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ORIGINAL PAPER

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Gene expression of the ericoid mycorrhizal fungus *Oidiodendron maius* in the presence of high zinc concentrations

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Abstract A heavy metal tolerant strain of the ericoid mycorrhizal species *Oidiodendron maius*, isolated from roots of *Vaccinium myrtillus* growing in soil heavily contaminated with zinc, was previously shown to tolerate high concentrations of zinc and cadmium ions in the growth medium. We have investigated the genetic basis of this fungal strain tolerance to high zinc concentrations by using an untargeted approach. From a cDNA library constructed by using mRNA from Zn-treated *O. maius* mycelia, 444 clones were randomly selected and 318 were sequenced. Sequence analysis identified 219 unique clones: 117 showed homology to previously identified genes, 26 matched unknown protein coding regions found in other organisms, and 76 were novel. Variation in the gene expression level after a 20-day treatment with high concentrations of Zn was monitored on 130 unigenes by reverse northern blot hybridisation. Sixteen unigenes were shown to be either up- (9) or down- (7) regulated. The putative function of these genes and their involvement in stress tolerance is discussed.

Keywords Expressed sequence tag · Heavy metal tolerance · Gene expression · Mycorrhizal fungi

Introduction

Ericoid fungi are a diverse group of soil fungi that establish a distinctive type of mycorrhizal association with ericaceous plants (Bonfante and Gianinazzi-Pearson 1979; Perotto et al. 2002). These fungi profusely colonise the fine hair roots typical of ericaceous plants and, due to their saprotrophic capabilities, play an important role in mineral cycling and plant nutrition (Read 1996). Thanks to their mycorrhizal status, host plants can grow on low-mineral, acidic, organic soils high in toxic metal ions, where they can become dominant species (Smith and Read 1997). The mechanisms that protect the host plants from heavy metal toxicity are, however, poorly understood (Perotto and Martino 2001). It is also unclear how the ericoid fungi themselves survive and tolerate toxic concentrations of heavy metals, and whether they rely on cellular and molecular mechanisms already described for other organisms.

A number of heavy metals—such as Cu, Fe, Zn—are essential micronutrients required for a wide variety of physiological processes. Zinc ions, for example, serve as cofactors for many enzymes, and a large number of protein sequences contain Zn^{2+}-binding structural domains. However, elevated concentrations of both essential and non-essential heavy metals can be toxic and cause growth inhibition of most organisms (Clemens 2001; Perego and Howell 1997). Symptoms of toxicity observed in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular/molecular level. Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to inhibition of activity or disruption of structures, or from the displacing of an essential element resulting in deficiency effects. In addition, excess of heavy metals may stimulate the formation of free radicals and reactive oxygen species, resulting in oxidative stress (Hall 2002). In order to maintain the concentration of essential metals within physiological limits and to minimise the detrimental effects of non-essential metals, living organisms have evolved a complex network of homeostatic mechanisms that serve to control the uptake, accumulation, trafficking and detoxification of metals. The main components of metal homeostasis involve transport, chelation and sequestration (Clemens 2001). Regulation of these activities ensures the proper delivery and distribution of metal ions at the cell and organism level, resulting in a basic level of metal tolerance. While basic metal tolerance is ubiquitous, some species and genotypes can grow on soil that, either naturally or due to human activities, contains growth-prohibiting concentrations of metals.

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Oidiodendron maius is one of the few fungal taxa identified as symbionts of ericaceous plants in Canada (Couture et al. 1983; Hambleton and Currah 1997) and Europe (Douglas et al. 1989; Perotto et al. 1996). Several strains of *O. maius* were isolated from the roots of *Vaccinium myrtillus* growing in heavy-metal-polluted plots in the Niepolomice forest (Poland), which were contaminated with industrial dusts containing different proportions of Zn, Cd, Al and Fe (Greszta 1988; Turnau 1988). These ericoid fungal strains have been investigated for their ability to grow in the presence of heavy metals (Bardi et al. 1999; Lacourt et al. 2000; Martino et al. 2000a,b, 2002; Perotto and Martino 2001), and *O. maius* strain Zn was selected as one of the isolates most tolerant to Zn and Cd.

Biochemical analyses indicate that high heavy metal concentrations induce profound changes in the profile of extracellular proteins in *O. maius* Zn (Martino et al. 2002). Thus, the presence of a set of genes coding for proteins involved in metal homeostasis and in stress-tolerance was envisaged.

In this report we present a pilot-scale cDNA sequencing project that provides a first insight into genes expressed by an endomycorrhizal fungus in the presence of high heavy metal concentrations, allowing us to identify a number of sequences that are likely candidates for long-term stress-tolerance determinants in *O. maius* Zn. Since only ribosomal genes have been cloned from *O. maius*, we chose to carry out random cDNA sequencing rather than an approach based on subtractive hybridisation. Therefore, we first isolated and sequenced expressed sequence tags (ESTs), an approach that is now routinely used for gene isolation from many organisms (Pandey and Lewitter 1999; Ohlrogge and Benning 2000). In order to highlight the specific genes regulated by a prolonged exposure to heavy metals, variation in the expression level of identified EST clones was monitored by reverse northern blot hybridisation in mycelia grown for 20 days under conditions of high Zn concentration.

Materials and methods

Fungal isolate

The fungal isolate investigated in this study is *O. maius* strain Zn, deposited in the MUT collection at the Department of Plant Biology, University of Turin (CLM1381.98). This strain was isolated in the Niepolomice Forest (25 km northeast of Krakow, Poland) from the roots of *Vaccinium myrtillus* growing in experimental plots treated with 5,000 t/km^2 of dusts derived from industrial electro-filters containing high concentrations of Zn, Cd and Al. The characteristics of the site and the identification of this fungal isolate are described in Martino et al. (2000b).

RNA isolation and cDNA library construction

The fungus was grown at 25–26°C in Czapek-pectin liquid medium ($NaNO_3$ 3 g/l, $K_2HPO_4 \cdot 3H_2O$ 1.31 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, KCl 0.5 g/l, pectin 8 g/l) with 7.6 mM Zn^{2+} added (as $ZnCl_2$; Merck, Darmstadt, Germany, 98% purity). Mycelia were harvested by filtration, washed with cold water and 10 mM EDTA, frozen in liquid nitrogen and used for RNA extraction following a modification of the method described in Shirzadegan et al. (1991). Mycelia were ground to a fine powder in liquid nitrogen using a mortar and pestle and transferred to tubes containing a 1:1 ratio of phenol and extraction buffer (100 mM LiCl, 100 mM Tris-Cl pH 8.0, 10 mM EDTA, 1% SDS) at 80°C. After vortexing for 1 min, tubes were incubated for 5 min in a dry block at 80°C. Then, one-half volume of chloroform/isoamyl alcohol (24:1 v:v) was added, the homogenate was thoroughly mixed by vortexing for 2 min and centrifuged at 3,000 rpm for 30 min at 4°C. The upper aqueous phase was extracted three times with one volume of cold phenol/chloroform/isoamyl alcohol (24:23:1 v:v:v) and once with one volume of chloroform/isoamyl alcohol (23:1) and centrifuged 3,000 rpm for 15 min at 4°C. The aqueous phase was finally mixed with one-third volume of 8 M LiCl and left overnight at 4°C. Subsequently, RNA was pelleted by centrifugation for 30 min at 13,000 rpm and the pellet was washed in 70% ethanol/30% 0.5 M NaCl for 1 h changing solution several times. The RNA was resuspended in an adequate amount of H_2O and a small amount was loaded on a gel to check the quality.

The cDNA library was constructed using the λ ZAPII cDNA Synthesis Kit (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. cDNA was synthesised from the mRNA isolated from total RNA using the PolyATtract Systems (Promega, Madison, Wis.). Double-stranded cDNA was ligated with *Eco*RI-*Xho*I oligonucleotide adapters for directional cloning in the Lambda ZAPII vector, and packaged using Gigapack III Gold. Recombinants were propagated in *Escherichia coli* strain XL1 Blue MRF′. The titre of the library was checked before and after the recommended amplification.

Sequencing

The phage library was converted to the plasmid form by mass excision, according to the protocol described by Stratagene. The phagemid library obtained was used to infect *E. coli* strain SOLR. Bacteria were grown for 15 min at 37°C and then plated at low density on medium containing Luria-Bertani (LB) broth and ampicillin (50 μg/ml). After incubation at 37°C overnight, individual colonies were selected randomly and manually transferred to new plates.

Insert size was checked for each colony through PCR reactions using the universal T3 and T7 primers. The re-

actions were performed in a final volume of 30 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 0.01% gelatine, 0.1 mM each dNTP, 1 unit (U) Red*Taq* DNA polymerase (Sigma, St. Louis, Mo.) and 50 pmol each primer, in a Perkin Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Foster City, Calif.) with the following program: 3 min at 95°C (1 cycle), 45 s at 94°C, 45 s at 55°C, 2 min at 72°C (30 cycles), 10 min at 72°C (1 cycle).

The chosen colonies were manually picked and transferred into individual wells of a 96-wells PCR plate containing 150 µl LB with 50 µg/ml ampicillin per well. After growth at 37°C, each cell culture was mixed with glycerol (12% final concentration) and stored at −80°C, ready for high throughput DNA sequencing.

Sequencing reactions were run and analysed by Genome Express (Meylan, France); all reactions contained the standard T7 sequencing primer and thus read into the presumed 3′ end of each cDNA.

Sequence editing

Original data analysis and assembly were performed on PC operating system-based computer. All the automated sequences were manually trimmed of vector and polylinker sequences and were truncated when ambiguous base calls increased in the sequence. Ambiguous and miscalled bases were corrected, when possible, after examination of the corresponding chromatogram files with the help of Sequencher software version 4.0.5 (Gene Codes, Ann Arbor, Mich.).

All sequences were examined for potential overlaps using the Sequencher software, with the following assembly parameters: alignment method = dirty data, minimum match percentage = 85%, minimum overlap = 20. Overlapping sequences were assembled into contigs, each one representing a different EST. The identification number of contigs adopted the ID of the first clone that appeared in the sequencing project.

The sequences have been deposited at the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers CK928136, CN200103–CN200319.

Similarity searches

The cDNA sequences obtained were compared with the nonredundant (nr) database available through the NCBI using the BlastX or tBlastX algorithms (Altschul et al. 1997). Blast searching was carried out automatically by the InterEST software developed in our laboratory (S. Abba', in preparation).

Sequences with an expected E value $\leq 1\times10^{-5}$ were considered to identify known genes or to have partial homology to known genes. ESTs showing almost 100% identity to *E. coli*, yeasts, or bacteriophage lambda at the amino acid and nucleotide level were eliminated as suspected contaminants of the library or artefacts. Putative identifications for the ESTs were assigned based on the results of the BLAST searches and, in some cases, on information contained in related abstracts in MEDLINE. The potential cellular roles for the matching sequence were determined using data of the *Saccharomyces cerevisiae* functional catalogue website (http://mips.gsf.de/proj/yeast/catalogues/funcat/).

RNA preparation and synthesis of ^{32}P-labelled cDNA probe

Total RNA was extracted from mycelia of *O. maius* Zn grown for 25 days at 25–26°C in Czapek-pectin liquid medium with 10 mM Zn^{2+} added (as $ZnSO_4$; Fluka, 99% purity) (treated sample—T) or not (control sample—C). Mycelia were harvested by filtration, washed with cold water and frozen in liquid nitrogen. Mycelia were then ground to a fine powder in liquid nitrogen using a mortar and pestle and transferred to tubes containing a 1:1 ratio of phenol and extraction buffer (100 mM Tris-Cl pH 8.0, 100 mM NaCl, 20 mM EDTA, 1% SLS, 0.1% PVP) on ice. The homogenate was thoroughly mixed by inverting the tubes for 1 min and then centrifuged for 5 min at 14,000 rpm. The upper aqueous phase was extracted with one volume of cold phenol/chloroform/isoamyl alcohol (24:23:1 v:v:v) and centrifuged at 14,000 rpm for 10 min. The aqueous phase was then mixed with one volume of isopropyl alcohol and precipitated for 30 min at −80°C. After centrifugation at 14,000 rpm for 30 min at 4°C, the pellet was redissolved in 500 µl H_2O and precipitated twice in 3 M LiCl for about 14 h at 4°C. Subsequently, RNA was pelleted by centrifugation for 30 min at 14,000 rpm at 4°C and the pellet was washed in 70% ethanol. The RNA was resuspended in an adequate amount of H_2O and a small aliquot was loaded on an agarose gel to check the quality and measured spectrophotometrically to determine the concentration.

The cDNA probes were prepared from 5 µg of each of the two RNA samples by reverse transcription in a 30 µl reaction consisting of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 3.3 mM DTT, 1 mM dNTP (without dCTP), 3.3 µM home-made AncT20 primers, 40 U RNaseOUT (Invitrogen-Life Technologies, Carlsbad, Calif.), 30 µCi [α-^{32}P]dCTP (NEN, Perkin Elmer) and 300 U SuperScript II RT (Invitrogen-Life Technologies). The reverse transcription was allowed to proceed for 90 min at 37°C and then Micro Bio-Spin P-6 columns (Bio-Rad, Hercules, Calif.) were used to remove the unincorporated ^{32}P. Water was added to the eluate to a volume of 100 µl and 1 µl was evaluated for [α-^{32}P]dCTP incorporation by liquid scintillation analysis (Packard).

Preparation of blots and hybridisation

The colonies of interest were picked and lysed by boiling in 10 µl H_2O. Using primers flanking the vector cloning site (T3 and T7), the cloned cDNA fragments were amplified

from the colony lysates in a 30 μl reaction as described above. To prepare for blotting, 12 μl of each PCR-amplified insert was loaded onto duplicate agarose gels, transferred to nylon membranes and UV cross-linked before being used for hybridisation. A total of 146 samples were loaded; each filter contained 17 clones (except the last, which contained only 11 clones) and, as control, the undiluted and 1:10 diluted PCR amplified insert of clone CB5 corresponding to a cDNA encoding elongation factor 1α (EF-1α).

Equal counts (3.5×10^6 cpm) of the ^{32}P-labelled cDNA probes C and T were heat denatured and used to probe the duplicate blots. The filters were pre-hybridised and hybridised at 65°C with 60 μl/cm^2 hybridisation solution containing 5×SSC, 5× Denhardt's solution (Sambrook et al. 1989), 0.5% SDS and 0.1 mg/ml ssDNA. Pre-hybridisation was carried out for 2 h; hybridisation for at least 15 h. After hybridisation, filters were washed at 65°C once in 3×SSC and 0.2% SDS for 25 min, once in 1.5×SSC and 0.2% SDS for 15 min, once in 0.6×SSC and 0.1% SDS for 15 min, and once in 0.24×SSC and 0.1% SDS for 15 min.

Detection, image acquisition and data analysis

All filters were exposed to the same phosphor screen for 30 min (short exposure), 3 h (medium exposure) and 3 days (long exposure). The screen was scanned using a PhosphoImager (Personal Molecular Imager FX, Bio-Rad) and the bands were quantified using Quantity One version 4.5.0 software (Bio-Rad). To calculate signal intensities, a rectangular box was drawn and overlaid on each band of the reverse northern blot image and the global background was subtracted.

Clones were manually flagged as P (present) or A (absent) according to the signal/background ratios: those with a ratio above the cut-off value of 3 were flagged as P; those with a ratio below 3 were flagged as A. Only clones flagged as P were analysed further.

Adjustments for differences in labelling and hybridisation were made based on the EF-1α signal. Normalised signals of the treated set of filters where then compared with those of the control set and the differences in expression were expressed as "fold change".

Results

RNA isolation and cDNA library

RNA was isolated from the ericoid fungus *O. maius* Zn grown for 21 days at 25–26°C in Czapek-pectin liquid medium with 7.6 mM of Zn^{2+} added. About 4 μg mRNA was used for the construction of the cDNA library. The titre of the primary cDNA library was about 450 pfu/ml, and 1.5×10^7 pfu/ml after the recommended amplification. The library was not subjected to further amplifications or modified prior to EST sequencing, except for those modifications that normally arise during the process of phagemid excision.

Sequencing and assembly of the ESTs

Initially, 444 cDNA clones were randomly selected and, prior to sequencing, the approximate size of the cDNA inserts were determined on agarose gels after PCR reactions using T3 and T7 primers. Of the clones tested, 318 (about 72%) contained an insert larger than 150 bp and were selected for sequencing. All the clones were sequenced using the T7 primer; since the cloning was directional, the sequencing was expected to generate the poly(T) tail and the 3′ end of the cDNA insert. The presence of a poly(A) tail in 41 clones suggested that in those cases the cDNA was inserted in the opposite orientation.

Ambiguous and miscalled bases were corrected, when possible, after examination of the corresponding chromatogram files with the help of Sequencher software version 4.0.5 (Gene Codes). The sequences were truncated when the number of ambiguous bases increased. After correction, 36 sequences (about 11%) still contained more than 3% of ambiguous bases and were discarded.

For 54 clones (17%), the entire sequence of the insert was determined in a single pass, indicating inserts smaller than 550–600 bp.

Four chimeric clones were identified by the presence of either two poly(T) tails or two poly(A) tails. The chimeric inserts were split to generate two single sequences marked with the addition of "a" and "b" to the ID number. After this process, the set of ESTs to be analysed was composed of 286 sequences. The size of the sequences ranged from 100 to 950 bp with an average size of ca. 670 bp; the distribution of sequence sizes is shown in Fig. 1.

To generate a set of unique sequences and to characterise transcript abundance in the library, the 286 ESTs were analysed for overlapping regions using the Sequencher software version 4.0.5 (Gene Codes). This allowed us to compare the full set of clones with itself and to construct a number of contigs so that a larger string of nucleotides could be used to search for homology in the public sequence databases. The relative abundance of overlapping clones is shown in Fig. 2, where 182 clones remained as singletons, as they did not overlap any other EST, and 104 clones, corresponding to 39 nonredundant sequences, occurred

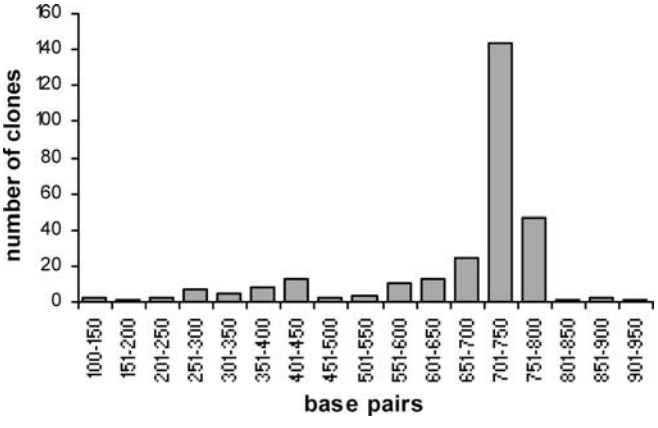

Fig. 1 Size distribution of the inserts of the 286 clones analysed from the *Oidiodendron maius* Zn cDNA library

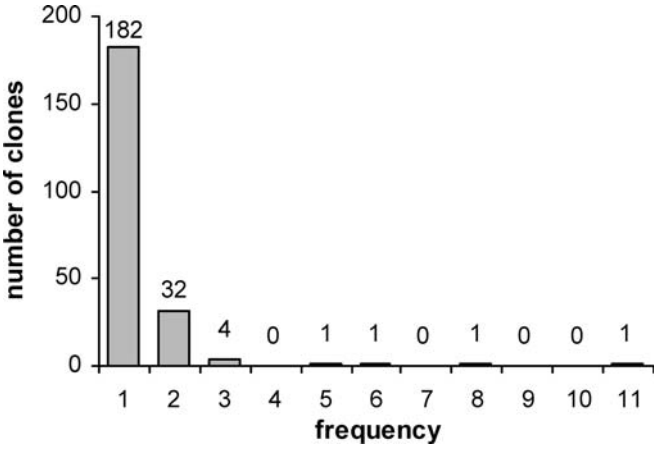

Fig. 2 Expressed sequence tag (EST) redundancy among the 286 sequences obtained from the *O. maius* Zn cDNA library

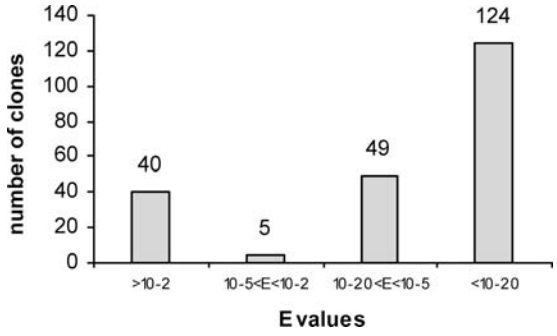

Fig. 3 Distribution of similarities between translated products of *O. maius* Zn EST sequences and the best match in the nr protein database: *>10-2* not significant, *10-5<E<10-2* weakly, *10-20<E<10-5* moderately, *<10-20* highly significant

repeatedly at frequencies ranging from 2 to 11. Thus, 221 different genes (unigenes) were obtained from the 286 cDNA clones analysed.

Sequence analysis and assignment of identities to the ESTs

To identify homologues of *O. maius* Zn genes, each edited EST was automatically queried against the NCBI databases using the BlastX algorithm (Altschul et al. 1997). The database sequence matches were arbitrarily classified as significant (E value $<10^{-5}$) or not significant (E value $>10^{-5}$).

Three sequences (AB8, BC5, BF1) exhibited 99–100% identity to *E. coli* proteins; a similarity search using the BlastN algorithm showed almost 100% identity to *E. coli* sequences also at the nucleotide level. These three clones were eliminated because they were clearly of non-fungal origin. Finally, we retained 218 sequences.

The frequency of highly ($E<10^{-20}$), moderately ($10^{-5}<E<10^{-20}$), weakly ($10^{-2}<E<10^{-5}$) and not significant ($E>10^{-2}$) E values is shown in Fig. 3. The percentage of amino acid identity for ESTs with significant E values ranged from 24% to 100%; for those considered as highly significant, the percentage was equal to or greater than 31%.

Of the 218 unigenes recovered, 173 (79%) fell into the moderately to highly significant range and were classified into 11 categories according to their putative function, using an adaptation of the classification scheme of the *S. cerevisiae* database as a reference (http://mips.gsf.de/proj/yeast/catalogues/funcat/). The proportion of predicted genes with an assigned cellular role is presented in Fig. 4.

Of the 218 unigenes, 45 (21%) showed no significant match to sequences in the protein database and they were queried against the fungal dbEST database, using the tBlastX algorithm. Seven of them had significant homologies (E value $<10^{-5}$) toward sequences isolated from cDNA libraries of Ascomycetes, and precisely *Aspergillus nidulans* (AH6; Ray et al. 2004), *Chaetomium globosum* (BB9), *Trichoderma reesei* (BC7; Diener et al. 2004), *Coccidioides posadasii* (BG5), *Cryphonectria parasitica* (CB6; Dawe et al. 2003), and *Sclerotinia sclerotiorum* (CE6, D26; Li et al. 2004). All other unigenes with no significant matches may represent novel fungal genes.

Overall, the library contained a broad range of genes encoding proteins involved in primary and secondary metabolism (23%) and protein synthesis and processing (15%). In the latter case, genes encoding ribosomal proteins were prevalent. Other genes have functions in the cell cycle, DNA processing and transcription (6%), energy production (2%), transport mechanisms and cell structure (7%), and cell defence (8%). Similarities to hypothetical or unknown proteins accounted for 38% of the genes identified.

The species showing highest homologies with *O. maius* translated genes were recorded. About 92% of the highest scoring protein homologues were derived from filamentous fungi, mainly *Gibberella zeae* (41), *Neurospora crassa* (39), *Aspergillus nidulans* (36) and *Magnaporthe grisea* (34). The rest were from a variety of organisms, such as yeasts, mammals, invertebrates, bacteria, and viruses (Fig. 5).

Two homologues of 30 kDa heat-shock proteins from *N. crassa* (AD6, AB11) were the most highly repeated ESTs in the library. In addition, homologues of a polyubiquitin from *Nicotiana tabacum* (AA6), a $NADPH_2$ dehydrogenase (AA7), an alcohol dehydrogenase (AA8), a metallothionein (AA5), a peptidyl-prolyl cis-trans isomerase (D7) from *Neurospora crassa* were also moderately redundant.

Identification of stress-related genes

According to the functional classification of the *S. cerevisiae* database, 13 of the 173 *O. maius* ESTs (8%) had significant similarities to genes implicated in cell defence. Five of these code for different heat shock proteins (HSP): 30 kDa HSP (clones AB6, AB11, AD6), 78 kDa HSP (clone BA3) and 104 kDa HSP (clone AB9). Five clones are homologous to genes involved in oxidative stress: Cu,Zn superoxide dismutase (AH9); epoxide hydrolase (BG11); ascorbate peroxidase (CC7); thioredoxin (AB4); cytochrome P450 (AF11). Two clones are similar to genes coding for toxic compound transporters: one (D30) showed high homology to the HMT1 vacuolar ABC transporter in-

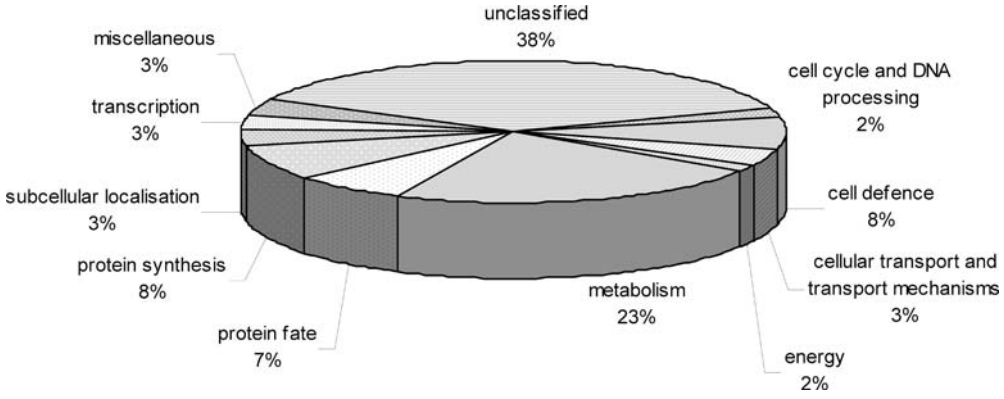

Fig. 4 Proportion of unigenes (173 total) coding for protein with assigned cellular function, obtained from *O. maius* Zn cDNA library

volved in *Schizosaccharomyces pombe* Cd tolerance; the other (BG1) to a major facilitator carrier of the plant-pathogenic fungus *Botryotinia fuckeliana*. Clone CH3 was similar to the large chain of a glutathione synthetase of *S.pombe*. Clone AA5 showed high similarity to a Cu-metallothionein of *N. crassa*.

Reverse northern blot hybridisation

To identify genes whose expression is differentially regulated in response to Zn treatment, cDNA inserts from 131 unique clones were loaded in duplicate onto agarose gels and transferred to nylon membranes. The first two samples of each filter consisted of elongation factor EF-1α, a housekeeping gene thought to be constitutively expressed. cDNA probes were prepared from total RNA extracted from *O. maius* Zn mycelia grown on Zn-containing (T) or on control (C) medium.

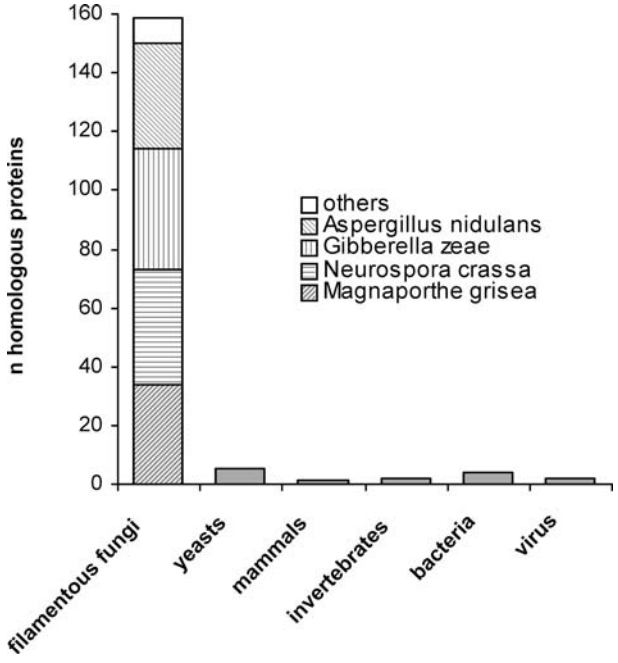

Fig. 5 Species groups from which the best matches to the *O. maius* Zn ESTs were identified

The results of hybridisation of 16 filters with the two probes is shown in Fig. 6. Of 130 clones, 93 (72%) produced, both in control and treated sample, a signal with intensity at least 3-fold above background. The signal of these clones was normalised against the housekeeping gene, and then the ratios normalised-signalT/normalised-signalC were calculated. Only clones with a ratio below 0.3 or above 3.0 (14 genes, corresponding to 15%) were considered as being differentially regulated: of these, seven were up-regulated and seven down-regulated (Table 1). The values of their relative expression ranged from a minimum of 0.04 (clone BC4) to a maximum of 108.35 (clone BA4).

Genes regulated in response to high Zn concentration

The 14 differentially regulated *O. maius* Zn genes belong to different functional categories (Table 1). BlastX searches of the seven Zn-induced clones gave, as best hits, hypothetical proteins of unknown function revealed during genome sequencing of *Magnaporthe grisea*, *Neurospora crassa*, *Gibberella zeae* and *Aspergillus nidulans*. Nevertheless, a putative function could be assigned to six of them based on significant E value. AC10 codes for a protein with high homology to a putative mitochondrial carrier of *Candida albicans*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. AC5 gave the best hits with conserved hypothetical proteins of *Pseudomonas putida* and *Streptococcus mutans* and a S-adenosylmethionine (SAM)-dependent methyltransferase of *Rhodospirillum rubrum* and *Shewanella oneidensis*. AD2 had homology with a transposase of *Aspergillus oryzae* and *Caenorhabditis elegans*. AE11 gene product had an amino acid identity of 67% and 64% with a diphosphomevalonate decarboxylase of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* respectively. A *Saccharomyces cerevisiae* Mak21p protein essential for 60S ribosome biogenesis was the protein with known function showing the best homology to BA4. CG8 has more than 80% amino acid identity with *A. nidulans* and *C. albicans* carboxypeptidase. For AG1, only homology with hypothetical proteins from genome sequencing of *A. nidulans*, *G. zeae*, *N. crassa* could be found.

The BlastX searches of three (AH4, BC4 and CA2) out of the seven Zn-repressed clones gave hypothetical proteins as first hits; in the other four cases the best results

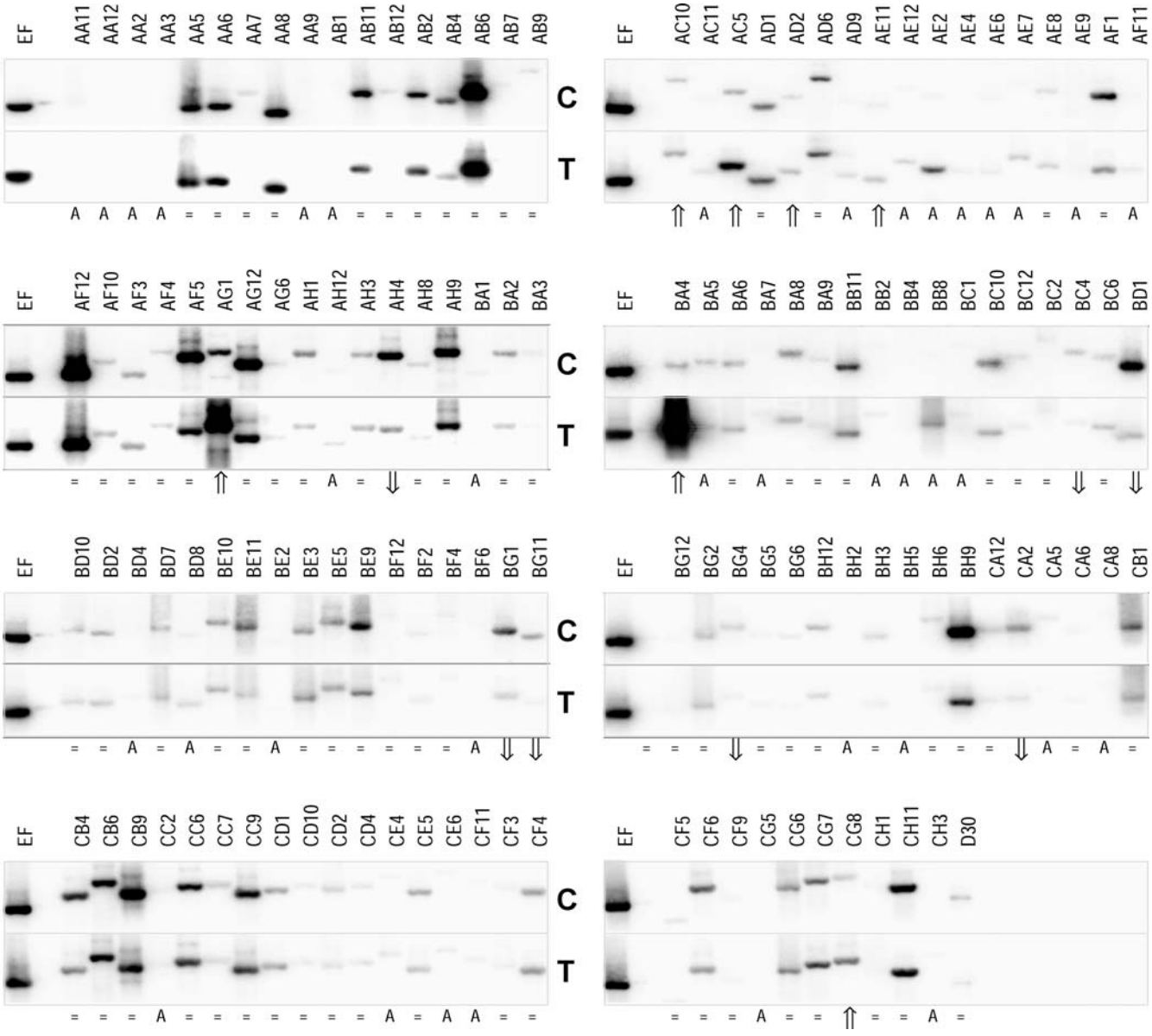

Fig. 6 Reverse northern blot hybridisation of 130 *O. maius* Zn EST unigenes with two probes obtained from mycelia grown for 20 days in the absence of Zn (*C*) or with addition of 10 mM Zn (*T*). *EF* Elongation Factor 1α (housekeeping gene used for data normalisation), *A* absent signal (ratio signal/background <3), ⇑ up-regulated genes (ratio treated/control >3.0), ⇓ down-regulated genes (ratio treated/control <0.3), = not regulated genes (ratio treated/control between 0.3 and 3.0)

were proteins with a specific function. The deduced amino acid sequence of BD1 showed more than 85% identity to the mitochondrial precursor of citrate synthase of *G. zeae*, *A. nidulans*, *N. crassa*. BG4 probably codes for a keto-acid reductoisomerase similar to those of *N. crassa* and *G. zeae*. The BG11 gene product is similar to the predicted epoxide hydrolases of *Clostridium acetobutylicum*, *Gallus gallus* and *Danio rerio*. The deduced amino acid sequence of BG1 had 73% identity with a DHA14-like major facilitator of *Botryotinia fuckeliana* and 50% with a transporter protein of *Phoma betae*. AH4 had homology with hypothetical proteins of *N. crassa*, *G. zeae*, *M. grisea* and 60–70% identity with alcohol dehydrogenases of *Hypocrea jecorina*, *A. oryzae* and *Puccinia triticina*.

Discussion

Genomic research for ericoid fungi is far less developed than for other mycorrhizal fungi such as arbuscular and ectomycorrhizal symbionts (Gianinazzi-Pearson et al. 2001; Franken and Requena 2001; Martin 2001; Wiemken and Boller 2002). At the time of submission, two chitin synthase genes identified in the sterile mycorrhizal endophyte PSIV (Lanfranco et al. 2004) were the only non-ribosomal sequences for ericoid fungi available in the public databases. We decided therefore to carry out a first foray into gene diversity of the ericoid fungus *O. maius* by means of random cDNA sequencing rather than an approach based on subtractive hybridisation.

Table 1 *Oidiodendron maius* Zn unigenes regulated by a 20-day treatment with 10 mM Zn

Clone ID	Accession number	Putative function	E-value	Expression level
AC10	CN200123	Mitochondrial carrier family protein	2.00E−17	3.31
AC5	CN200128	SAM-dependent methyltransferases	1.00E−37	9.38
AD2	CN200136	Transposase	4.00E−17	3.26
AE11	CN200141	Diphosphomevalonate decarboxylase	2.00E−76	5.39
AE2	CN200143	Hypothetical protein MG02330.4	2.00E−35	Up[a]
AG1	CN200161	Hypothetical protein	8.00E−37	3.63
AH4	CN200170	L-Arabinitol 4-dehydrogenase	4.00E−78	0.27
BA4	CN200178	Essential for 60 S ribosome biogenesis	1.00E−22	108.35
BB8	CN200191	Oxidoreductase	2.00E−43	Up[a]
BC4	CN200197	Putative oxidoreductase protein	9.00E−29	0.04
BD1	CN200201	Citrate synthase	1.00E−76	0.23
BG1	CN200223	DHA14-like major facilitator	7.00E−48	0.27
BG11	CN200225	Alpha/beta hydrolase superfamily	2.00E−21	0.14
BG4	CN200229	Ketol-acid reductoisomerase precursor	3.20E−113	0.32
CA2	CN200243	Hypothetical protein AN6605.2	5.00E−39	0.17
CG8	CN200297	Serine-type carboxypeptidase homolog precursor	1.00E−119	3.96

[a]For these up-regulated clones the signal in the control membrane was too low, therefore an expression value could not be assigned to them (see text)

The 286 ESTs clones sequenced from the *O. maius* strain Zn cDNA library were assembled in 218 unigenes, which should represent ca. 2.6% of the total complement of genes, since it has been estimated that the genome of ascomycetes contains about 8,000–9,000 genes (Kupfer et al. 1997). The relative abundance of cDNAs among the EST sequences did not correlate with the expression pattern of the genes in vivo. In fact, clones with a relatively high abundance, such as clones AD6 (11), AB11 (7), AA6 (6), AA8 (5), showed a low relative expression (0.27, 0.48, 0.78 and 0.79, respectively), whereas genes found only once in the EST analyses, like clones AB6, AF12, AG1 and BA4, showed a high relative expression (4.92, 3.48, 5.25, 24.05, respectively). The discrepancy between "in silico northern" and "in vitro northern" observed here, and also by Zhu et al. (2001), is not surprising as the number of sequences obtained and analysed in our work is probably too small to provide a representative picture of gene expression.

About 21% of the unisequences showed no similarity to protein sequences in the databases and may represent novel fungal genes, although this could be due in some cases to the sequence being derived from either the 3′ or the 5′ untranslated cDNA regions. Of the ESTs showing significant similarity to sequences cloned from other organisms, 95% were similar to fungal sequences (92% filamentous fungi and 3% yeasts) and 5% to sequences from diverse organisms (mammals, invertebrates, bacteria, and viruses).

Among the EST sequences of *O. maius* Zn, clone AA5 codes for a protein showing high similarity to a Cu-metallothionein of *N. crassa* (CAA26793). The deduced protein of clone D30 had high homology (E value 2e−73, 78% similarity) with the *Schizosaccharomyces pombe* vacuolar transporter *hmt1* (CAA78419), whose activity is fundamental for Cd tolerance because it transfers the Cd-phytochelatin complex from the cytosol to the vacuole (Ortiz et al. 1992, 1995). Metallothioneins (MTs) and phytochelatins (PCs) are of primary importance in buffering the intracellular concentration of free thiophilic metal ions, such as Cu, Zn, and Cd (Clemens 2001; Sanita' di Toppi et al. 2002, and references therein). Fungal MTs have been characterised mainly in yeasts but there are also few reports on filamentous fungi like *Podospora anserina* (Averbeck et al. 2001), *N. crassa* (Munger et al. 1985), *Agaricus bisporus* (Nishiyama et al. 1990), and *Gigaspora margarita* (Lanfranco et al. 2002). To date, only two fungi have been reported to be able to synthesise both MTs and PCs: *Candida glabrata*, which produces MTs when exposed to toxic concentrations of Cu but produces mainly PCs in response to Cd stress (Mehra et al. 1988, 1989) and *Schizosaccharomyces pombe*, which produces HM-chelating PC peptides through a plant-like PC-synthase enzyme (SpPCS; Clemens et al. 1999; Ha et al. 1999) and has a putative MT (deposited under accession no. CAB57404 and obtained by genomic sequencing). The finding in *O. maius* Zn of genes encoding both an MT and a protein involved in PC function suggests that this mycorrhizal fungus may use both compounds for heavy metal detoxification. We have carried out some pilot experiments to detect PC synthase genes in *O. maius* Zn by using two different approaches: degenerate primer-based PCR, and screening of a genomic library with heterologous probes. Although more experiments are needed to exclude the presence of a PCS gene, both techniques gave negative results. In parallel, we are putting some efforts into characterising the gene corresponding to clone D30 to establish whether the sequence homology with *S. pompe hmt1* corresponds also to a similar function.

Expression analyses of genes corresponding to EST clones with significant homology to sequences in databases, carried out in *O. maius* Zn mycelia grown for 25 days in the

presence or absence of 10 mM Zn, showed that some of these genes do respond to Zn treatment. For two genes, the change in the expression level was quite dramatic (108-fold up and 25-fold down), whereas in all other cases it ranged between 3- to 9-fold up and between 3- to 7-fold down; 37 clones gave no detectable hybridisation signals, either in both treatments or in one treatment only. For example, clones AE2 and BB8 (see Table 1) provided a clear signal only in the treated membrane suggesting that they may correspond to Zn-responsive genes with an off/on regulation.

Among the differentially regulated genes, we could not find any previously reported heavy metal-responsive or stress-related genes. The fact that most of the genes included in the cell defence category (e.g. Cu-metallothionein, Cu,ZnSOD, ascorbate peroxidase, thioredoxin, heat shock proteins) were not affected by the Zn treatment may be because the tolerant strain *O. maius* Zn does not perceive 10 mM Zn in the growth medium as a serious stress condition. This hypothesis is supported by the observation that *O. maius* Zn was still able to grow at even higher metal concentrations (15.4 mM $ZnCl_2$) in the culture medium (Martino et al. 2000b). A second possible explanation is that in *O. maius* Zn stress-related genes may be involved in a earlier than 25 days response to heavy metal stress. Studies on superoxide dismutase (SOD) gene induction in *Saccharomyces cerevisiae* (Cyrne et al. 2003; Garay-Arroyo et al. 2003), *Schizosaccharomyces pombe* (Jung et al. 2002; Lee et al. 2002) and on the plant *Nicotiana plumbaginifolia* (Tsang et al. 1991) show a rapid transcript increase after 3–50 h of exposure to various stresses (osmotic, light, oxidative, heat and heavy metals). Similarly, a rapid increase in the transcription of MT genes was described in *Candida glabrata* 1 h after induction with Cu (Thorvaldsen et al. 1993), in *Arabidopsis* 8–48 h after treatment with a number of metals (Murphy and Taiz 1995), and in *Gigaspora margarita* symbiotic mycelia exposed for 24 h to Cu (Lanfranco et al. 2002).

The differentially regulated genes identified in *O. maius* Zn have diverse functions and cannot be connected to a single metabolic network. In most cases they do not appear to be directly related to heavy metal tolerance. The gene showing the most dramatic change in expression level (BA4) codes for a protein similar to *S. cerevisiae* Mak21p, which is essential for 60S ribosomal subunit biogenesis (Edskes et al. 1998), but whose putative target remains to be identified. Yeast Mak21p is homologous to the human and murine CAATT-binding protein (CBF), that regulates Hsp70 production (Lum et al. 1990). Similarly, the *O. maius* Zn Mak21p homologue may be a transcription factor overproduced under Zn stress, but we have no evidence of its involvement in ribosome biogenesis. Further analysis will be necessary to define the target promoter or promoters of this putative, highly expressed CBF in order to confirm its function.

One of the Zn-induced genes in *O. maius* Zn, which codes for a putative mitochondrial carrier (clone AC10) showing 50% similarity (E value = 7E−18) with *S. cerevisiae* MTM1 transporter, could potentially be involved in oxidative stress. An excess of heavy metals may stimulate the formation of free radicals and reactive oxygen species, resulting in oxidative stress (Hall 2002; Schützendübel and Polle 2002). Deletion studies (Luk et al. 2003) have shown that the yeast mitochondrial carrier functions in the activation of SOD2 by specifically facilitating insertion of the essential manganese cofactor. Recent biochemical analyses have demonstrated an increase in intracellular MnSOD in response to Zn treatment in *O. maius* Zn (E. Martino, University of Torino, personal communication), suggesting a possible role for this mitochondrial carrier in the maintenance of the cellular redox balance.

Likewise, we can only speculate on the possible significance of the increased transcription level observed for the *O. maius* Zn gene that codes for a putative SAM-dependent methyltransferase (clone AC5). SAM-dependent methyltransferases catalyse the transfer of methyl groups from SAM to diverse types of substrates. A SAM-dependent methyltransferase has been shown to be involved in the methylation of stress-induced pinosylvin in Scots pine needles (Chiron et al. 2000). In a study on protein accumulation during the senescence of *Podospora anserina*, Averbeck et al. (2000) identified a 27-kDa protein, PaMTH1, with significant homology to a SAM-dependent methyltransferase. The authors suggest that the protein is involved in age-related methylation reactions that protect aging cultures against increasing oxidative stress. It is not unlikely that prolonged exposure to heavy metals may cause a similar effect in mycelial cultures of *O. maius* Zn.

An important process in the maintenance of metal homeostasis is chelation by extracellular and intracellular organic compounds (Gadd 1993; Clemens 2001). Experiments with tobacco and papaya transgenic plants highly expressing citrate synthase have shown that increased production and secretion of citric acid confers tolerance to Al (de la Fuente et al. 1997). An increased release of organic acids (citric, fumaric and malic acids) in the medium following exposure to heavy metals was demonstrated biochemically for *O. maius* Zn (Martino et al. 2000b, 2003). However, expression analysis of clone BD1, coding for *O. maius* Zn citrate synthase, shows that this gene is repressed by Zn treatment. Although this finding seems to be in discordance with the biochemical data, some reports in the literature demonstrate that production of citric acid is not necessarily connected with the level of expression of the enzyme. For example, Ruijter et al. (2000), studying *A. niger* citrate synthase, showed that up to 11-fold overproduction of citrate synthase did not increase the rate of citric acid production by the fungus, suggesting that citrate synthase contributes little to flux control in the pathway involved in citric acid biosynthesis. The significance of repression of *O. maius* Zn citrate synthase gene transcription is currently unclear, as well as the exact role of the secreted citric acid in this strain (Martino et al. 2003).

A gene coding for a carboxypeptidase in *O. maius* Zn is up-regulated in the presence of high Zn concentration. Carboxypeptidases Y (CPY) are proteases widely distrib-

uted in fungi, as well as in higher plants and animals, which release C-terminal amino acids with a broad specificity. The involvement of CPY in the cellular response to an excess of unfolded proteins has been shown in some studies, based mainly on the detection of protein level and enzymatic activity (Lahav et al. 2004). It is possible that heavy metal stress in *O. maius*, like in other organisms, may boost the proteolytic system for the elimination of denatured proteins. Jungmann et al. (1993) showed in *S. cerevisiae* that the expression of components of the proteolytic pathway increased after exposure to Cd, and that mutants for one of these components were hypersensitive to the metal.

High concentrations of Zn ions repressed the transcription of a ketol-acid reductoisomerase homologue in *O. maius* Zn. Ketol-acid reductoisomerase is a key enzyme involved in the biosynthetic pathway of the amino acids isoleucine, valine, and leucine. This enzyme is of interest in agrochemical research because it is present only in plants and microorganisms, making it a potential target for specific herbicides and fungicides (Dumas et al. 2001).

Expression of the *O. maius* Zn gene encoding a carrier belonging to the major facilitator superfamily (MFS) was down-regulated in response to Zn treatment. The MFS, also called the uniporter-symporter-antiporter family, is a family of carriers capable of transporting small solutes in response to chemiosmotic ion gradients. MFS functions in the uptake of sugars, in drug efflux systems, Krebs cycle metabolites, and in organophosphate:phosphate exchange. The gene in the database with the highest similarity with the *O. maius* Zn gene is *B. fuckeliana* Bcmfs1. Hayashi et al. (2002) suggest that Bcmfs1 may be involved in protection of *B. fuckeliana* against plant defence compounds during the pathogenic phase of growth on host plants, and against fungitoxic antimicrobial metabolites during its saprophytic phase of growth.

Other regulated genes (AD2, BB8, AE11, BG11) code for proteins whose function is difficult to relate to a cellular role in *O. maius* Zn metal tolerance (Table 1); some others (AG1, BC4, CA2) code for proteins with unknown function and are potentially very interesting as they may represent novel genes related to heavy metal tolerance.

In conclusion, the sequence information and clones resulting from this study represent, to our knowledge, the first EST collection of an endomycorrhizal fungus in the presence of heavy metals. These data open the possibility to use *O. maius* as a model system for molecular genetic studies of heavy metal tolerance in ericoid fungi, and may also provide a useful tool for researchers studying heavy metal tolerance in other filamentous fungi.

It will now be crucial to further characterise the involvement of the differentially expressed genes in metal tolerance, and to establish their precise role not only for the fungus, but also for the host plant. Biochemical evidence (Martino et al. 2002, 2003) suggests that some fungal products are released into the extracellular environment, thus extending their potential protective role also to the plant.

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