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ORIGINAL PAPER

Gene expression profiling of a Zn-tolerant and a Zn-sensitive *Suillus luteus* isolate exposed to increased external zinc concentrations

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Abstract Complementary DNA (cDNA)-amplified fragment-length polymorphism (AFLP) was applied to analyze transcript profiles of a Zn-tolerant and a Zn-sensitive isolate of the ectomycorrhizal basidiomycete *Suillus luteus*, both cultured with and without increased external zinc concentrations. From the obtained transcript profiles that covered approximately 2% of the total expected complement of genes in *S. luteus*, 144 nonredundant, differentially expressed transcript-derived fragments (TDFs), falling in different classes of expression pattern, were isolated and sequenced. Thirty-six of the represented genes showed homology to function-known genes, whereas 6 matched unknown protein coding sequences, and 102 were possibly novel. Although relatively few TDFs were found to be responsive to the different zinc treatments, their modulated expression levels may suggest a different transcriptional response to zinc treatments in both isolates. Among the identified genes that could be related to heavy-metal detoxification or the tolerance trait were genes encoding for homologues of a heat-shock protein, a putative metal transporter, a hydrophobin, and several proteins involved in ubiquitin-dependent proteolysis.

Keywords *Suillus luteus* · Zn tolerance · Zn toxicity · Gene expression profiling · cDNA–AFLP

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Introduction

Some heavy-metal elements (e.g., Cu, Fe, and Zn) are essential micronutrients that are required for a wide variety of cellular processes, but may become cytotoxic at elevated concentrations. Various other metal elements (e.g., Hg and Pb) have not been shown to be essential for living organisms and can be toxic even at very low concentrations. Hence, soils that contain high concentrations of heavy metals, whether from natural origin or from anthropogenic activity, may pose a considerable challenge to exposed biota, and this selective pressure may lead to the evolution of tolerant ecotypes. The ectomycorrhizal basidiomycete *Suillus luteus* (L.:Fr.) is a common root symbiont found in pioneer conditions and has been shown to alleviate heavy-metal toxicity for its host plant in metal-contaminated environments. In vitro dose–response experiments have revealed the adaptive nature of the heavy-metal tolerance in this fungal species: populations inhabiting heavy-metal contaminated soils characteristically displayed a higher level of tolerance to those metals that were enriched in the soil of origin when compared with populations from noncontaminated soils (Colpaert et al. 2000, 2004; Adriaensen et al. 2005). Although the exact mechanism remains unclear, this tolerance trait is believed to be due to adaptations of mechanisms that are involved in the general homeostasis of and the constitutive tolerance to essential metal elements.

Possible strategies for preventing the build-up of toxic heavy-metal concentrations at sensitive sites within the cell include reduced uptake or enhanced efflux pumping of heavy metal ions, extracellular chelation, and cell-wall binding, intracellular chelation, and compartmentation by transport into the vacuole or other cell organelles. Additionally, heat-shock proteins and metallothioneins may be involved in the repair of stress-damaged proteins, and antioxidative detoxification mechanisms may reduce the accumulation of reactive-oxygen species initiated by metal ions (Hall 2002). In *S. luteus*, Zn exclusion mechanisms are most likely involved in the naturally selected adaptive Zn resistance (Colpaert et al. 2005).

In the present study, complementary DNA-amplified fragment length polymorphism (cDNA–AFLP) was performed to obtain a first insight into genes that are differentially expressed in Zn-tolerant and sensitive *S. luteus* isolates, both in the presence and absence of high external zinc concentrations, and that may be involved in stress relief and tolerance. cDNA–AFLP is an efficient method for the isolation and identification of differentially expressed genes, which gives reliable results when confirmed by RNA gel blot analysis (Bachem et al. 1996, 1998). It is a genome-wide expression analysis tool that does not require prior sequence information and, therefore, constitutes a useful tool for gene discovery (Ditt et al. 2001). In comparison with other techniques used for this purpose (e.g., suppression subtractive hybridization), cDNA–AFLP shows a very high specificity and allows distinguishing between genes with a high degree of sequence homology (Breyne and Zabeau 2001). The obtained information is useful for a better understanding of the mechanisms of heavy-metal toxicity and tolerance in ectomycorrhizal fungi, and because these fungi protect their host against toxic effects of heavy metals, it may eventually contribute to the development of more efficient phytoremediation strategies of polluted soils (Adriaensen et al. 2005, 2006).

Material and methods

Fungal material

Two isolates of *S. luteus* were used in this study: one Zn-tolerant isolate (UH–Slu–Lm8) obtained from a heavy metal polluted site in Lommel (Belgium) and one Zn-sensitive isolate (UH–Slu–P13) obtained from a nonpolluted site in Paal (Belgium). In a previous study, these isolates have been shown to reside at opposite extreme ends of the tolerance spectrum (Colpaert et al. 2004). The in vitro EC_{50} value for biomass production on solid Fries medium was 3.1 mM Zn for the UH–Slu–P13 isolate. The Zn-tolerant UH–Slu–Lm8 from the Lommel population had an in vitro EC_{50} value of 27.0 mM Zn.

For the present investigation, both isolates were cultured on the same solid modified Fries medium (Colpaert et al. 2004). The basic solution contained 28 mM glucose, 5.4 mM ammonium tartrate, 1.5 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.3 mM NaCl, 0.2 mM $CaCl_2 \cdot 2H_2O$, 56 μM *myo*-inositol, 4 μM $FeCl_3 \cdot 6H_2O$, 6 μM $MnSO_4 \cdot H_2O$, 0.8 μM $CuSO_4 \cdot 5H_2O$, 0.8 μM nicotinamide, 0.7 μM *p*-aminobenzoic acid, 0.5 μM pyridoxine, 0.3 μM riboflavin, 0.3 μM thiamine, 0.2 μM Ca-pantothenate, 0.1 μM biotin, and 0.8% agar. Zinc was added to the nutrient medium as $ZnSO_4 \cdot 7H_2O$ at three different concentrations of 0.02, 1.5, and 3 mM Zn^{2+} for the different treatments. The pH of the final medium was adjusted to 4.5.

Uniform inocula (0.5-cm^2 plugs of fungal mycelium) were prepared from 1-week-old cultures of both isolates by preincubation of various plugs of mycelium on cellophane-covered agar plates with basic medium for 2 or 3 days. Single inocula were transferred to the test plates covered with cellophane and were allowed to grow in the dark for 7 days at 23°. Three replicates were made for each treatment.

RNA extraction and cDNA synthesis

Cultured mycelial tissue from the three replicates was pooled and ground in liquid nitrogen. Total RNA was extracted from 60-mg ground tissue using the RNeasy plant mini-kit (Qiagen, France) and poly(A)$^+$ RNA was isolated from 250-μg samples of total RNA using Oligotex columns (Qiagen), both according to the manufacturer's instructions. Double-stranded cDNA was synthesized starting from 0.5–1 μg of poly(A)$^+$ RNA using the Smart™ cDNA library construction kit (Clontech, USA) following the manufacturer's instructions and ligation-dependent polymerase chain reaction (LD-PCR) products were purified by a QIAquick PCR purification kit (Qiagen).

cDNA–AFLP analysis

cDNA–AFLP analysis was performed following Bachem et al. (1996). In brief, approximately 0.5 μg of double-stranded cDNA was digested with the restriction enzymes *Bst*YI and *Mse*I in a two-step reaction. After adaptor ligation, the preamplifications were performed with the *Mse*I and *Bst*YI primers without selective nucleotides (*Bst*YI primers had C as 3′ nucleotide). From a tenfold dilution of preamplification product, 5 μl was used for the subsequent selective amplification using *Mse*I and ^{33}P-labelled *Bst*YI primers with two selective nucleotides (all possible combinations of *Bst*YI primers+CN and *Mse*I primers+NN were used). Amplification products were separated on 5% denaturing polyacrylamide gels using the SequiGen system (BioRad, USA). Vacuum-dried gels were exposed to Fuji super RX medical X-ray film (Fujifilm

Medical Systems, Benelux NV, Belgium) and scanned with a Storm 860 PhosphoImager (Molecular Dynamics, USA).

Characterization of cDNA–AFLP fragments

Bands corresponding to transcripts of which the steady-state expression levels differed between both isolates in at least one of the three zinc treatments were cut out from gel, and the DNA fragments were extracted according to Frost and Guggenheim (1999). The gel fragments were rehydrated in 100 µl of 2× GoTaq PCR buffer (100 mM KCl, 20 mM Tris–HCl at pH 9.0 and 25°C, 3 mM $MgCl_2$, and 0.2% Triton® X-100, Promega Benelux, The Netherlands) for 10 min at room temperature. The remaining buffer was removed, 100 µl of fresh buffer was added, and the samples were incubated at 94°C for 90 min.

The eluted DNA was purified using a Purelink™ 96 PCR purification kit (Invitrogen, USA) and reamplified under the same conditions as used for selective amplification. The amplified fragments were ligated into pCR®2.1-TOPO® vectors (Invitrogen) according to the manufacturer's instructions, and the plasmids were transformed into *Escherichia coli*, TOP10 strain (Invitrogen). Plasmid inserts from four positive clones were PCR amplified in 20-µl reactions containing 0.5 µM M13f-primer, 0.5 µM M13r-primer, 1.5 U *Taq* DNA polymerase (Amersham Pharmacia Biotech, USA), 10 mM Tris–HCl at pH 9, 15 mM $MgCl_2$, 50 mM KCl, and 2 mM of all four deoxyribonucleotide triphosphates (dNTPs). After an initial cell lysis at 95° for 5 min, PCR was performed with the following temperature profile: 35 cycles of 95° for 20 s, 50° for 20 s, and 72° for 1.5 min. The PCR products were diluted 20 times, and 1 µl of each was used in a separate 20-µl sequencing reaction with the M13f-primer using ABI BigDye® terminator reaction mix (Applied Biosystems, USA), following the manufacturer's instructions. The sequences were run on an ABI PRISM® 3100-avant genetic analyzer. The local basic local alignment search tool (BLAST) function in the BioEdit software package (Hall 1999) was used to identify redundant transcript-derived fragments (TDFs), and database searches were performed using the BLAST network service (NCBI, National Center for Biotechnology Service; http://www.ncbi.nlm.nih.gov/BLAST). The functions of function-known genes by BLASTX and TBLASTX sequence alignments (Altschul et al. 1997) with the nonredundant (nr) databases were classified according to their putative function as described in the *Saccharomyces cerevisiae* functional catalogue (http://mips.gsf.de/genre/proj/yeast/).

RT-PCR analysis

To verify cDNA–AFLP expression patterns, RT-PCR analysis of selected differentially regulated genes and a nonregulated control gene was performed using cDNA from the original *S. luteus* isolates as template. Based on the sequence of the recovered TDFs, gene-specific primers were designed using primer3 software (Rozen and Skaletsky 1996) and tested for PCR amplification of a fragment of the same size with equal efficiency in both isolates using genomic DNA as template and the same PCR conditions as described below. Confirmation of the cDNA–AFLP expression patterns was performed with equal amounts of template cDNA (approximately 25 ng) in 20-µl reactions containing 0.5 µM of each primer (see Table 1 for primer combinations), 1 U

Table 1 Primer combinations used for RT-PCR verification of differentially expressed cDNA–AFLP fragments in a Zn-tolerant and a Zn-sensitive *Suillus luteus* isolate

TDF	Primer sequence (5′ → 3′)	T_a (°C)	Product size (bp)
C	F: TGAAGGCAAAGTCACGAATG R: TTCCCGAGCATTGATGTCTAC	56	247
16 Putative copper ion transporter	F: CCCGTCTCCTCAGCATTATC R: GGAACACCCAAATCATCGAC	56	250
27 Glutathione–*S*-transferase	F: GCAGTGGTATCAACGCAGAG R: TGCTCGACGAACATCCATAC	55	245
61 Ketol-acid reductoisomerase	F: ATCCTGGAAGACGGCTACAC R: GCACAATCACAAACGTGGTC	56	222
66 Predicted protein	F: CAACCCAAGATCATTCATGC R: GCCTGGACTGAAACCATGTC	56	214
89 Metallothionein	F: ACTTGCAGGCGCCTTTACAC R: TTCCCAAGTCCCTGTTTCTG	57	213
128 G protein subunit alpha	F: CGCACTGTATTCGTAGTTGA R: GCGTGGTTATGCGATGTTTA	53	139
134 Heat-shock protein HSP60	F: ACTCCTGAACGACCCAACC R: AAGGCTGGCCTTGAGAAGC	57	223
147 Manganese superoxide dismutase	F: ATCCGATCGTCACGACTGC R: TTAAGGTACTGGAGGTAGAAAGCA	55	100

T_a Annealing temperature

Taq DNA polymerase (Amersham Pharmacia Biotech), 10 mM Tris–HCl at pH 9, 15 mM $MgCl_2$, 50 mM KCl, and 200 μM of all four dNTPs. The temperature profile of the PCR was the following: initial denaturation at 95° for 2 min, 30 cycles of 95° for 30 s, primer-specific annealing temperature for 30 s and 72° for 1 min, followed by a final extension at 72° for 10 min. Amplified PCR products (10 μl) were electrophoresed on a 2% (*w*/*v*) agarose gel and visualized by ethidium bromide staining.

Results

cDNA was synthesized from RNA extracted from a Zn-tolerant and a Zn-sensitive *S. luteus* isolate, cultured on solid medium with three different concentrations of zinc. A clear difference in the response to elevated zinc concentrations was apparent between the two isolates, as growth rate of the nontolerant isolate was significantly more restricted at zinc concentrations of 1.5 and 3 mM when compared with the growth rate of the tolerant isolate. However, both isolates maintained a continuous growth rate at the applied zinc concentrations, and total RNA quantification indicated that the amounts of RNA did not vary significantly between the control and the zinc-treated mycelia, which indicates that the zinc treatments were not lethal. The fact that only a limited number of genes were affected in the Zn-treated UH–Slu–P13 isolate also suggests a relatively mild level of the Zn stress.

cDNA–AFLP expression patterns were obtained by selective amplification using 64 of the 128 possible primer combinations (*Bst*YI primers+CN, *Mse*I primers+NN), and 59 of these revealed TDFs with differential expression patterns (Fig. 1). In total, 3,793 TDFs were screened, and 213 (6%) were found to be expressed at different levels in both isolates in at least one of the three zinc treatments. Most of these TDFs were exclusively and constitutively expressed either in the tolerant isolate or in the sensitive isolate (70 TDFs in the tolerant isolate and 67 in the sensitive isolate). Two of the TDFs that were exclusively expressed in the tolerant isolate showed reduced expression levels at elevated zinc concentrations, and four of the TDFs that were exclusively expressed in the nontolerant isolate showed increased expression levels at higher zinc concentrations. The remaining TDFs were expressed in the tolerant and the nontolerant isolate, either constitutively in both but with different expression levels (38 TDFs) or modulated in response to an elevated zinc concentration (32 TDFs). In the Zn-tolerant isolate, expression levels of four TDFs increased at elevated zinc concentrations and expression levels of ten TDFs decreased, whereas in the sensitive isolate, expression levels of seven TDFs increased and expression levels of three TDFs decreased.

One hundred ninety-seven TDFs (92%) were successfully recovered from the gel matrix and PCR amplified. The recovered fragments, ranging in length between 50 and 450 bp, were cloned before sequencing to prevent problems associated with direct sequencing of PCR products (Durrant et al. 2000; Ditt et al. 2001), and correct sequences were obtained for 156 TDFs. Due to the presence of comigrating fragments, the correct sequences of 41 TDFs could not be identified unambiguously.

Based on pairwise comparisons among the 156 sequences of recovered fragments, 144 TDFs were found to represent potentially different genes (Table 2; GenBank accession numbers AM085154–AM085297). Redundancy was due to the presence of highly homologous sequences, resulting from mispriming during PCR amplification or

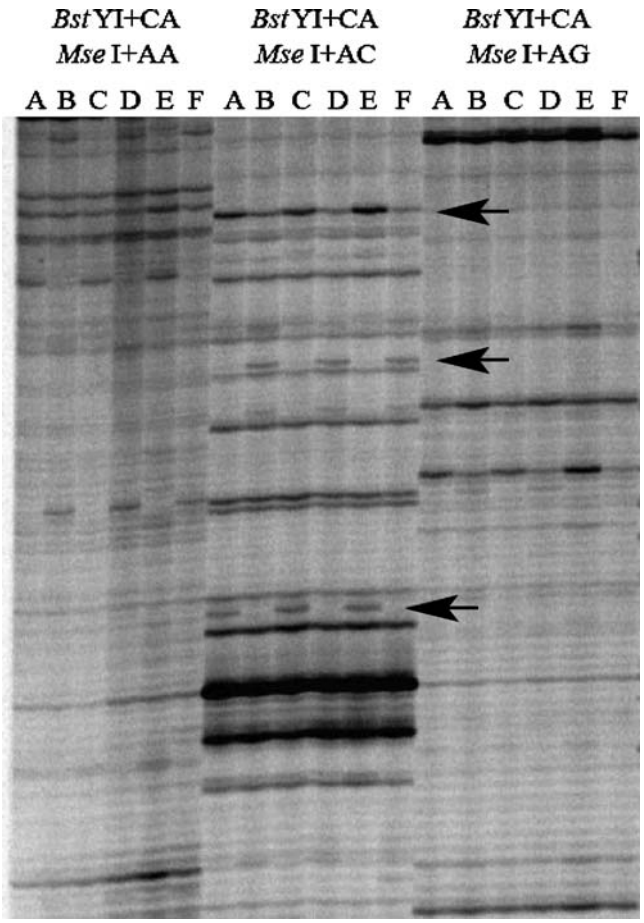

Fig. 1 Part of an autoradiograph obtained by cDNA–AFLP analysis of a Zn-tolerant and a Zn-sensitive *S. luteus* isolate. The primer combinations that were used to generate the gene expression profiles are indicated *above the lanes*; *A* tolerant isolate grown on Fries medium with 0.02 mM Zn^{2+}, *B* sensitive isolate grown on Fries medium with 0.02 mM Zn^{2+}, *C* tolerant isolate grown on Fries medium with 1.5 mM Zn^{2+}, *D* sensitive isolate grown on Fries medium with 1.5 mM Zn^{2+}, *E* tolerant isolate grown on Fries medium with 3 mM Zn^{2+}, *F* sensitive isolate grown on Fries medium with 3 mM Zn^{2+}, *arrows* indicate TDFs with differential expression patterns

Table 2 Sequence homology, expression patterns, and functional classification of differentially expressed transcript-derived fragments (TDFs) obtained by cDNA–AFLP of a Zn-tolerant and a Zn-sensitive *Suillus luteus* isolate cultured on solid media with different zinc concentrations; the expression levels per gene are described as *highest*, *reduced*, or *none*

			Zn treatments										
			UH–Slu–Lm8			UH–Slu–P13							
	TDF	Size (bp)	0.02 mM	1.5 mM	3 mM	0.02 mM	1.5 mM	3 mM	Homology	*E* value	GenBank accession no.	BLAST hit	Functional classification
	Tolerant isolate exclusive TDFs—constitutive expression												
	28	159	Highest	Highest	Highest	None	None	None	*Cryptococcus neoformans* ARF small monomeric GTPase	6.00E-20	AM085226	AAW44725	Transport
	31	139	Highest	Highest	Highest	None	None	None	*Neurospora crassa* G protein subunit alpha	2.00E-12	AM085229	AAA02559	Signal transduction
	44	193	Highest	Highest	Highest	None	None	None	*Cryptococcus neoformans* predicted protein	1.00E-12	AM085241	EAL20604	Unknown
	50	153	Highest	Highest	Highest	None	None	None	*Ustilago maydis* predicted protein	3.00E-07	AM085247	EAK84662	Unknown
	51	123	Highest	Highest	Highest	None	None	None	*Cryptococcus neoformans* predicted protein	3.00E-11	AM085248	AAW43861	Unknown
	59	159	Highest	Highest	Highest	None	None	None	*Agaricus bisporus* glyceraldehyde-3-phosphate reductase	3.00E-20	AM085255	AAA32634	Metabolism and energy
	73	59	Highest	Highest	Highest	None	None	None	*Cryptococcus neoformans* predicted protein	4.00E-12	AM085268	AAW46744	Unknown
	81	266	Highest	Highest	Highest	None	None	None	*Saccharomyces cerevisiae* cytoplasmic arginyl–tRNA synthetase	9.00E-22	AM085275	NP_010628	Protein synthesis
	85	362	Highest	Highest	Highest	None	None	None	*Pisolithus tinctorius* hydrophobin-3 precursor	2.00E-12	AM085279	AAC95356	Cellular organization
	87	148	Highest	Highest	Highest	None	None	None	*Cryptococcus neoformans* 60S ribosomal protein	4.00E-10	AM085281	AAW41987	Protein synthesis
	98	219	Highest	Highest	Highest	None	None	None	*Ustilago maydis* predicted protein	5.00E-07	AM085291	EAK82359	Unknown
	107	118	Highest	Highest	Highest	None	None	None	*Neurospora crassa* dihydroxy-acid dehydratase	6.00E-10	AM085166	CAD70774	Metabolism and energy
	116	295	Highest	Highest	Highest	None	None	None	*Arabidopsis thaliana* postsynaptic protein cript	6.00E-12	AM085175	AAM67128	Signal transduction
	134	232	Highest	Highest	Highest	None	None	None	*Cryptococcus neoformans* heat shock protein HSP60	1.00E-30	AM085191	AAW41904	Cell rescue and defense
	137	212	Highest	Highest	Highest	None	None	None	*Cryptococcus neoformans* 40S ribosomal protein s12	4.00E-13	AM085194	AAW42305	Protein synthesis
	Tolerant isolate exclusive TDFs—modulated expression												
	16	371	Reduced	Reduced	Highest	None	None	None	*Cryptococcus neoformans* putative copper ion transporter	6.00E-13	AM085214	AAW46226	Transport
	Nontolerant isolate exclusive TDFs—constitutive expression												
	47	280	None	None	None	Highest	Highest	Highest	*Paxillus involutus* glutathione *S*-transferase	8.00E-31	AM085244	AAT91250	Cell rescue and defense
	60	315	None	None	None	Highest	Highest	Highest	*Cryptococcus neoformans* hydrolase	2.00E-12	AM085256	AAW42079	Metabolism and energy
	62	174	None	None	None	Highest	Highest	Highest	*Schizosaccharomyces pombe* transcription factor btf3 homologue	4.00E-09	AM085258	NP_594757	Transcription
	100	254	None	None	None	Highest	Highest	Highest	*Cryptococcus neoformans* arginine–tRNA ligase	1.00E-13	AM085293	AAW44508	Protein synthesis
	111	217	None	None	None	Highest	Highest	Highest	*Saccharomyces cerevisiae* threonine synthase	1.00E-06	AM085169	CAA35157	Metabolism and energy
	132	313	None	None	None	Highest	Highest	Highest	*Cryptococcus neoformans* mitochondrial translocase subunit tim8	5.00E-20	AM085189	AAW43967	Transport
t2.29	155	213	None	None	None	Highest	Highest	Highest	*Neurospora crassa* cyanate lyase	6.00E-12	AM085211	CAD70840	Metabolism

Table 2 (continued)

TDF	Size (bp)	Zn treatments						Homology	*E* value	GenBank accession no.	BLAST hit	Functional classification
		UH–Slu–Lm8			UH–Slu–P13							
		0.02 mM	1.5 mM	3 mM	0.02 mM	1.5 mM	3 mM					
												and energy
Shared TDFs—constitutive expression												
2	160	Highest	Highest	Highest	Reduced	Reduced	Reduced	*Schizosaccharomyces pombe* aconitate hydratase	1.00E-09	AM085155	NP_594031	Metabolism and energy
10	106	Highest	Highest	Highest	Reduced	Reduced	Reduced	*Candida albicans* farnesyl diphosphate synthetase	2.00E-06	AM085163	EAK93751	Metabolism and energy
30	226	Highest	Highest	Highest	Reduced	Reduced	Reduced	*Cryptococcus neoformans* proteasome subunit β type 2	1.00E-20	AM085228	AAW42482	Metabolism and energy
129	120	Highest	Highest	Highest	Reduced	Reduced	Reduced	*Cryptococcus neoformans* proteasome component pts1	3.00E-11	AM085186	AAW44255	Metabolism and energy
133	276	Highest	Highest	Highest	Reduced	Reduced	Reduced	*Cryptococcus neoformans* ARF small monomeric GTPase	1.00E-44	AM085190	AAW44725	Transport
152	283	Highest	Highest	Highest	Reduced	Reduced	Reduced	*Cryptococcus neoformans* retinal short-chain dehydrogenase	1.00E-12	AM085208	AAW45076	Metabolism and energy
9	240	Reduced	Reduced	Reduced	Highest	Highest	Highest	*Aspergillus flavus* taka-amylase	1.00E-13	AM085162	AAS89890	Metabolism and energy
27	183	Reduced	Reduced	Reduced	Highest	Highest	Highest	*Arabidopsis thaliana* glutathione *S*-transferase	2.00E-12	AM085225	NP_193723	Cell rescue and defense
89	259	Reduced	Reduced	Reduced	Highest	Highest	Highest	*Paxillus involutus* metallothionein	4.00E-06	AM085283	AAS19463	Cell rescue and defense
104	99	Reduced	Reduced	Reduced	Highest	Highest	Highest	*Aspergillus nidulans* enolase	2.00E-07	AM085297	EAA62839	Metabolism and energy
106	110	Reduced	Reduced	Reduced	Highest	Highest	Highest	*Cryptococcus neoformans* CTP synthase	2.00E-12	AM085165	AAW42085	Metabolism and energy
Shared TDFs—modulated expression												
66	223	None	None	Highest	Highest	Highest	Highest	*Cryptococcus neoformans* predicted protein	1.00E-08	AM085262	EAL19218	Unknown
136	237	Reduced	Highest	None	None	Reduced	Reduced	*Suillus pictus* translation elongation factor EF1-alpha	2.00E-37	AM085193	AAW80842	Protein synthesis
109	146	Highest	Highest	Reduced	Highest	Highest	Highest	*Glomus intraradices* myc1	5.00E-08	AM085168	AAF14349	Transcription
115	99	Highest	Highest	Reduced	Highest	Highest	Highest	*Cryptococcus neoformans* 60S ribosomal protein l11	1.00E-09	AM085173	AAW45899	Protein synthesis
128	142	Highest	Highest	None	Highest	Highest	Highest	*Cryptococcus neoformans* thioredoxin peroxidase	5.00E-17	AM085184	AAW46264	Cell rescue and defense
147	102	Highest	Highest	None	Reduced	Reduced	Reduced	*Paxillus involutus* manganese–superoxide dismutase	6.00E-10	AM085202	AAD25353	Cell rescue and defense
61	261	Highest	Highest	Reduced	Reduced	Highest	Highest	*Cryptococcus neoformans* ketol-acid reductoisomerase	2.00E-38	AM085257	AAW44038	Metabolism and energy

representing either different alleles from the same gene or different instances of multicopy genes (Bachem et al. 1996).

Using a threshold value of 1E-05 for the *E* value returned by the BLASTX or the TBLASTX search algorithm, about 25% of the nonredundant TDFs showed homology with genes of known function, 4% showed homology with function-unknown genes, and 71% showed no homology (Table 2). In most cases, highest homology scores corresponded to sequences from fungal species, in particular *Cryptococcus neoformans* (20 sequences). The species showing highest homologies with *S. luteus* translated gene sequences included the closely related species *S. pictus*, but high homology scores were also obtained for sequences from bacterial, animal, and plant species. Some TDFs showed homology with different parts of the same gene and are either derived from the same transcript or represent different alleles or different instances of a multicopy gene: TDFs 27 and 47, homologous with a gene encoding for glutathione *S*-transferase, TDFs 28 and 133, homologous with an ARF small monomeric GTPase gene, and TDFs 81 and 100, homologous with a gene encoding for arginine–transfer RNA (tRNA) ligase. Of the 36 nonredundant function-known genes, 15 are involved in cellular metabolism and energy production, 6 in cell rescue/defense and 6 in protein synthesis. Other genes have functions in cellular transport (four), signal transduction (two), transcription (two), and cellular organization (one).

To validate the cDNA–AFLP expression patterns, eight differentially expressed TDFs were selected for RT-PCR analysis using specific primer sequences and cDNA from the original isolates as template. The comparisons showed that six of the eight cDNAs examined had the same expression profiles as revealed by cDNA–AFLP (Fig. 2). A different expression pattern was obtained for TDFs 89 and 134. In case of TDF 134, the homologous band was absent in the transcript profile of the nontolerant isolate, whereas RT-PCR showed the corresponding gene to be expressed. This apparent absence of a homologous band in the transcript profile could be due to sequence divergence of the corresponding genes in both isolates. In case of TDF 89, RT-PCR did not confirm the observed presence of a band in the tolerant isolate treated with 3 mM Zn^{2+}, which may indicate a failed RT-PCR or the presence of nonhomologous fragments of the same size. However, in general, cDNA–AFLP is shown to be a reliable method for identifying differentially expressed genes in *S. luteus* exposed to heavy-metal stress.

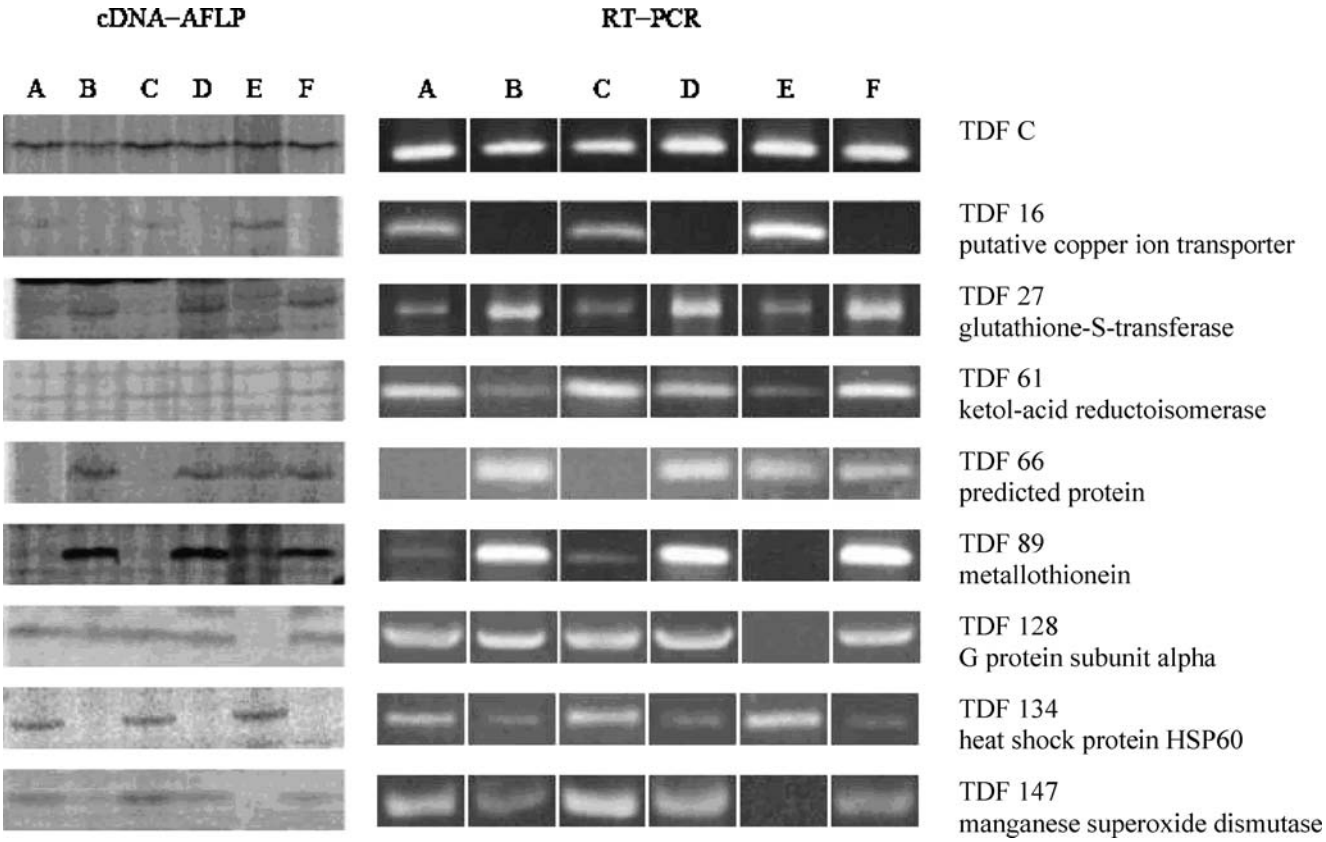

Fig. 2 Comparison of expression patterns of eight differentially expressed genes and one constitutively expressed control gene (TDF C) obtained by cDNA–AFLP and RT-PCR; *A*/*C*/*E* transcript profile of Zn-tolerant isolate treated with 0.02, 1.5, and 3 mM Zn^{2+}, respectively; *B*/*D*/*F* transcript profile of Zn-sensitive isolate treated with 0.02, 1.5, and 3 mM Zn^{2+}, respectively

Discussion

Estimations suggest that the genome of filamentous fungi, including ectomycorrhizal fungi, usually varies between 20 and 40 Mb, with a complement of about 8,000 genes (Kupfer et al. 1997; Le Quéré et al. 2002). Using this estimation for the number of genes in *S. luteus*, the present set of 140 nonredundant differentially expressed TDFs corresponds to approximately 2% of the total expected complement of genes. A total of 42 TDFs (30%) corresponded to genes homologous to GenBank entries, including genes of known function as well as hypothetical proteins. The remaining TDFs (70%) did not have significant matches within the GenBank databases and may represent undescribed fungal genes, although this could also be partly due to the fragments being derived from either the 5′ or the 3′ untranslated messenger RNA (mRNA) regions. This relatively high proportion of TDFs showing no homology to GenBank entries is compatible with the range of 50–65% reported for expressed sequence tags (ESTs) in other filamentous fungi (Lee et al. 2002; Ospina-Giraldo et al. 2000; Skinner et al. 2001) and might reflect the low number of database entries describing sequences of fungal origin.

In concordance with the high levels of genomic divergence found between *S. luteus* isolates (Muller et al. 2004), considerable variation in steady-state transcript profiles was revealed between the tolerant and the nontolerant *S. luteus* isolate, both following treatments with physiological external zinc concentrations and increased concentrations. Although, in several cases, the differential expression pattern was shown to be caused by sequence divergence of the corresponding transcripts, RT-PCR analysis confirmed that the differential TDF profiles were mainly due to differential gene expression in both isolates. Adaptive evolution of heavy-metal tolerance by natural selection may explain this difference in gene expression to a certain extent, but other causes, including genetic hitchhiking and natural regulatory variation, may be equally important. In genomic regions with low recombination rates, positive selection of alleles responsible for the tolerance trait and modifiers that enhance the tolerance may be associated with a reduction of the genetic variation around the selected sites (Maynard Smith and Haigh 1974), and this signature of selection may be important at the genome-wide level and more common than usually accepted (Quesada et al. 2003). Besides the variation due to this genetic hitchhiking effect, part of the observed differential expression in both *S. luteus* isolates will be due to variation in gene expression levels present in natural populations. Townsend et al. (2003) reported extensive natural regulatory variation in *Saccharomyces cerevisiae* and showed that the majority of these differences in gene expression are below the twofold level. Although most of the differences in expression profiles between both *S. luteus* isolates were due to TDFs exclusively observed in either one of the isolates and could, therefore, be expected not to result from natural regulatory variation, a larger number of isolates should be screened to assess the importance of natural regulatory variation in *S. luteus*.

Relatively few genes were found to be responsive to the zinc treatments at the transcript level, but an apparent difference in the TDF profiles of these genes may reflect a difference in response to elevated external zinc concentrations between the tolerant and the nontolerant isolate. In the Zn-tolerant isolate, a modulation of the TDF profiles was, in general, only observed at 3 mM zinc, whereas in the Zn-sensitive isolate, a response was already observed at 1.5-mM external zinc. Both isolates also differed in the set of genes responsive to zinc treatment and in the way that TDF expression levels were affected, a decrease being the main effect in the tolerant isolate and an increase in the nontolerant isolate. In the nontolerant isolate, elevated external zinc concentrations increased the TDF expression levels of ketol-acid reductoisomerase and translation elongation factor 1 respectively involved in amino acid biosynthesis and protein synthesis. Increased transcript levels have been shown for ketol-acid reductoisomerase after heat shock and for translation elongation factor 1 in response to heavy-metal-induced stress, but the functional significance of this increase remains unclear (Joseph et al. 2002; Rosen et al. 2002). In the tolerant isolate, differentially expressed genes of which the TDF expression levels were down regulated at the 3-mM zinc concentration encoded for homologues of ketol-acid reductoisomerase, thioredoxin peroxidase, manganese superoxide-dismutase, and translation elongation factor 1, as well as homologues of Myc1 and a 60S ribosomal protein, the latter respectively involved in transcription and protein synthesis.

Among the identified genes, five showed significant similarity with genes encoding proteins involved in cell rescue and defense: glutathione *S*-transferase (TDFs 27/47), metallothionein (TDF 89), thioredoxin peroxidase (TDF 128), heat-shock protein HSP60 (TDF 134), and manganese superoxide-dismutase (TDF 147). Three of these proteins are known to be responsive to oxidative stress: thioredoxin peroxidase, which reduces organic hydroxyperoxides and H_2O_2 to harmless products using thioredoxin as an electron donor (Godon et al. 1998; Lee et al. 1999); glutathione *S*-transferase, which metabolizes toxic products of lipid peroxidation and DNA damage caused by reactive oxygen species and detoxifies H_2O_2 by oxidizing reduced glutathione (Marrs 1996; Roxas et al. 2000); and manganese superoxide-dismutase, which catalyzes the conversion of superoxide radicals ($\cdot O_2^-$) to hydrogen peroxide and oxygen (Jacob et al. 2001). Although zinc is not a redox-active

metal, exposure to zinc is connected with the accumulation of reactive oxygen species because of its capability to displace bounded iron, a redox-active metal, from DNA and proteins, and because of its stimulatory effect on ferri-reductase activity, which may also generate superoxide radicals (Fujs et al. 2005). Nevertheless, an increase of the TDF expression levels of the three genes involved in the protection against oxidative stress was not observed in either *S. luteus* isolate after zinc treatment. On the contrary, a reduction of the expression level was observed in the tolerant isolate for manganese superoxide-dismutase and thioredoxin peroxidase.

An increase in TDF expression levels of heat-shock protein HSP60 or metallothionein after zinc treatment was not observed either. Heat-shock proteins are known to be induced under a variety of stress factors that cause protein unfolding, misfolding, or aggregation (e.g., heat, UV radiation, and heavy-metal ions), and are capable of re-establishing the balance between protein synthesis, assembly, and degradation. HSP60 is believed to protect enzymes possessing Fe/S clusters, such as aconitase and succinate dehydrogenase, from oxidative inactivation and release of their iron, thus, preventing additional oxidative stress (Cabiscol et al. 2002). Metallothioneins are small proteins containing cysteine clusters that have a high affinity for heavy metals, in particular copper, cadmium, and zinc. Their gene expression is induced by a great number of stimuli, among which are heavy-metal load, viral infection, and UV radiation, and they have been implicated in heavy-metal detoxification, metal homeostasis, and radical scavenging (Heuchel et al. 1994).

Vallino et al. (2005) reported a similar observation in the ericoid mycorrhizal fungus *Oidiodendron maius*, where EST analysis revealed no transcriptional response of stress-related genes after zinc treatment, and proposed this to be due to the applied zinc concentrations not being high enough to induce severe toxic effects. However, growth of the nontolerant *S. luteus* isolate was clearly inhibited by both zinc treatments, thus, rendering their nontoxicity unlikely. Possibly, other defense systems dominate the stress response, or posttranslational mechanisms control these defense systems. Jacob et al. (2001) showed that the transcript level of manganese superoxide-dismutase was not altered by cadmium exposure of *Paxillus involutus* isolates and postulated this result to be due to posttranslational mechanisms that activate a pre-existing pool of manganese superoxide-dismutase in response to cadmium-induced stress.

In conclusion, the results from this study proved cDNA–AFLP to be an efficient and high fidelity method to screen the effects of heavy-metal-induced stress on the steady-state transcript profiles of *S. luteus*. One hundred forty-four nonredundant and differentially expressed TDFs were isolated, and RT-PCR analysis confirmed their differential expression to be mainly due to differences in gene expression levels. Although a clear difference at the transcript level in response to the zinc treatments was found between the tolerant and the sensitive isolate, the relationship between the affected genes and the metal-induced stress was not always clear. In some cases, however, the difference in expression profiles between both isolates indicated a possible role in the cellular defense against heavy metals of the genes involved, including genes encoding for a putative metal transporter, a hydrophobin (Pawlik-Skowrońska et al. 2002), and several proteins involved in the ubiquitin-dependent degradation of proteins (Chondrogianni et al. 2005), but further experiments are necessary to confirm their roles. These data provide a basis for the use of *S. luteus* as a model system for molecular genetic studies of heavy-metal tolerance in ectomycorrhizal fungi and may aid researchers studying heavy-metal tolerance in other filamentous fungi. It will now be necessary to characterize the involvement of the identified genes in heavy metal tolerance and to establish their precise role.

References

Adriaensen K, Vrålstad T, Noben JP, Vangronsveld J, Colpaert JV (2005) Copper-adapted *Suillus luteus*, a symbiotic solution for pines colonising Cu mine spoils. Appl Environ Microbiol 11:7279–7284

Adriaensen K, Vangronsveld J, Colpaert JV (2006) Zinc-tolerant *Suillus bovinus* improves growth of Zn-exposed *Pinus sylvestris* seedlings. Mycorrhiza 16:553–558

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Bachem CW, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RG (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J 9:745–753

Bachem CW, Oomen RJF, Visser RG (1998) Transcript imaging with cDNA–AFLP: a step-by-step protocol. Plant Mol Biol Rep 16:157–173

Breyne P, Zabeau M (2001) Genome-wide expression analysis of plant cell cycle modulated genes. Curr Opin Plant Biol 4:136–142

Cabiscol E, Belli G, Tamarit J, Echave P, Herrero E, Ros J (2002) Mitochondrial Hsp60, resistance to oxidative stress, and the labile iron pool are closely connected in *Saccharomyces cerevisiae*. J Biol Chem 277:44531–44538

Chondrogianni N, Tzavelas C, Pemberton AJ, Nezis IP, Rivett AJ, Gonos ES (2005) Overexpression of a proteasome β_5 subunit increases the amount of assembled proteasome and confers ameliorated response to oxidative stress and higher survival rates. J Biol Chem 280:11840–11850

Colpaert JV, Vandenkoornhuyse P, Adriaensen K, Vangronsveld J (2000) Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. New Phytol 147:367–379

Colpaert JV, Muller LAH, Lambaerts M, Adriaensen K, Vangronsveld J (2004) Evolutionary adaptation to Zn toxicity in populations of Suilloid fungi. New Phytol 162:549–560

Colpaert JV, Adriaensen K, Muller LAH, Lambaerts M, Faes C, Carleer R, Vangronsveld J (2005) Element profiles and growth in Zn-sensitive and Zn-resistant Suilloid fungi. Mycorrhiza 15:628–634

Ditt RF, Nester RW, Comai L (2001) Plant gene expression response to *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 98:10954–10959

Durrant WE, Rowland O, Piedras P, Hammond-Kosack KE, Jones JDG (2000) cDNA–AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. Plant Cell 12:963–977

Frost MR, Guggenheim JA (1999) Prevention of depurination during elution facilitates the re-amplification of DNA from differential display gels. Nucleic Acids Res 27:e6

Fujs S, Gazdag Z, Poljsak B, Stibilj V, Milacic R, Pesti M, Raspor P, Batic M (2005) The oxidative stress response of the yeast *Candida intermedia* to copper, zinc, and selenium exposure. J Basic Microbiol 45:125–135

Godon C, Langniel G, Lee J, Buhler JM, Kieffer S, Perrot M, Boucherie H, Toledano MB, Labarre J (1998) The H_2O_2 stimulon in *Saccharomyces cerevisiae*. J Biol Chem 273:22480–22489

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1–11

Heuchel R, Radtke F, Georgiev O, Stark M, Aguet M, Schaffner W (1994) The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. EMBO J 13:2870–2875

Jacob C, Courbot M, Brun A, Steinman HM, Jacquot JP, Botton B, Chalot M (2001) Molecular cloning, characterization and regulation by cadmium of a superoxide dismutase from the ectomycorrhizal fungus *Paxillus involutus*. Eur J Biochem 268:3223–3232

Joseph P, Lei Y-X, Whong W-Z, Ong TM (2002) Oncogenic potential of mouse translation elongation factor-1δ, a novel cadmium-responsive proto-oncogene. J Biol Chem 277:6131–6136

Kupfer DM, Reece CA, Clifton SW, Roe BA, Prade RA (1997) Multicellular ascomycetous fungal genomes contain more than 8000 genes. Fungal Genet Biol 21:364–372

Lee J, Godon C, Langniel G, Spector D, Garin J, Labarre J, Toledano MB (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J Biol Chem 274:16040–16046

Lee SH, Kim BG, Kim KJ, Lee JS, Yun DW, Hahn JH, Kim GH, Lee KH, Suh DS, Kwon ST, Lee CS, Yoo YB (2002) Comparative analysis of sequences expressed during the liquid-cultured mycelia and fruit body stages of *Pleurotus ostreatus*. Fungal Genet Biol 35:115–134

Le Quéré A, Johansson T, Tunlid A (2002) Size and complexity of the nuclear genome of the ectomycorrhizal fungus *Paxillus involutus*. Fungal Genet Biol. 36:234–241

Marrs KA (1996) The function and regulation of glutathione *S*-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol 47:127–158

Maynard Smith J, Haigh J (1974) The hitch-hiking effect of a favourable gene. Genet Res 23:23–35

Muller LAH, Lambaerts M, Vangronsveld J, Colpaert JV (2004) AFLP-based assessment of the effects of environmental heavy metal pollution on the genetic structure of pioneer populations of *Suillus luteus*. New Phytol 164:297–303

Ospina-Giraldo MD, Collopy PD, Romaine CP, Royse DJ (2000) Classification of sequences expressed during the primordial and basidiome stages of the cultivated mushroom *Agaricus bisporus*. Fungal Genet Biol 29:81–94

Pawlik-Skowrońska B, Sanità di Toppi L, Favali MA, Fossati F, Pirszel J, Skowroñski T (2002) Lichens respond to heavy metals by phytochelatin synthesis. New Phytol 156:95–102

Quesada H, Ramiréz UEM, Rozas J, Aguadé M (2003) Large-scale adaptive hitchhiking upon high recombination in *Drosophila simulans*. Genetics 165:895–900

Rosen R, Büttner K, Becher D, Nakahigashi K, Yura T, Hecker M, Ron EZ (2002) Heat shock proteome of *Agrobacterium tumefaciens*: evidence for new control systems. J Bacteriol 184:1772–1778

Roxas VP, Lohdi SA, Garrett DK, Mahan JR, Allen RD (2000) Stress tolerance in transgenic tobacco seedlings that overexpress glutathione *S*-transferase/glutathione peroxidase. Plant Cell Physiol 41:1229–1234

Rozen S, Skaletsky JH (1996) Primer3. http://www-genome.wi.mit.edu/genome_software/other.primer3.html

Skinner W, Keon J, Hargreaves J (2001) Gene information for fungal plant pathogens from expressed sequences. Curr Opin Microbiol 4:381–386

Townsend JP, Cavalieri D, Hartl DL (2003) Population genetic variation in genome-wide gene expression. Mol Biol Evol 20:955–963

Vallino M, Drogo V, Abba' S, Perotto S (2005) Gene expression of the ericoid mycorrhizal fungus *Oidiodendron maius* in the presence of high zinc concentrations. Mycorrhiza 15:333–344