1997). Notably, H_2O_2 has been shown to accumulate in the outer mantle during the compatible interaction between Maj and poplar, but not in the incompatible association between Nau and poplar (Gafur *et al*. 2004). Small GTPases, including Rho, Rab and cdc42, are important regulators for cell growth, cell cycle progression and organization of the actin cytoskeleton and they may be involved in the morphological changes accompanying the formation of ectomycorrhizae (Johansson *et al*. 2004). A more detailed phylogenetic analysis revealed that the *rabA* is homologous to members of a Rab family including Ypt31and Ypt32 from *Saccharomyces cerevisiae* (Pereira-Leal & Seabra 2001) (Fig. 8). Ypt31 and Ypt32 are probably involved in intra-Golgi transport or in the formation of transport vesicles at the most distal Golgi compartment (Jedd *et al*. 1997).

We also obtained data indicating that the lack of compatibility with birch and poplar in Nau has been associated with an enhanced rate of sequence evolution in two of the differentially expressed genes, *cipC1* and *cchA*. Although the d_n/d_s ratio of these genes exceeded one, which would indicate positive selection (Nei & Kumar 2000), d_n was not significantly different from d_s. More probably, the sequence divergence of *cipC1* and *cchA* reflects a decreased selection pressure associated with the lower functional constraints on these proteins in Nau.

The *cipC1* gene product of *P. involutus* displayed high sequence identity to a conconamycin-induced protein (cipC) first identified in *Aspergillus nidulans*. The function of this protein is not known but its abundance is significantly increased in the presence of the antibiotic concanamycin,

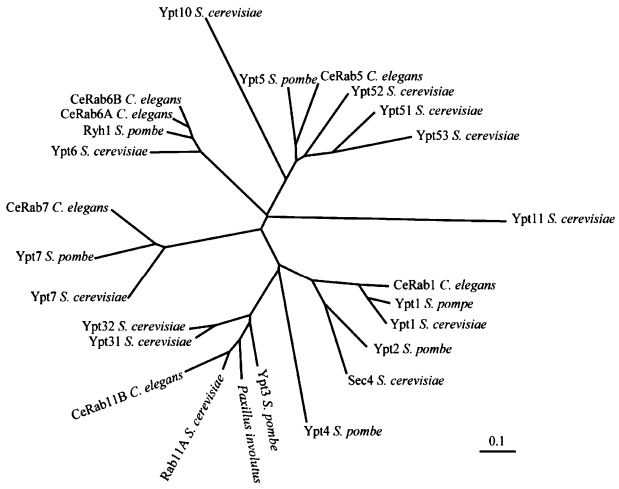

Fig. 8 Phylogeny of *rab* genes. Sequences from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are included together with the *Paxillus involutus* sequence and the *S. cerevisiae* orthologues found in *Caenorhabditis elegans*. Values at nodes indicate per cent bootstrap support of 500 replicates for the orthologue groups defined by Pereira-Leal & Seabra (2001). The *P. involutus* sequence is positioned in the group at the bottom of the figure. The scale unit is substitutions per site. Accession numbers running clockwise starting with *P. involutus* are: AY585950, AAB54158, CAB07678, NP_010948, NP_011305, NP_013713, O94655, CAA91357, NP_013363, S12789, AAC69020, CAA77590, NP_009823, S34729, CAB04205, NP_012939, S43399, NP_014306, NP_014095, AAC69218, S04590, NP_116615, S12790, NP_116650, CAB11239 and S10026.

which stimulates hyphal hyper-branching (Melin *et al*. 2002). CipC homologues have also been reported in several EST studies of ectomycorrhizal fungi (Peter *et al*. 2003). The second gene with an enhanced rate of evolution is *cchA* which translates into a protein of 70 amino acids and displays significant sequence similarity to a number of fungal metallochaperones including the antioxidant 1 (atx1p) protein of *S. cerevisiae* (identity 44%). The *ATX1* gene was originally identified as a gene conferring protection against reactive oxygen species (Lin & Culotta 1995). Subsequently, it was shown that atx1p is a cytosolic copper chaperone that transfers copper from the plasma membrane copper transporter Ctr1p to the intracellular copper-transporting P-type ATPase Ccc2p, located in the Golgi compartment of the secretory pathway (Pufahl *et al*. 1997). Ccc2p translocates copper ions to secreted copper-containing enzymes such as the iron oxidase Fet3p. Other enzymes that can be provided with copper by this pathway are the copper-containing laccases (Uldschmid *et al*. 2003), which are thought to be involved in a number of processes related to the function of the ectomycorrhizal symbiosis (Burke & Cairney 2002).

The underlying reasons for the differences in the expression of the symbiosis-regulated genes are not known. However, duplications and/or deletions of genes are probably not involved, because all of the differentially expressed genes were found in the same copy number in Maj and Nau. More probably, the observed differences in expression are the result of changes in promoter elements and levels of transcription factors.

Acknowledgements

This study was supported by grants from the Swedish Research Council, The Foundation for Strategic Research (Genome Research Programme), and The Swedish Council for Environment, Agricultural Sciences and Spatial Planning. Andres Schützendübel received financial support through a Marie-Curie-Fellowship. Custom microarrays were produced at the SWEGENE DNA Microarray Resource Centre at the BioMedical Centre B10 in Lund, and DNA sequencing was performed at the SWEGENE Center of Genomic Ecology at the Ecology Building in Lund, supported by the Knut and Alice Wallenberg Foundation through the SWEGENE consortium. We thank Dr R. Langenfeld-Heyser and Dr Eva Friman for their help with the microscopic investigations and DNA sequencing, respectively.

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The paper is one in a series of ongoing studies of the functional and evolutionary genomics of the ectomycorrhizal fungus *Paxillus involutus*. The work described in this study was part of the PhD programs of Antoine LeQuéré, Balaji Rajashekar and Jenny Hedh. Andres Schützendübel with a PhD in Forest Botany (Georg-August-University) and Björn Canbäck with a PhD in Molecular Biology (Uppsala University) participated in this project as postdocs. Susanne Erland is associate professor at Lund University. Her main research interests are on the community structure and genetic diversity of ectomycorrhizal fungi. Tomas Johansson is assistant professor at Lund University. His research focused on gene expression during the development of ectomycorrhizal association. Anders Tunlid is professor of microbial ecology, Lund University.
