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ORIGINAL PAPER

Transcriptome analysis by cDNA-AFLP of *Suillus luteus* Cd-tolerant and Cd-sensitive isolates

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Abstract The ectomycorrhizal basidiomycete *Suillus luteus* (L.:Fr.), a typical pioneer species which associates with young pine trees colonizing disturbed sites, is a common root symbiont found at heavy metal contaminated sites. Three Cd-sensitive and three Cd-tolerant isolates of *S. luteus*, isolated respectively from non-polluted and a heavy metal-polluted site in Limburg (Belgium), were used for a transcriptomic analysis. We identified differentially expressed genes by cDNA-AFLP analysis. The possible roles of some of the encoded proteins in heavy metal (Cd) accumulation and tolerance are discussed. Despite the high conservation of coding sequences in *S. luteus*, a large intraspecific variation in the transcript profiles was observed. This variation was as large in Cd-tolerant as in sensitive isolates and may help this pioneer species to adapt to novel environments.

Ruytinx and Craciun contributed equally to this work.

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Introduction

Trace elements such as Cu, Fe, Mn and Zn are essential micronutrients that are required for a wide variety of cellular processes, but eventually these metals become toxic at elevated concentrations. Other heavy metals (e.g. Cd, Hg and Pb) are not 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 activities, may pose considerable challenge to exposed biota, and this selective pressure may lead to the evolution of metal-tolerant populations.

Suillus luteus (L.:Fr.), an ectomycorrhizal basidiomycete, is a typical pioneer fungus in primary forest succession. It is a common root symbiont of young pine trees (*Pinus sylvestris*) and evolves metal-tolerant ecotypes on heavy metal contaminated sites (Colpaert et al. 2000). Its basidiospores are most likely dispersed by wind and mammals (Ashkannejhad and Horton 2006), and its frequent and early sexual reproduction with the release of billions of basidiospores are a trade mark of its pioneer behaviour and may favour rapid selection for genotypes adapted to specific soil conditions.

In the ectomycorrhizal symbiosis, most mineral nutrients pass through fungal cells and tissues before reaching the root cells of their host plants. Movement of minerals and trace elements from soil solution towards the root exodermis is certainly affected by the mycobionts although such effects depend on the molecular structure of the compounds and on fungal characteristics (Taylor and Peterson 2005; Vesk et al. 2000). *S. luteus* produces large well differentiated hyphal networks in soils, but relatively few mycorrhizas with thick mantles surrounding the absorption roots of *P. sylvestris*. The

external mycelial system can be classified into the long-distance exploration type (Agerer 2001).

Inoculation of tree seedlings with ectomycorrhizal fungi usually ameliorates fitness of heavy metal treated host plants. Pines inoculated with *Laccaria bicolor* while exposed to Cd grew better than non-mycorrhizal plants and maintained a higher nutrient status (Ahonen-Jonnarth and Finlay 2001), an effect that might be further strengthened by ectomycorrhiza associated bacteria (Kozdroj et al. 2007). The beneficial effect induced by the fungus is nevertheless species and isolate specific. Cd-tolerant *S. luteus* isolates perform much better in safeguarding a host plant against Cd toxicity than isolates that are more sensitive to Cd (Krznaric et al. 2009).

Although the exact mechanisms of the adaptive Cd tolerance remain unclear, evolution towards higher metal tolerance, both in plants and fungi, are believed to be due to modifications of existing genetic networks controlling metal homeostasis and detoxification (Bellion et al. 2006, Clemens 2006, Verbruggen et al. 2009). *S. luteus* populations from contaminated soils displayed a higher level of tolerance to those metals that were enriched in the soil of origin when compared with populations from non-contaminated soils (Adriaensen et al. 2005; Colpaert et al. 2004; Muller et al. 2004). Tolerance mechanisms to heavy metals in ectomycorrhizal fungi seem to include reduced uptake of metals into the cytosol by extracellular chelation through extruded ligands and binding onto cell-wall components, enhanced intracellular chelation of metals in the cytosol, increased efflux from the cytosol out of the cell or into sequestering compartments and enhanced free-radical scavenging capacities (Blaudez et al. 2000; Courbot et al. 2004; Jacob et al. 2004; Bellion et al. 2006, 2007). Tolerance mechanisms are little-known in *S. luteus* but enhanced Zn tolerance seems to rely, at least partially, on enhanced Zn exclusion (Colpaert et al. 2005).

In the present study, we have compared the transcriptomic profiles of Cd-tolerant and Cd-sensitive *S. luteus* isolates in the presence and absence of non-lethal Cd concentrations in order to identify genes potentially involved in Cd tolerance. We have used the cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis, an efficient method for the isolation and identification of differentially expressed genes, which does not require prior sequence information (Bachem et al. 1996, 1998; Craciun et al. 2006; Ditt et al. 2001; Muller et al. 2007a).

Material and methods

Fungal material

Six isolates of *S. luteus* were selected for a transcriptomic analysis. Three Cd-tolerant isolates (Lc4, Sl24, Lm2) were isolated from sporocarps collected from a heavy metal contaminated site in Lommel-Maatheide (NE-Belgium), and three Cd-sensitive isolates (P13, Sl34, Sl33) were collected from a non-polluted site in Paal (Colpaert et al. 2004).

Isolates were cultured on solid modified Fries medium as described by Colpaert et al. (2004). Uniform inocula (0.5 cm^2 plugs of fungal mycelium) were prepared by 2 or 3 days preincubation of various plugs of mycelium on cellophane-covered agar plates. Single inocula were transferred to cellophane-covered test plates containing nutrient solution with 0, 9 and 18 μM cadmium added as $CdSO_4$ and were allowed to grow in the dark for 7 days at 23°C. Five replicates were made for each treatment.

RNA extraction and cDNA synthesis

Total RNA was extracted from the mycelia from the 0 and 9 μM Cd exposure; in the 18 μM Cd-exposed mycelia, not enough biomass was produced by the Cd-sensitive isolates. The RNeasy Plant Mini Kit (Qiagen, France) was used for the extraction, poly $(A)^+$ RNA was isolated from 250 μg samples of total RNA using Oligotex columns (Qiagen, France). Double-stranded cDNA was synthesized starting from 100 ng of poly$(A)^+$ RNA using the SMART™ cDNA Library Construction Kit (Clontech, USA) following the manufacturer's instructions and ligation-dependent polymerase chain reaction (PCR) products were purified by a QIAquick PCR purification Kit (Qiagen, France).

cDNA-AFLP analysis

cDNA-AFLP analysis was performed following Bachem et al. (1996). In brief, approximately 0.5 μg of ds cDNA was digested with the restriction enzymes *Bst*YI and *Mse*I in a two-step reaction. After adaptor ligation, the pre-amplifications were performed with the *Mse*I and *Bst*YI primers without selective nucleotides. From a 2-fold dilution of pre-amplification product, 5 μl were used for the subsequent selective amplification using *Mse*I and ^{33}P-labelled *Bst*YI primers with two selective nucleotides. Amplifications using 88 out of 128 possible BstYI +T/C+N + MseI +NN primer combinations were performed on each genotype and condition. Amplification products were separated on 5% denaturing polyacrylamide gels using the SequiGen system (BioRad, Belgium). Vacuum dried gels were exposed two days to Fuji Super RX Medical X-ray film (Fujifilm Medical Systems, Benelux N.V., Belgium) to obtain the autoradiographs, or one day to PhosphorImager screens and scanned in a Storm 860 PhosphorImager (Molecular Dynamics).

Isolation and sequencing of amplified cDNA products

Bands corresponding to transcripts of which the steady-state expression levels differed between the Cd-sensitive and Cd-

tolerant isolates in at least two of the three tolerant or sensitive isolates, with or without Cd exposures, were cut out from denaturing gels (from both tolerant and sensitive isolates) 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, pH 9.0 at 25°C, 3 mM $MgCl_2$ and 0.2% Triton® X-100, Promega Benelux, Leiden, The Netherlands) for 10 min at room temperature. The remaining buffer was removed and replaced with a fresh 100 µl aliquot of buffer, samples were incubated at 94°C for 90 min. Ten microlitres containing the eluted DNA in 2× PCR buffer were used in a 20 µl PCR reaction under the same conditions as used for selective amplification. The successfully amplified fragments were ligated into pTZ57R/T vectors by using the InsT/Aclone™ PCR Product Cloning Kit (Fermentas MBI, Germany) according to the manufacturer's instructions. Ligated plasmids were used to transform *Escherichia coli* DH5α strain. Eight positive bacterial colonies were screened by PCR using plasmid specific primers Lac1 (5′-agtcacgacgttgtaaaacgacg gccagt-3′) + M13Rev (5′-cacacaggaaacagctatgaccatgattac-3′), to verify the presence and the correct size of the insert. Plasmids containing an insert having the expected size were extracted using GeneJET™ Plasmid Miniprep Kit (Fermentas MBI) and 1 µg was sent to be sequenced by MWG Biotech, Germany. Overall, for each transcript derived fragment (TDF) we ended up with six to nine DNA sequences.

The insert sequences were compared with NCBI non-redundant databases (nr) using Blastn, Blastx and tBlastx sequence alignment programs (Altschul et al. 1997) (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 non-redundant databases were classified according to their putative function as described in the *Saccharomyces cerevisiae* functional catalogue (http://mips.gsf.de/genre/proj/yeast/). Using the ClustalW2 algorithm (Larkin et al. 2007) insert sequences representing the same TDF were aligned to estimate inter- and intra-population variation. Sequences of all TDFs were submitted to GenBank (accession numbers GR975618–GR976128).

Real-time quantitative PCR

The expression of the TDFs 9, 41, 55, 67, 74 and 81 - corresponding genes was determined by real-time quantitative PCR on an ABI Prism 7000 sequence detection system (Applied Biosystems). As reference genes, with an equal expression in all isolates and growth conditions, actin 1 (*ACT1*) and TDF 1 - corresponding gene, a homologue of a *L. bicolor* predicted protein (possibly involved in translation, ribosomal structure and biogenesis), were selected. Specific primers (Table 1) with a melting temperature of 65±1°C were designed to amplify fragments of approximately 100 bp. The efficiency of the primers was controlled on genomic DNA, until equal amplification efficiencies were obtained for all Cd-tolerant and Cd-sensitive *S. luteus* isolates. Real-time PCR reactions were performed in a total volume of 25 µl, containing 2× Plexor Master Mix (Promega), 0.3 µM specific forward and reverse primer, and 5 µl cDNA; "no template controls" contained 5 µl RNase free water instead. Standard cycling conditions were used (2′ at 95°C; 40×5″ 95°C, 35″ at 60°C) and each reaction was run in duplicate. A dissociation curve was generated to assure specificity of amplification.

The expression levels were determined using the formula $2^{-\Delta Ct}$, and expressed relatively to the sample with the highest expression level before input into geNorm (reference genes) or before normalization (genes of interest). Using the geNorm algorithm described by Vandesompele et al. (2002), the expression stability of *ACT1* and TDF1 - corresponding gene was examined and approved. The gene expression normalization factor for each sample was calculated as the geometric mean of the two reference genes and used to normalize the data.

Results

Three Cd-tolerant and three Cd-sensitive *S. luteus* isolates were selected based on their contrasting growth in an in vitro dose response experiment (Fig. 1). Growth of the Cd-sensitive isolates (S133, S134, P13) was significantly inhibited at 18 µM $CdSO_4$, whereas no growth inhibition was observed on control conditions, or among Cd-tolerant isolates (Lc4, Lm2, S124) exposed to 18 µM $CdSO_4$ (Fig. 1).

To analyse differential responses in the transcript profiles of Cd-tolerant and Cd-sensitive *S. luteus* isolates, the improved cDNA-AFLP protocol of Breyne et al. (2003) was used. A total of 125–175 AFLP bands ranging from 90 to 800 bp were detected on autoradiography in one primer combination for one Cd exposure per isolate. With the exception of constitutively expressed TDFs, very few TDFs presented a similar profile among Cd-sensitive or Cd-tolerant isolates. Some Cd-TDFs seemed missing in one or in two isolates of the same population (sensitive or tolerant). Therefore, TDFs with a similar tendency in at least two of the three isolates of one population were selected for identification.

As a result of the cDNA-AFLP analysis a total of 111 Cd-TDFs meeting the above criteria were selected. Obtained TDFs corresponded to 261 potentially different genes (Table 2 and Electronic supplementary material, Supplementary Table). Highest homology scores of the Cd-TDFs corresponded to sequences from *L. bicolor* and *Coprinopsis cinerea*. High homology scores were also obtained for sequences belonging to other fungal, bacterial,

Table 1 Primer combinations used for qPCR analysis

TDF/gene	Sequence	5′ modification
ACT1: actin 1	F: GGCCACACGAAGCTCATTATAGAAT R: CTTGACCCTAAAGTACCCTATCGAG	HEX, iso-dC
TDF1: hypothetical protein CNBM1630, Cryptococcus	F: CCGGTTTCAGTAATACAGAGTCCT R: GGTCTGTTTACCTTACTTTATTATGTCCACC	FAM, iso-dC
TDF9: actin binding protein	F: GCTCCAAGGTAGTCGTAATGTGAT R: TGCGTAGTGATCTTGGCCAGAGA	FAM, iso-dC
TDF41: Gti/Pac2 superfamily protein	F: TCCGACGACATACAGCCCATTATC R: GGGACGGGGCGAAGTACTATAAT	HEX, iso-dC
TDF55 unknown function	F: GGCCTCTACCGATAGCCCAT R: CGTAGTGATCCGGTCGATGC	HEX, iso-dC
TDF67: copper amine oxidase	F: CGAGTCTGTGGGACGTACTTTCCA R: ACAAGACGTTGTGGGTTGTAAGAG	FAM, iso-dC
TDF74: glutathione-S-transferase	F: CTTTGAGGGCGAGGATGGATTC R: TGTTTCCAAGAAGCCCAGACTCAG	FAM, iso-dC
TDF81: heat shock protein	F: CATCTTGGCATCCTTGAGTACCTT R: AGGACTTCTCTGCCAACATCAC	FAM, iso-dC

animal and plant species. Based on alignments of homologous sequences originating from the same PAGE fingerprint but from a different clone, an equal inter- and intra-isolate homology of 99.5% was estimated. The length of the sequences ranged from 116 to 700 bp, with an average of 315 bp. Most TDFs were identified only in one primer combination.

For many Cd-TDFs there were no common sequences found between the analysed sequences belonging to different isolates. As a result, we only kept 49 Cd-TDFs, presented in Table 2. These Cd-TDFs could be unambiguously identified by sequences corresponding to the same gene and originating from at least two different isolates. We were unable to classify the remaining 40 Cd-TDFs (45%) due to the fact that EST sequences belonging to different genes (Electronic supplementary material, Supplementary Table) were obtained because of co-migration of TDFs having approximately the same size. Genes corresponding to the dominant TDF sequences obtained were classified according to their putative function. Among the 49 classified Cd-TDFs, two were constitutively overexpressed in both populations, ten were constitutively overexpressed only in the Cd-tolerant population (↑ T) in both Cd exposures and eight were overexpressed only in the sensitive population (↑ S) in both Cd exposures. Fifteen TDFs were modulated by the presence of Cd in the culture medium as follows: ten Cd-TDFs were Cd-regulated only in the sensitive (S ↓↑), three Cd-TDFs only in the Cd-tolerant (T ↓↑), and two were modulated both in the Cd-tolerant and the Cd-sensitive population (S,T ↓↑). The remaining 14 Cd-TDFs did not show a clear, consistent pattern. For these TDFs a different behaviour was observed between isolates of the same population.

In order to validate the cDNA-AFLP expression patterns, six genes (Cd-TDF 9, 41, 55, 67, 74, 81) were selected for Real-Time quantitative PCR analysis (qPCR). By using cDNA-AFLP analysis, for these six genes an equal expression level was observed within the population (tolerant or sensitive) and a different expression level was observed between the two populations. Expression patterns of the six genes were similar when analysed by qPCR although expression levels were more easily to quantify. Due to the real-time detection and high sensitiveness of qPCR also very low expressed genes which seemed missing (Cd-TDF 41, 81) or showed a near to background expression (Cd-TDF 55) for the Cd-sensitive isolates in the PAGE fingerprints could be detected.

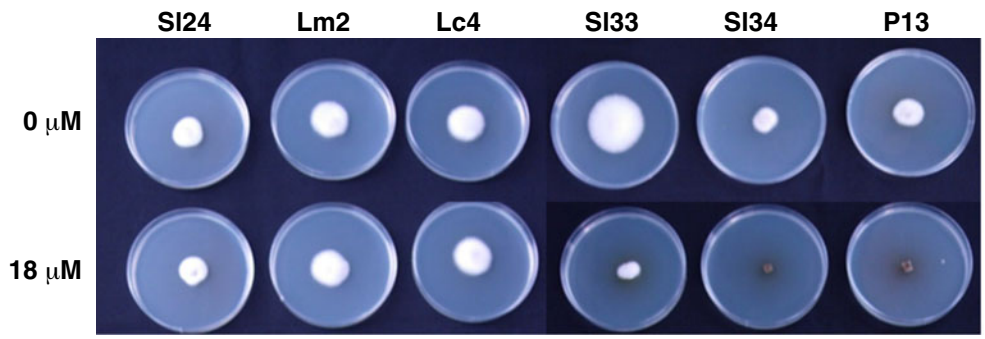

Fig. 1 *Suillus luteus* Cd-tolerant (Sl24, Lm2, Lc4) and sensitive (Sl33, Sl34, P13) isolates exposed to 0 and 18 μM Cd during 7 days

Table 2 Summary of the sequence analysis of the differentially expressed TDFs and their expression patterns obtained by cDNA-AFLP, of three Cd-tolerant and sensitive *Suillus luteus* isolate cultured for 7 days on solid media in the presence (+) or absence (−) of 9 μM Cd

		Expression profile																							
		Cd-sensitive						Tolerant																	
		P13		Sl34		Sl33		Lc4		Sl24		Lm2													
TDF	Size (bp)	-	+	-	+	-	+	-	+	-	+	-	+	Homology	Id. %	Pos%	*E*-value	GenBank accession	BLAST hit
House keeping genes																			
1	398	■	■	■	■	■	■	■	■	■	■	■	■	*predicted protein [Laccaria bicolor S238N-H82], Translation, ribosomal structure and biogenesis*	58	80	2.27E-20	GR975618	EDR04688
47	406	■	■	■	■	■	■	■	■	■	■	■	■	*predicted protein [Laccaria bicolor S238N-H82], Translation, ribosomal structure and biogenesis*	58	80	2.30E-20	GR975910	EDR04688
Metabolism and energy																			
16	183	■	■	■	■	■	■	■	■	■	■	■	■	*hypothetical protein CC1G_09654 [Coprinopsis cinerea okayama7#130] Succinyl-CoA synthetase; Tricarboxylic acid cycle*	64	73	4.62E-05	GR975706	EAU82052
30	289	■	■	■	■	■	■	■	■	■	■	■	■	*hypothetical protein CC1G_02033 [Coprinopsis cinerea okayama7#130] Mannose-1-phosphate guanylyltransferase. Cell envelope biogenesis, outer membrane*	79	87	2.49E-30	GR975757	EAU91544
35	129	□	■	□	■	□	■	□	□	□	□	□	□	*predicted protein [Laccaria bicolor S238N-H82] Protein phosphatase 2A homologues, catalytic domain*	77	83	4,8E-5	GR975798	EDR14080
38	231	□	□	□	□	□	□	■	■	■	■	■	■	*hypothetical protein CC1G_01684 [Coprinopsis cinerea okayama7#130] Src homology 3 domain protein*	73	85	1.83E-17	GR975827	EAU92639
39	204	■	■	■	■	■	■	■	■	■	■	■	■	*predicted protein [Postia placenta Mad-698-R] F-box domain, mediating protein-protein interaction*	47	62	2.39E-01	GR975839	EED81851
41	239	□	□	□	□	□	□	■	■	■	■	■	■	*predicted protein [Coprinopsis cinerea okayama7#130]; Gti/Pac2 superfamily*	58	75	2,734	GR975856	EAU87471-
43	587	□	■	□	■	□	■	□	■	■	■	■	■	*predicted protein [Postia placenta Mad-698-R] Nucleotide transport and metabolism*	79	88	2.16E-67	GR975871	EED83915
44	511	■	■	■	■	■	■	■	■	■	■	■	■	*predicted protein [Laccaria bicolor S238N-H82]; Ubiquitin – conjugating enzyme E2 catalytic domain*	94	96	9.99E-60	GR975888	EDR03704
50	141	□	□	□	□	□	□	■	■	■	■	■	■	*predicted protein [Laccaria bicolor S238N-H82] MmsB domain, Lipid metabolism*	51	70	0,01	GR975931	EDR11339
63	612	■	■	■	■	■	■	□	□	□	□	□	□	*hypothetical protein MPER_09043 [Moniliophthora perniciosa FA553] Aspartate kinase, Amino acid transport and metabolism*	75	82	3.75E-72	GR975981	EEB92445
67	698	■	■	■	■	■	■	■	■	■	■	■	■	*hypothetical protein CC1G_00597 [Coprinopsis cinerea okayama7#130] Copper amine oxidase, catalyse the oxidation of primary amines to aldehydes*	44	58	3.14E-29	GR976013	EAU92378
72	173	□	■	□	■	□	■	□	■	□	■	□	■	*candidate 1,4-alpha-glucan branching enzyme from glycoside hydrolase family GH13 [Postia placenta Mad-698-R]; Carbohydrate transport and metabolism*	80	93	2.07E-13	GR976043	EED84701
76	123	■	■	■	■	■	■	□	□	□	□	□	□	*predicted protein [Laccaria bicolor S238N-H82]; Provisional amidophosphoribosyltransferase*	58	73	4,677	GR976066	EDR14233
78	197	□	□	□	□	□	□	■	■	■	■	□	□	*hypothetical protein CC1G_10805 [Coprinopsis cinerea okayama7#130];Mitochondrial aconitases; Energy production and conversion (metabolism)*	82	96	2.40E-17	GR976084	EAU86914
82	188	■	■	■	■	□	□	■	■	■	■	■	■	*polyprotein [Bovine viral diarrhea virus genotype 2]; Ubiquitin*	95	95	5.76E-19	GR976114	AAD44046
Protein synthesis																			
2	368	■	■	■	■	■	■	■	■	■	■	■	■	*hypothetical protein CC1G_00309 [Coprinopsis cinerea okayama7#130]; Ribosomal_L7Ae family, ribosome biogenesis*	83	90	8.19E-42	GR975624	EAU84790
13	367	■	■	□	■	■	■	□	■	□	■	□	■	*hypothetical protein CC1G_10325 [Coprinopsis cinerea okayama7#130]; SYF2 splicing factor*	61	75	6.79E-28	GR975685	EAU83920
14	296	■	■	□	□	■	■	□	□	□	□	□	□	*hypothetical protein CC1G_02986 [Coprinopsis cinerea okayama7#130]; Ribosomal_P0_like subfamily, Translation, ribosomal structure and biogenesis*	78	84	1.25E-13	GR975694	EAU85963
22	225	■	■	■	■	■	■	■	■	■	■	□	□	*hypothetical protein CC1G_11396 [Coprinopsis cinerea okayama7#130]; Putative snoRNA binding domain, Translation, ribosomal structure and biogenesis*	60	80	7.51E-11	GR975720	EAU80596
74	395	■	■	■	■	■	■	□	□	□	□	□	□	*hypothetical protein CC1G_01604 [Coprinopsis cinerea okayama7#130]; GST domain of elongation factor 1B*	75	85	8.64E-12	GR976056	EAU87957
Cell rescue and defense																			
75	498	■	■	■	■	■	■	□	□	□	□	□	□	*hypothetical protein CC1G_06380 [Coprinopsis cinerea okayama7#130] Survival factor 1 like; involved in oxidative stress response*	59	76	4.37E-41	GR976060	EAU85479
81	531	□	□	□	□	□	□	■	■	■	■	■	■	*predicted protein [Laccaria bicolor S238N-H82] Hsp 70 protein; Protein folding*	95	97	3.98E-84	GR976103	EDR11285
89	284	■	■	■	■	■	■	■	■	■	■	□	□	*thioredoxin h BAC21264 Cucurbita maxima; participate in various redox reactions*	57	73	1.00E-17	GR976150	BAC21264
Transport																			
12	554	■	■	■	■	■	■	■	■	■	■	■	■	*hypothetical protein LACBIDRAFT_293419 [Laccaria bicolor S238N-H82] Band 7 family protein; many band 7 domain-containing proteins are lipid raft-associated*	68	88	1.49E-22	GR975676	EDR11283
79	171	□	■	□	■	■	■	□	□	■	■	□	■	*Predicted flavoprotein involved in K+ transport AN8582.2 Aspergillus nidulans; Inorganic ion transport and metabolism*	49	69	8.17E-02	GR976090	EAA60616
Transcription and chromatine remodeling																			
10	439	□	□	□	□	□	□	■	■	■	■	■	■	*predicted protein [Laccaria bicolor S238N-H82] PHD zinc finger nucleus protein, regulation of transcription, DNA-dependent, Zn & ion binding*	66	81	3.04E-64	GR975664	EDR11026

Expression levels per gene are indicated by "■" (highest level), "■" (reduced expression) and "□" (no expression or near to the background)
Id BLAST identities, *Pos* BLAST positives

Table 2 (continued)

77	512	■	■	■	■	■	■	■	■	■	■	■	■	*transcription initiation factor [Laccaria bicolor S238N-H82;] transcriptional activator activity*	30	59	5.00E-03	GR976069	EDR12858
Signal transduction																			
11	579	■	■	■	■	■	■	□	□	□	□	□	□	*hypothetical protein CC1G_07086 [Coprinopsis cinerea okayama7#130] Endonuclease/Exonuclease/phosphatase family; involved in intracellular signalling*	56	70	5.99E-21	GR975670	EAU85392
Cellular organization																			
9	138	■	■	■	■	■	■	□	□	□	□	□	□	*actin-binding protein SLA2/Huntingtin-interacting protein Hip1 [Laccaria bicolor S238N-H82]*	88	88	3.66E-02	GR975659	EDR15786
57	340	■	■	□	□	□	□	□	□	■	■	■	■	*WiSP family protein [Tropheryma whipplei TW08/27]*	26	44	1,583	GR975969	CAD67236
Unknown function																			
6	521	■	■	□	□	■	■	□	□	□	□	□	□	*predicted protein [Laccaria bicolor S238N-H82]*	33	44	1.19E-06	GR975640	EDR13713
24	172	■	■	■	■	■	■	□	□	□	□	□	□	*predicted protein [Postia placenta Mad-698-R]*	44	69	5.29E-01	GR975730	EED82794
33	430	□	□	□	□	□	□	■	■	■	■	■	■	*predicted protein [Coprinopsis cinerea okayama7#130]; IGR protein motif, fungal protein with unknown function*	59	72	2.45E-30	GR975776	EAU85889
54	186	■	□	■	□	□	□	■	□	■	□	□	□	*predicted protein [Postia placenta Mad-698-R]*	58	73	9.22E-09	GR975955	EED81259
55	145	□	□	□	□	□	□	■	■	■	■	■	■	*predicted protein [Laccaria bicolor S238N-H82]; DUF2461; Conserved hypothetical protein*	68	80	1,53E-6	GR975960	EDR00070
88	158	□	□	□	□	□	□	■	■	■	■	□	□	*hypothetical protein CC1G_09360 [Coprinopsis cinerea okayama7#130]*	46	59	4,71	GR976145	EAU89778
No close homology																			
7	217	□	□	□	□	□	□	■	■	■	■	■	■	*no close homology*	-	-	-	GR975646	-
29	183	■	■	■	■	■	■	■	■	■	■	■	■	*no close homology*	-	-	-	GR975742	-
40	148	□	□	■	■	■	■	■	■	■	■	■	■	*no close homology*	-	-	-	GR975844	-
42	118	■	■	■	■	■	■	□	□	□	□	□	□	*no close homology*	-	-	-	GR975862	-
48	236	□	□	□	□	■	■	■	■	□	□	■	■	*no close homology*	-	-	-	GR975919	-
70	204	□	■	□	■	□	□	□	■	□	□	□	□	*no close homology*				GR976032	
73	146	□	■	□	■	□	■	□	■	□	□	□	■	*no close homology*	-	-	-	GR976051	-
80	146	■	■	□	■	■	■	□	□	■	■	□	■	*no close homology*	-	-	-	GR976096	-
84	168	□	□	□	□	□	□	■	■	■	■	□	□	*no close homology*	-	-	-	GR976124	-
85	116	□	□	□	□	□	□	□	□	■	■	■	■	*no close homology*	-	-	-	GR976128	-
86	125	■	■	■	■	■	■	■	■	■	■	■	■	*no close homology*	-	-	-	GR976132	-

The glutathione-S-transferase family gene (Cd-TDF 74) was on average higher expressed in the Cd-sensitive isolates. A 1.5 to 3-fold induction of expression by Cd was observed for this gene in the Cd-sensitive isolates whereas the Cd exposure had no effect on the expression level in the Cd-tolerant population. A higher expression compared to the Cd-tolerant population and an induction of expression (2–8-fold) by Cd in the Cd-sensitive population was also observed for the actin binding protein SLA2 (Cd-TDF 9). The Gti/Pac2 superfamily gene (Cd-TDF 41), the DUF2461 conserved hypothetical protein gene (Cd-TDF 55), the copper amine oxidase gene (Cd-TDF 67) and the heat shock protein (hsp) 70 gene (Cd-TDF81) show an on-average higher abundance in the Cd-tolerant isolates (Fig. 2). There was no consistent effect of Cd on the expression of the Gti/Pac2 superfamily gene (Cd-TDF 41). An induction was observed for the isolates Sl33 and Sl24 while the others show an equal level (Sl34, Lm2) or even a repression (P13, Lc4) upon Cd exposure. When looking more in detail the copper amine oxidase gene (Cd-TDF 67) and the hsp70 gene (Cd-TDF 81) present almost the same expression pattern. Cd induces the expression of these two genes in four out of the six isolates (Sl34, Sl33, Lc4, Sl24). The expression pattern of the DUF2461 conserved hypothetical protein gene (Cd-TDF 55) is very alike the two former genes (Cd-TDF 67 and 81) with the exception of the lower expression level upon Cd exposure in Sl34 and under control circumstances in Lm2.

Discussion

A cDNA-AFLP analysis, whether or not upon Cd exposure, was performed on Cd-tolerant and Cd-sensitive isolates of

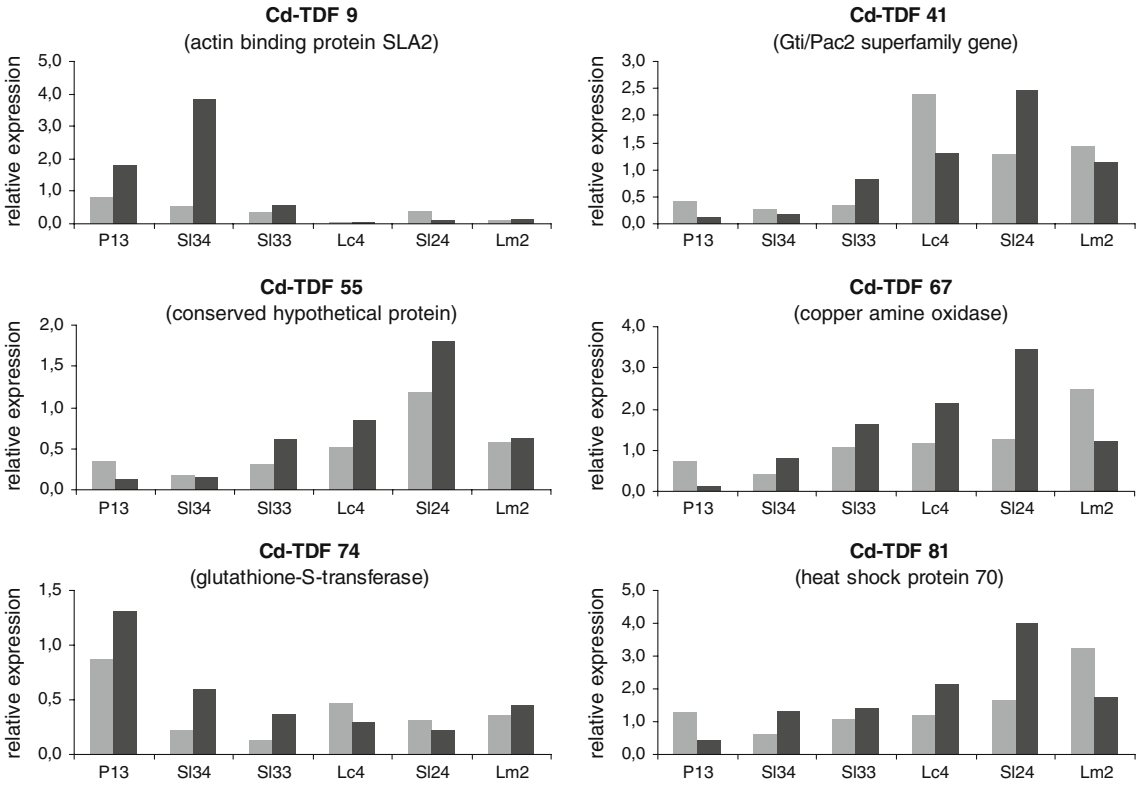

Fig. 2 Expression profile of selected Cd-TDFs in Cd-sensitive (P13, Sl34, Sl33) and Cd-tolerant (Lc4, Sl24, Lm2) *Suillus luteus* isolates, following 0 (■) and 9 (■) μM Cd exposure

S. luteus in order to investigate the molecular determinants of adaptive Cd tolerance in this species. This study is the first attempt to compare at the global level the transcriptome of different isolates. In general, rather unexpected, a high heterogeneity in expression patterns was observed. Even in control circumstances few TDFs presented a similar profile among isolates of the same population (sensitive or tolerant). Some TDFs seemed missing in one or two isolates. This could be caused by an expression below the detection limit or sequence divergence of the corresponding transcript although it is not likely that sequence divergence would explain all observed variations. Coding sequences seem to be relatively highly conserved in *S. luteus* in contrast to the high overall genetic variation reported by Muller et al. (2007b). Based on the transcript sequences obtained in this study a similar inter- and intra-population homology of 99.5% was estimated. Consequently, most of the observed heterogeneity in expression level is probably due to regulatory variations in gene expression which are present in natural populations. High variation in gene expression of individuals in the same population was already reported in human (Storey et al. 2007) and can be important in evolution (Oleksiak et al. 2002). The effectiveness in colonizing disturbed ecosystems and the ability to evolve adaptive tolerance for a range of metals of *S. luteus* could profit on the huge variation in gene expression among the individuals of a population.

For further analysis, 89 TDFs differentially expressed upon Cd exposure or between populations were selected. However, 40% of these Cd-TDFs could not be unambiguously identified because of co-migration of different transcripts of approximately the same length. In cDNA-AFLP, the cDNA mixture is divided in pools as a result of digestion with restriction enzymes and amplification of the obtained fragments using primers extended with selective nucleotides. Several transcripts can produce fragments with approximately the same length in which case these fragments end up in the same gel band and consequently do not give any useful information about expression. The chance by which this occurs depends on the total amount of fragments in a pool and thus on the total amount of genes transcribed, the occurrence of the restriction enzyme recognition sites and the amount of selective nucleotides used. We used two different restriction enzymes in combination with four selective nucleotides, two flanking each restriction site. The use of the same restriction sites with the same amount of, or even less, selective nucleotides resulted in apparently satisfying results for organisms having a transcript set which run up to twice the size of the transcript set of *L. bicolor*, the closest relative of *S. luteus* with an available genome sequence (Fusco et al. 2005; Craciun et al. 2006; Rohde et al. 2007; Molesini et al. 2009). However, for most of the published cDNA-AFLP analyses there is no indication of the amount

of TDFs that could not be identified due to co-migration of several fragments because the total amount of fragments subjected to sequencing (Molesini et al. 2009) or the amount of failing sequencing reactions in case of direct sequencing (Fusco et al. 2005) is not mentioned. Moreover the change of detecting co-migrating fragments is reduced in several studies by cutting out a band for only one of the experimental conditions (Craciun et al. 2006; Muller et al. 2007a) or by picking up only one colony for sequencing (Fusco et al. 2005). If bands of more than one experimental condition were cut out and a reasonable amount of colonies was picked up, as in this study, a substantial part of TDFs could not be identified because of co-migration (e.g. 32% in a study of Rohde et al. 2007). Therefore it is obvious to use more selective nucleotides to obtain a higher percentage of identifiable TDFs leading to a larger return of cDNA-AFLP analyses.

Out of the 49 identified TDFs, two were more expressed upon Cd exposure in the Cd-tolerant and Cd-sensitive population. Both genes are involved in energy metabolism. The glycan branching enzyme from glycoside hydrolase family GH13 (Cd-TDF 72) is involved in the glycogen metabolism, the succinyl CoA synthetase (Cd-TDF 16) is one of the enzymes of the TCA cycle. Besides energy supply the TCA cycle is also important in organic acid production. Jacob et al. (2004) detected a higher aconitase expression in *Paxillus involutus* upon Cd exposure and asserted a higher activity of TCA cycle was mainly needed to produce organic acids for sequestration of Cd. Sequestration by organic acids could also be a mechanism of Cd detoxification in *S. luteus*. Nevertheless, the higher need for energy to drive other defence mechanisms, particularly in the Cd-sensitive population should not be underestimated. Moreover, the aim of a higher activity of the TCA cycle and an altered energy metabolism in general, is not necessarily identical in both populations.

Three TDFs are found to be Cd responsive in the Cd-tolerant population only. Two of them show homology to genes involved in gene regulation on the transcriptional (Cd-TDF 10, PHD zinc finger protein) or posttranscriptional level (Cd-TDF 13, SYF2 splicing factor); one shows no close homology. In the Cd-sensitive population considerably more genes (factor 3.33) with very diverse functions were up- or downregulated by Cd. None of these genes has a known role in the direct defence against Cd. The cellular metabolism of isolates in this Cd-sensitive population is probably severely disturbed in distinct aspects, whereas Cd-tolerant isolates are able to better maintain cellular homeostasis. The ability to maintain cellular homeostasis in tolerant populations can be due to a constitutive expression rather than an inducible expression of genes involved in stress response as shown for adaptive metal tolerant populations of *Orchesella cincta* and *Viola baoshanensis* (Roelofs et al. 2009; Zhang et al. 2009). In this study, ten TDFs were constitutively more expressed in the isolates of the Cd-tolerant population. Four were studied in more detail by qPCR and an induction by Cd in some of the Cd-sensitive isolates was observed for three of them (Cd-TDF 55, 67, 81) which could indicate a possible role in Cd response and detoxification. Cd-TDF 81, homologous to a heat shock protein 70 (Hsp70) gene could play a role in the protection of proteins upon Cd exposure. Hsp proteins are chaperones involved in protein folding which were often shown to be upregulated in response to stress (Wang et al. 2004; Muller et al. 2007a; Kieffer et al. 2009). Cd-TDF 67 corresponding gene encodes a copper amine oxidase (CAO). CAO catalyzes the oxidative amination of primary amines to aldehydes, with production of hydrogen peroxide. Reactive oxygen species (ROS) can function as signalling molecules in the response to stress (Neill et al. 2002; Smeets et al. 2009). In plants, hydrogen peroxide induced by CAO is involved in wound healing (Angelini et al. 2008). The potential role of CAO in response to Cd in Cd-tolerant *S. luteus* needs to be further investigated; kinetics of early responses, measurements of enzyme activities and ROS production are required. Cd-TDF 55 shows homology to a protein with unknown function.

A substantial part of the TDFs was constitutively less expressed in the Cd-tolerant population. Among these TDFs is the survival factor 1 (Cd-TDF 75) and a glutathione-S-transferase family protein (Cd-TDF 74), both known to be involved in oxidative stress response (Kieffer et al. 2009; Semane et al. 2010). When studied more in detail by qPCR, Cd-TDF 74 showed an on average higher expression and induction upon Cd exposure in Cd-sensitive isolates. This observation suggests that a 7-day Cd exposure induces less oxidative stress in the Cd-tolerant population. Physiological experiments indicate a role for Cd exclusion in the adaptive Cd tolerance in *S. luteus* (Krznaric 2009). If adaptive Cd tolerance in Suilloid fungi is due to an exclusion mechanism stress and cellular damage are expected to be reduced due to lower intracellular Cd concentrations.

In the present study approximately 13 000 transcripts were screened for differential expression in *S. luteus* isolates whether or not treated with Cd. *L. bicolor* the closest relative of *S. luteus* with an available genome sequence disposes of ±20,000 genes (Martin et al. 2008). Even if we take into account that only 60% of the obtained TDFs are giving useful information about gene expression (because of co-migration in the other TDFs) a still reasonable part of about 8,000 transcripts was tested. If *S. luteus* and *L. bicolor* would have a similar amount of genes, this would correspond to approximately 40% of the total gene set. Nevertheless, no clear indication of Cd exclusion or compartmentalization (e.g. metal transporter) which could lead to a sustainable cellular protection against

Cd was detected. In the Cd-tolerant population transcript levels (Cd-TDF 74 and 75) corresponding to two anti-oxidative proteins were lower while the level of transcript encoding a chaperone (Cd-TDF 81) was higher than in the sensitive population, suggesting that toxic effects of Cd exposure are less pronounced and damage is more efficiently repaired. The high intraspecific variation in gene expression profiles together with the Cd-responsiveness of a transcription factor (Cd-TDF 10) in the Cd-tolerant population let us assume that variation in gene expression is important for adaptation in *S. luteus*. The possibility that adaptive Cd tolerance evolved because of a selection for an altered expression of a key factor (e.g. metal transporter) cannot be excluded from this study, but a regulation of the tolerance trait at the posttranscriptional level may not be neglected and a splicing factor (Cd-TDF 13) was upregulated upon Cd exposure. A study of the proteome of several Cd-sensitive and Cd-tolerant *S. luteus* isolates after Cd application is planned.

Finally, this study generated a database of 537 entries containing 261 potentially different genes. Since Genbank entries of Suilloid fungi are relatively scarce, the sequences obtained in this study are certainly of significant value. Moreover, Genbank entries of ectomycorrhizal fungi are mainly restricted to the sequenced species *L. bicolor*. A representation of sequences reflecting the diversity of ectomycorrhizal fungi is desired and needs in particularly an enrichment of sequences of Boletales.

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