Analysis of manganese-regulated gene expression in the ligninolytic basidiomycete *Ceriporiopsis subvermispora*

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Abstract In this work, we explore the use of the unbiased cDNA–AFLP strategy to identify genes involved in Mn^{2+} homeostasis in *Ceriporiopsis subvermispora*. In this ligninolytic white-rot fungus, whose genome has not yet been sequenced, three Mn peroxidase genes responding to Mn^{2+} have been characterized. Using cDNA–AFLP to identify transcript-derived fragments (TDFs), a total of 37 differentially expressed cDNA fragments were identified by comparing band intensities among cDNA–AFLP patterns obtained from mycelia from cultures supplemented with different concentrations of Mn^{2+}. Of 21 differentially expressed TDFs, nine were classified as upregulated, five as downregulated and seven as unregulated. Of these, six upregulated and two downregulated TDFs were selected for further characterization. The expected TDFs for the known Mn peroxidases were not isolated, but several genes encoding proteins related to protein sorting, storage and excretion of excess Mn^{2+} were identified. Transcripts induced under Mn^{2+} supplementation exhibited homologies to the elongation factor eEF3, a HDEL sequence binding protein and the ARD1 subunit of the *N*-acetyltransferase complex, among others. Overall, the results obtained in this study suggest a complex picture of Mn^{2+} homeostasis and provide the possibility to search for common regulatory elements in the promoters of the novel putatively identified genes.

Keywords Manganese · Transcription · cDNA–AFLP · *Ceriporiopsis subvermispora* · Regulation

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Introduction

Manganese-dependent gene expression is not fully understood in eukaryotic organisms. In turn, the homeostasis of this metal appears to involve a complex network of proteins. In this work, we use the ligninolytic basidiomycete *Ceriporiopsis subvermispora* as a model system to analyze the effect of Mn^{2+} on gene expression, since this microorganism produces lignin-degrading Mn-dependent peroxidases. Among the experimental strategies available to search for novel genes in a nonannotated genome, we chose cDNA–AFLP, since this is an unbiased and robust method to iden-

tify differentially expressed genes as transcript-derived fragments (TDFs) under varying metabolic conditions.

Manganese (Mn) is the 12th most abundant element on earth and the third in abundance among transition elements. This essential trace element plays an important role in several physiological processes as almost every compartment of the cell carries at least one enzyme whose activity is dependent on Mn^{2+}. This metal acts as a cofactor for oxidases, dehydrogenases, DNA and RNA polymerases, kinases, decarboxylases and sugar transferases (Crowley et al. 2000; Culotta et al. 2005; Keen et al. 2000). Mn^{2+} is directly involved in the detoxification of reactive oxygen species (ROS) as a cofactor of the Mn^{2+}-dependent superoxide dismutase (SOD) and by its own redox potential, which allows it to scavenge superoxide radicals (Cho et al. 2005).

Mn^{2+} homeostasis is better understood in prokaryotes, where different players have been described. Among these are the members of the ferric uptake regulator (Fur) family with functional specialization, such as the Mn^{2+} uptake regulator Mur, which regulates the expression of Mn^{2+} homeostatic factors (Lee and Helmann 2007). Another protein involved in Mn^{2+} homeostasis is MntR, a transcription factor known to regulate the expression of natural resistance-associated macrophage protein (NRAMP)-like Mn^{2+} transporters. Both Mur and MntR evolved from iron regulators (Moore and Helmann 2005).

There have been numerous efforts to achieve a comparable level of knowledge in eukaryotes (Aschner et al. 2007; Lei et al. 2007; Malecki et al. 1999; Pittman 2005). In contrast to prokaryotes where manganese homeostasis is regulated at the transcriptional level, in eukaryotes a post-translation regulatory mechanism operates (Moore and Helmann 2005). This is the case of the eukaryotic NRAMP-like Mn^{2+} transporters Smf1p and Smf2p in *Saccharomyces cerevisiae*. Orthologs of these proton-metal symporters are widely conserved from bacteria to humans and are able to transport various bivalent cations such as manganese, iron, cadmium and copper (Culotta et al. 2005). Smf1p is a high-affinity Mn^{2+} transporter localized at the plasma membrane; however, mutants lacking this transporter accumulate Mn^{+2} in a similar fashion to wild-type cells (Luk and Culotta 2001; Supek et al. 1996). In contrast, defects on Smf2p cause a cell-wide effect on Mn^{2+} accumulation and bioavailability, as strains containing a *smf2*Δ deletion accumulate very low levels of manganese (Culotta et al. 2005). Smf2p has been shown to localize to Golgi vesicles, in a process that does not involve Smf2p endocytosis from the cell surface (Luk and Culotta 2001; Portnoy et al. 2000). Mn^{2+} starvation causes an accumulation of both Smf1p and Smf2p transporters; however, no increase in their transcript levels during Mn^{2+} starvation is observed. This response to Mn^{2+} is post-transcriptional, due to an increase of the transporter proteins stabilization and shift of their cellular localization (Liu and Culotta 1999; Culotta et al. 2005).

Another mediator of Mn^{+2} homeostasis in eukaryotes is Pmr1p. This protein is a P-type Ca^{2+}/Mn^{2+}ATPase that pumps cytosolic Ca^{2+} and Mn^{2+} into the Golgi in yeast. In worms, Δ*pmr-1* Δ*sod1* double mutants show a fourfold to fivefold increase in cytosolic Mn^{2+} (Cho et al. 2005). Pmr1p is required for secretory pathway glycosylation, sorting and endoplasmic reticulum-associated protein degradation. Pmr1 apparently supplies the Mn^{2+} ions required for *N*- and *O*-linked glycosylation, as *pmr1* mutants show unbalanced intralumenal Mn^{2+} levels that alter processes in the early secretory pathway (Dürr et al. 1998).

Mn^{2+} is also crucial for the activity of manganese peroxidases from the fungal species *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, two ligninolytic basidiomycetes that have been intensively studied due to their ability to degrade lignin. (Otjen and Blanchette 1987; Blanchette et al. 1992). In *P. chrysosporium*, the most extensively studied ligninolytic fungus, iron-responsive genes have been isolated using differential display reverse transcription-PCR (Assman et al. 2003). For over a decade, our research group has been interested in these white rot fungi, and many of our previous findings indicate that Mn^{2+} plays a central role in ligninolysis as a substrate for manganese peroxidase, one of the key enzymes involved in lignin degradation (Larrondo et al. 2001; Lobos et al. 1998; Tello et al. 2000; Urzúa et al. 1995; Urzúa et al. 1998). *C. subvermispora* was chosen for this study, since its Mn peroxidases are believed to be the main component in their lignin-degrading machinery, as this organism does not have functional lignin peroxidases like other lignin-degrading fungi (Lobos et al. 1994; Rüttimann et al. 1992). Experimental evidence from our research group suggests an involvement not only in the activity of Mn peroxidases, but also in transcriptional regulation, which sparked our interest to further study this issue (Manubens et al. 2003; Rüttimann et al. 1992).

It was originally suggested that Mn^{2+} might induce the expression of manganese-dependent peroxidases through metal-responsive elements (MREs) located in the promoters of the corresponding genes (Brown et al. 1991). To date, there is no evidence whatsoever supporting this hypothesis. MREs have been identified in animals and plants as the target site of transcription factors responding to cadmium, zinc and copper (Thiele 1992). However, a novel promoter sequence required for Mn^{2+} regulation of the manganese peroxidase 1 gene (*mnp1*) in the lignin-degrading fungus *P. chrysosporium* has been described (Ma et al. 2004). To clarify the role of Mn^{2+} in the regulation of transcription, we undertook a systematic screening of differentially expressed genes in *C. subvermispora* cultures subjected to various Mn^{2+} conditions.

The *C. subvermispora* genome has not been sequenced yet, which complicates transcriptome-wide analysis. We chose the cDNA–AFLP technique, which allowed us to overcome this difficulty and screen for genes that are differentially expressed (1) in stationary cultures with increasing Mn^{2+} concentrations or (2) during a time course after supplementation of cultures grown at a fixed (160 μM) Mn^{2+} concentration. cDNA–AFLP is a robust and reproducible technique that has been used for differential gene expression profiling (Bachem et al. 1996). This technique has recently attracted great interest, especially for studies in plants whose genomes have not been sequenced (Kadota et al. 2007; Leymarie et al. 2007; Sarosh and Meijer 2007; Yao et al. 2007).

In this work, we identified 37 differentially expressed transcript-derived fragments (TDFs), and 21 were further analyzed. Bioinformatics analysis of TDF sequences allowed us to assign a putative identity to 13 of these and propose that these genes might be involved in Mn^{2+} homeostasis through proteins related to the compartmentalization, storage, sorting and detoxification of Mn^{2+}, as well as to Fe–S cluster biosynthesis.

Materials and methods

Strains and growth conditions

C. subvermispora FP-105752 was obtained from the Centre for Mycology Research (Forest Products Laboratory, Madison, WI, USA). Stationary cultures grown in standard minimal medium for the preparation of inocula were as described by Rüttimann et al. (1992). The fungus was grown for 10–14 days at 30°C in liquid cultures with constant agitation (200 rpm) in standard minimal medium containing 0, 5, 20, 160, 320, 1,000, 3,000 and 5,000 μM of Mn^{2+}. In addition, cultures containing 5 μM Mn^{2+} were supplemented with Mn^{2+} until a final concentration of 160 μM was reached, and the mycelium was harvested after 60 and 90 min of incubation.

RNA isolation

Total RNA was isolated from 150 mg of mycelium by phenol:chloroform extraction following a protocol described before (Manubens et al. 2003). Seventy-five micrograms total RNA was used for mRNA poly(A)$^+$ extractions using Oligo(dT)/Dynabeads (Dynal, Norway). The RNA was resuspended in diethylpyrocarbonate-treated deionized water and stored at −70°C.

Oligonucleotides

All oligonucleotides used were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Primers used for the real-time RT-PCR were designed using PrimerQuest^{SM} Software from IDT's SciTools (Rozen and Skaletsky 2000).

cDNA–AFLP analysis

Double-strand cDNA was synthesized using Superscript reverse transcriptase (Invitrogen) and primer dTxADAPT. cDNA–AFLP protocol using *Vsp I* and *Taq I* endonucleases was carried out according to Bachem et al. (1996). To identify the best primer pairs, 256 primer combinations were analyzed using cDNA from cultures containing 0 and 160 mM of Mn^{2+}. The best ten primer pairs were selected for the specific PCR amplifications of all experimental conditions.

Selective amplification products were denatured in formamide at 95°C, and separated by electrophoresis polyacrylamide (6% acrylamide, 7% urea) sequencing gels. Gels were dried on 3MM Whatman paper (Whatman, Maidstone, UK) and analyzed after overnight exposure of Kodak X-OMAT AR films at −70°C.

Isolation and sequencing of TDFs

Bands of interest were selected, extracted from the dried gels and soaked in TE buffer for 60 min at 65°C. Isolated TDFs were amplified using the preamplification primers. PCR products were purified, ligated to pCR-II TOPO TA plasmid (Invitrogen, USA) and cloned in *Escherichia coli* DH5α. The DNA of purified plasmid was sequenced by the Automatic Sequencing Service of Macrogen (Seoul, Korea).

Real-time PCR

cDNAs from seven different culture conditions, where Mn^{2+} concentrations were 0, 5, 5 + 60 min 160 μM, 5 + 90 min 160 μM, 20, 160 and 320 μM, were used to compare expression levels of selected fragments. The amplification reaction contained 2 μL of cDNA, 10 μL of Brilliant® SYBR® Green 2X QPCR Master Mix from Stratagene (La Jolla, CA, USA), 100 nM forward and reverse primers, and 300 nM ROX reference dye from Stratagene in a final reaction volume of 20 μL. The MX3000P QPCR system from Stratagene, along with its software, was used to obtain threshold cycles for all amplification reactions. The thermal cycling protocol consisted of one hot start step of 94°C for 10 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. A melting curve was added at the end of every amplification reaction, and measurements were recorded between 55 and 94°C. Real-time PCR reactions were conducted in parallel in triplicate, and standard deviations were calculated from these results. Each run was repeated at least twice for each TDF, and

normalization was performed using the transcript levels of the constitutively expressed *Csgapd* gene as control in all samples. Relative expression ratios were calculated according to Pfaffl (2001).

Transmission electron microscopy

Samples from growth conditions 0, 160 and 320 μM Mn^{2+} were fixed overnight at room temperature in 0.134 M pH 7.2 sodium cacodylate buffer containing 3% (v/v) glutaraldehyde. Samples were then washed three times with 1 volume of buffer for 20 min, and postfixed for 1 h at room temperature in a 1% aqueous solution of OsO_4. A final washing step was performed three times with double-distilled water before staining for 1 h in 1% uranyl acetate solution and then dehydrated with increasing concentrations of acetone (50, 70, 95, 100%) for 20 min each one. The samples were pre-embedded in epon/acetone 1/1 overnight (Medcast, Pelco, Inc.), and finally embedded in epon the next day. Samples were polymerized at 60°C for 24 h. Ultrathin sections of hyphae from the periphery and central parts of pellets were sectioned with a Sorvall MT2-B ultramicrotome and stained with 4% uranyl acetate in methanol and lead citrate (Reynolds 1963). Observations were made with a Philips Tecnai 12 transmission electron microscope operated at 80 kV.

Identification of TDFs

Transcript-derived fragment sequences were analyzed using BLASTX and TBLASTX algorithms against either NCBI nonredundant database (NCBI, National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov) or the *P. chrysosporium* BestModels v2.1 gene models database on the *P. chrysosporium* Genome Project website (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). Sequences that had no significant homology (E value $< e^{-7}$) were left out of the identification process, while sequences that had high homology to putative proteins were further studied using the hypothetical protein sequence and the BLASTP algorithm on NCBI nonredundant protein database. The results of the latter study are presented as the putative identity of the protein following the protein reference.

Results

Defining a classification criterion for Mn^{2+} altered gene expression

C. subvermispora is able to grow in culture media containing up to 5 mM Mn^{2+} (data not shown), indicating the existence of a robust homeostatic machinery that proficiently deals with excess manganese. *C. subvermispora* mycelium is white either when Mn^{2+} concentration in culture media is under 160 μM or when it grows in the wild, while mycelia growing from 320 μM Mn^{2+} to 5 mM show very little growth and a characteristic dark color. We avoided high Mn^{2+} concentrations (320 μM and over) due to this anomaly, thus trying to study the effect of Mn^{2+} on gene expression within limits where cells reach homeostasis without growth defects or dark pigmentation.

To identify differentially expressed TDFs as an effect of increasing Mn^{2+} concentration in the culture medium, we designed two experimental approaches: the first consisted of a time course, where at time zero, to the 14 days old cultures containing 5 μM Mn^{2+}, a supplement of 160 μM of Mn^{2+} was added during 60 and 90 min. Because of the relatively short time involved, changes in these assays were named "transcriptional response." The second approach consisted of cultures with a constant Mn^{2+} (0, 5, 20 or 160 μM) concentration and changes in gene expression in these assays were called "physiological response." Based on the time course approach of the transcriptional response, TDFs that showed a tendency to increase their relative transcript expression level compared to the 5 μM Mn^{2+} control culture were classified as upregulated, those that tended to decrease as downregulated, while fragments that showed no change were classified as unregulated (see Table 1). TDFs that showed no obvious tendency were not included in further studies.

A total of 37 differentially expressed transcript-derived fragments (TDFs) were isolated, cloned and sequenced. Of these, 21 were classified under one of the three categories based on their transcriptional levels during the time course: Nine were classified as upregulated, five as downregulated and seven as unregulated (see Table 1). The 14 remaining TDFs showed an irregular pattern and were not included in further studies. All 21 classified TDFs were searched for homology of known genes; however, bioinformatics analysis could assign a putative identity to only 13 of these sequences. Six upregulated and two downregulated TDFs were selected for further characterization. To confirm the relative expression levels by an independent experimental approach, five upregulated genes (CsMn09, CsMn42, CsMn04, CsMn41 and CsMn36) were compared to the expression of three unregulated genes (CsMn02, CsMn06 and CsMn34) using real-time PCR (see Fig. 1). In the following sections, the findings for these selected TDFs and their possible involvement of the putative encoded proteins in Mn^{2+} homeostasis are discussed.

Upregulation in response to Mn^{2+}

TDFs CsMn09 and CsMn42 showed a threefold increase in transcript levels 90 min after the supplementation of

Table 1 Transcriptional expression profiles of TDFs in response to time course (transcriptional) and stationary (physiological) Mn^{+2} concentration

Transcriptional response	Physiologica1 response	Fragment	Method	NCBI dbEST GenBank Acc	Length (bp)	Protein homology	Protein acc. N[a].	Gene ontology	Score	E value	Identities (%)
Upregulated	Upregulated	CsMn04	Real-time RT-PCR	FK939102	370	Putative ortholog to *Saccharomyces cerevisiae* protein SSD1 [*P. chrysosporium*]	139421	Cell wall organization and biogenseis, response to drug	66.6	23E−27	73
	Upregulated	CsMn09	Real-time RT-PCR	FK939107	128	Putative ortholog to *S. cerevisiae* N-terminal acetyltransferase complex ARD1 subunit [*P. chrysosporium*]	140132	N-terminal protein amino acid acetylation	93.0	7.0E−21	90
	Upregulated	CsMn33	cDNA–AFLP	FK939126	186	Putative Zn-dependent hydrolase/ oxidoreductase family protein [*P. chrysosporiam*]	3076	Hydrolase activity	43.2	2.0E−10	77
	Upregulated	CsMn36	cDNA–AFLP, Real-time RT-PCR	FK939129	410	Hypothetical protein CCLG_00086 [*Coprinopsis cinerea* Okayama 7#130]-putative acyl-CoA dehydrogenase	EAU84567	Oxidoreductase activity, acting on the CH–CH group of donors	225.0	7.0E−58	81
	Upregulated	CsMn37	cDNA–AFLP	FK939130	334	No significant homology	–	–	–	–	–
	Upregulated	CsMn39	cDNA–AFLP	FK939132	336	No significant homology	–	–	–	–	–
	Upregulated	CsMn40	cDNA–AFLP	FK939133	288	No significant homology	–	–	–	–	–
	Upregulated	CsMn41	cDNA–AFLP, real-time RT-PCR	FK939134	414	Hypothetical protein CC1G_05681 [*C. cinerea* Okayama 7#130]-putative cytosolic Fe–S cluster assembling factor NBP35	EAU90143	Nucleotide binding	248.0	6.0E−65	86
	Up-regulated	CsMn42	cDNA–AFLP, real-time RT-PCR	FK939135	221	HDEL sequence binding protein [*Cryptococcus neoformans var. neoformans* JEC21]	XP_568027	Protein retention in the ER, receptor activity, ER retention sequence binding	47.0	3.0E−08	80
Downregulated	Unregulated	CsMn01	cDNA–AFLP	FK939099	297	Phenylalanine ammonia-lyase [*P. chrysosporium*]	124439	L-phenylalanine catabolic process	58.0	2.0E−16	58
	Unregulated	CsMn25	cDNA–AFLP	FK939120	194	No significant homology	–	–		–	–
	Downregulated	CsMn26	cDNA–AFLP	FK939121	154	Elongation factor 3 [*C. neoformans var. neoformans* JEC21]	AAK26245	Translation elongation factor activity	72.0	1.0E−11	70
	Downregulated	CsMn27	cDNA–AFLP	FK939122	136	Hypothetical protein [*P. chrysosporium*]–putative cystathionine β-lyase	35886	Amino acid biosynthetic process	84.0	4.0E−18	72
	Downregulated	CsMn29	cDNA–AFLP	FK939124	136	No significant homology	–	–	–	–	–
Upregulated	Upregulated	CsMn02	Real-time RT-PCR	FK939100	142	Putative maturase/reverse transcriptase [*Bacillus sp.* NRRL B-14911]	ZP_01171048	RNA-dependent DNA replication	55.8	8.0E−7	57
	Upregulated	CsMn06	Real-Time RT-PCR	FK939104	198	Hypothetical protein CC1G_07903 [*C. cinerea* Okayama 7#130]–putative glutathione S-transferase	EAU82621	Glutathione transferase activity	62.8	6.0E−9	39
	Unregulated	CsMn28	cDNA–AFLP	FK939123	157	No significant homology	–	–	–	–	–
	Unregulated	CsMn31	cDNA–AFLP	FK939125	87	No significant homology	–	–	–	–	–
	Upregulated	CsMn34	cDNA–AFLP, Real-Time RT-PCR	FK939127	248	Hypothetical protein [*P. chrysosporium*]–putative nonribosomal peptide synthetase-like enzyme	135156	Ligase activity, metabolic process	90.9	3.0E−24	77
	Unregulated	CsMn35	cDNA–AFLP	FK939128	166	No significant homology	–	–	–	–	–
	Unregulated	CsMn38	cDNA–AFLP	FK939131	272	Hypothetical protein [*P. chrysosporium*]	4889	–	33.1	3.0E−12	65

This table includes TDFs' classification and bioinformatics identification using BLASTX and TBLASTX. Protein accession numbers refer to the NCBI protein database or to the *Phanerochaste chrysosporiam* genome database

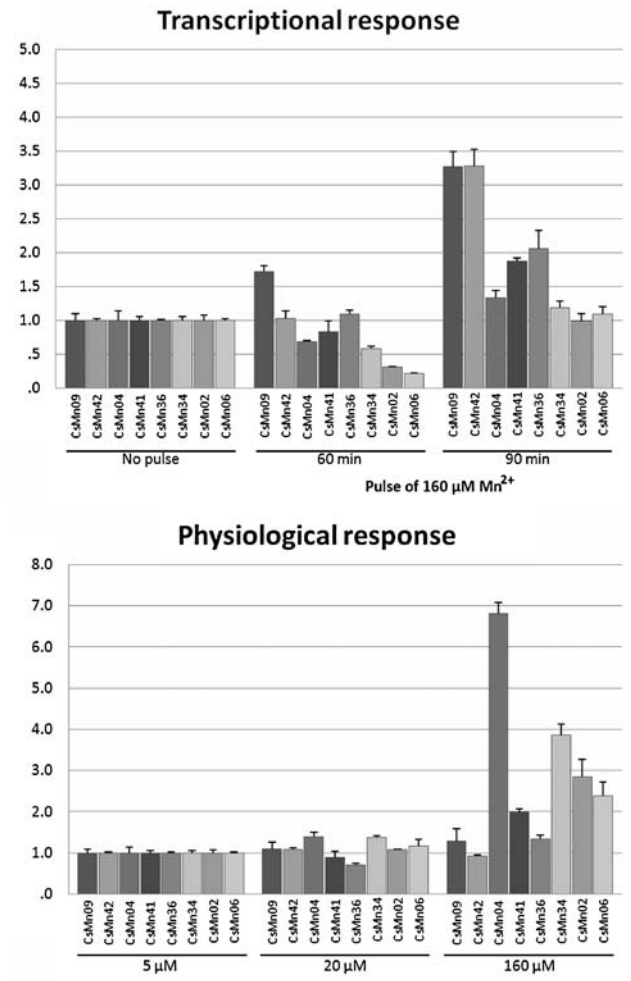

Fig. 1 Relative expression of selected TDFs assayed by real-time PCR. *Upper panel* response of TDFs after submitting cultures incubated at 5 µM Mn^{+2} to pulses of 0 (control), 60 and 90 min. *Lower panel* TDF expression in cultures incubated at 5, 20 and 160 µM Mn^{+2} during 14 days at 28°C ($n = 3$)

cultures with 160 µM Mn^{2+} (see Fig. 1). CsMn09 was tentatively identified as the putative ARD1 subunit of the *N*-acetyltransferase complex (see Table 1). This complex catalyzes the N-terminus acetylation of nascent polypeptides in a cotranslational fashion and is responsible for acetylation of about 85% of all eukaryotic proteins (Driessen et al. 1985; Polevoda and Sherman 2003).

In turn, TDF CsMn42 was identified as a putative homolog to the HDEL sequence binding protein or the ER lumen-retaining receptor from *Cryptococcus neoformans* (see Table 1 and Fig. 1). This protein is a receptor responsible for the recognition of the peptide sequence HDEL, which is found on the C-terminal region of proteins that reside in the endoplasmic reticulum (ER) (Semenza et al 1990).

CsMn04 was identified as a putative homolog to the *S. cerevisiae* SSD1 protein (see Table 1). This protein has been associated with cell wall biogenesis and organization (Ibeas et al. 2001; Kaeberlein and Guarente 2002; Tsuchiya et al. 1996). After Mn^{2+} supplementation, the levels of mRNA encoding this protein decreased 31% after 60 min and then rose 33% relative to the control condition after the 90-min incubation (net change of 64% in 30 min) (see Fig. 1).

BLAST analysis revealed high homology between the nucleotide-binding protein 35 (Nbp35) from *S. cerevisiae* and TDF CsMn41 (see Fig. 1). Its expression originally fell 17% at 60 min, yet 90 min after the Mn^{2+} supplementation, it was clearly upregulated with an expression increase of 88% (net increase of 105% in 30 min) (see Fig. 1). Nbp35 is an essential protein that plays a central role in the assembly of cytosolic and nuclear Fe–S clusters.

CsMn36 is another interesting TDF. It was putatively identified as acyl-coenzyme A dehydrogenase, an enzyme responsible for the oxidation of fatty acids in the mitochondria and peroxisomes in a process known as β-oxidation (see Table 1) (Wanders 2004). This TDF was found to increase 10% after 60 min of the Mn^{2+} supplementation and to double after a 90-min pulse (see Fig. 1).

TDF CsMn33 was identified as a putative member of the Zn-dependent hydrolase/oxidoreductase family of proteins (see Table 1). The hydrolase/oxidoreductase domain is conserved through at least eighty-one proteins from different organisms, mainly prokaryotes (Marchler-Bauer et al. 2007). The only two connections found between this protein family and Mn^{2+} are as follows: (1) the bivalent cation-binding domain and that (2) some of the proteins containing this conserved domain have been characterized as metal-dependent hydrolases. The observed induction is also present in the constant Mn^{2+} concentration study (see Table 1).

An ultrastructural study in mycelia incubated in cultures with high Mn^{2+} concentration showed the presence of multivesicular vesicular bodies that can be identified by their characteristic low density electro-lucent central regions (Fig. 2), which are indicative of secretion processes. Similar membranous structures have been observed in *P. chrysosporium* (Daniel et al. 1992).

Downregulation in response to Mn^{2+}

TDF CsMn01 was identified as a putative homolog of phenylalanine ammonia-lyase (PAL) from *P. chrysosporium* (see Table 1). This enzyme participates in the catabolism of L-phenylalanine through its nonoxidative deamination.

TDF CsMn26 was identified as a putative homolog to the fungal-specific elongation factor 3 (EF3) from *C. neoformans* (see Table 1). This TDF's expression is repressed both after the short-time transcriptional response assays with Mn^{2+} and in stationary cultures.

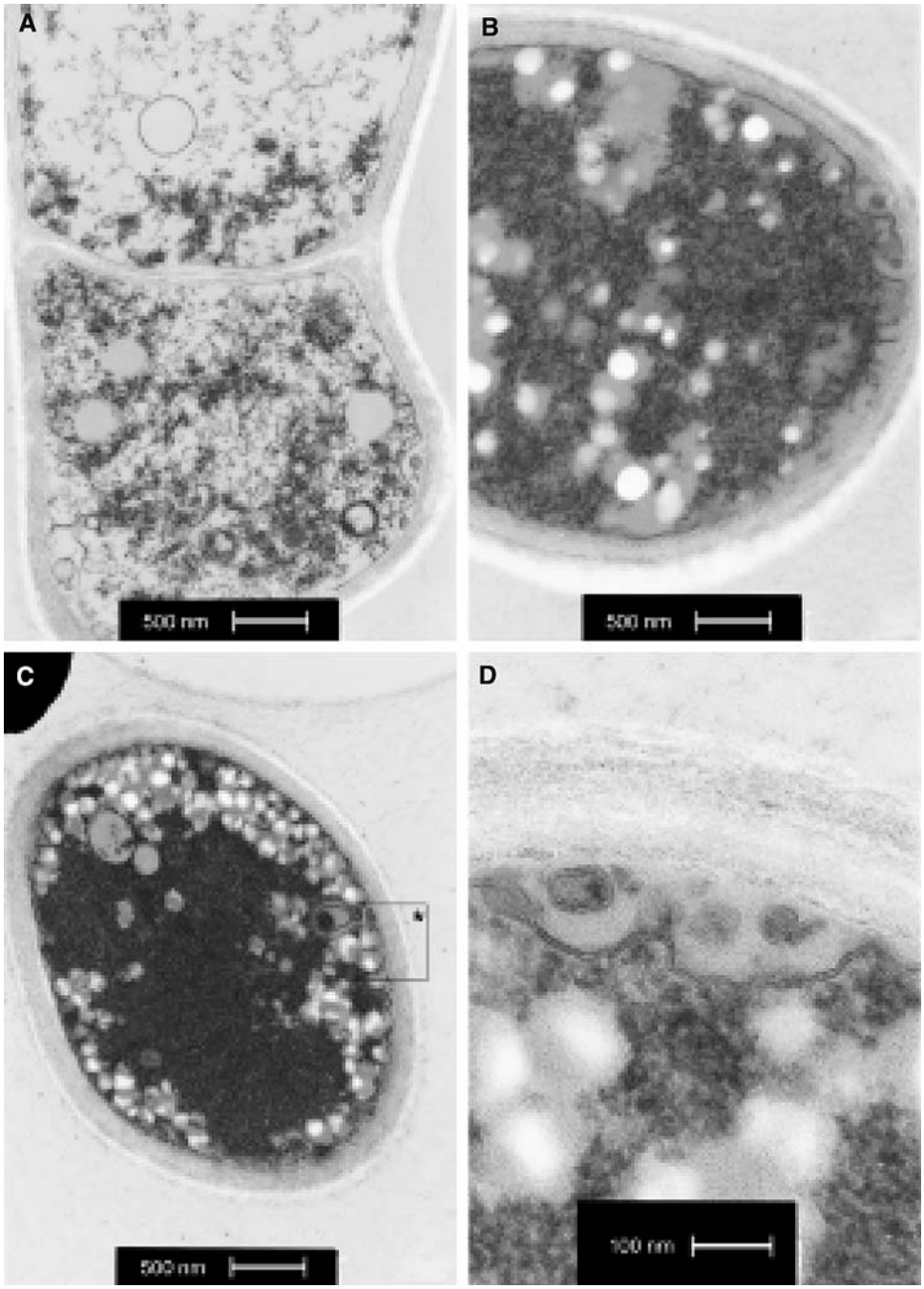

Fig. 2 Effect of Mn^{+2} in the accumulation of intracellular vesicles. *C. subvermispora* was cultured for 10 days in **a** Mn^{+2} deficient media, **b** 160 μM Mn^{+2} or **c** 320 μM Mn^{+2}. **d** An amplification of an area similar to that marked on **c** with an *asterisk*

Another fragment found to be repressed by the Mn^{2+} supplementation and constant Mn^{2+} concentration approaches was TDF CsMn27. This fragment was identified as a homolog to a hypothetical protein from *P. chrysosporium*, a putative cystathionine *β*-lyase (see Table 1). This enzyme participates in the metabolism of sulphur amino acids, specifically in the biosynthesis of L-methionine and in the conversion from homocysteine to L-cysteine (Thomas and Surdin-Kerjan 1997).

Discussion

In this work, we found a general correlation between the cDNA–AFLP expression levels and the transcript levels detected independently by real-time PCR, both for upregulated genes and for those nonregulated genes assayed as controls. For those TDFs where a putative homolog could be assigned, a surprising variety of proteins was found. We expected to find proteins related to the transport and storage

of this metal, in a similar manner as previously described for iron-related genes. However, a bewildering assortment of processes appeared to be somehow related to Mn^{2+} metabolism.

Several of the Mn^{2+}-dependent upregulated TDFs constitute interesting findings. As mentioned previously, TDFs CsMn09, CsMn42 and CsMn04 correspond to a putative ARD1 subunit of the *N*-acetyltransferase complex, a receptor of the peptide sequence HDEL and the SSD1 protein. ARD1 is needed for N-terminus acetylation together with NAT1, another subunit of the complex (Mullen et al. 1989). In the stationary cultures, the TDF CsMn09 was also induced, yet to a lesser extent (see Fig. 1).

On the other hand, Erd2 is homologous to the ER lumen-retaining receptor in *S. cerevisiae*. There is no change in expression levels of this TDF under stationary culture conditions (see Fig. 1). However, the enhanced expression of CsMn42 after a 90-min exposure to 160 μM Mn^{2+} may be indicative of a higher activity of the secretory pathway as a homeostatic response to the increase in Mn^{2+}. In this scenario, the putative receptor would be induced to retain resident proteins of the ER in its lumen once the activity of the secretory pathway increases. This receptor has also been related to diverse cell responses like cell differentiation, development and ER stress through the modulation of kinases such as MAPK (Yamamoto et al. 2003). This TDF CsMn42 could be associated with the activity of TDF CsMn04 (*vide infra*). In turn, one of the first studies aimed to characterize the function of SSD1 showed that this protein conferred cellular tolerance to high Ca^{2+} concentrations, a bivalent cation related to Mn^{2+} homeostasis through the Pmr1p pump (Dürr et al. 1998).

Strongly linked to ionic stress response, SSD1 was described as a regulator of *S. cerevisiae* resistance to the plant defence protein osmotin (PR-5). SSD1 mediates the proper sorting/synthesis of osmotin-resistance factors onto the cell wall in a post-transcriptional manner and is necessary to maintain cellular integrity during osmotin-induced osmotic stress (Ibeas et al. 2001). Notably, when *C. subvermispora* was grown at stationary 160 μM Mn^{2+}, there was an almost sixfold increase of its mRNA levels, the highest observed in this study (see Fig. 1). This evidence suggests that SSD1 may participate in an active Mn^{2+} detoxification process. This protein has been reported to possess a RNA-binding activity, which had not been linked previously to a role in cell wall morphology or high Ca^{2+} concentration tolerance (Uesono et al. 1997). Ibeas et al. (2001) reported that deletion of SSD1 alleles impeded the sorting of osmotin-resistance factors to the cell wall without affecting their mRNA levels. Taken together, our findings on the relative induction of this post-transcriptional regulator suggest that SSD1 may in fact be a Mn^{+2} homeostatic regulator.

Other upregulated TDFs are CsMn41 (homolog to Nbp35) and CsMn36 (homolog to acyl-CoA dehydrogenase). As recently reviewed, Nbp35 participates as part of a scaffold system for the assembly and transfer of Fe–S clusters into apoproteins together with Cfd1, Nar1 and other members of the cytosolic Fe–S protein assembly (CIA) machinery (Lill and Mühlenhoff 2006). Mn^{2+} not only converges with iron in the regulation of cellular processes in bacteria, but has also been shown to participate in the regulation of the iron regulatory protein 1 (Irp1), also known as cytosolic aconitase. Irp1 is a central regulator of iron homeostasis and senses iron levels through a 4Fe–4S cluster whose Fe in the fourth labile position can be replaced by other metals to form the holoenzyme. Irp1 is an active cytosolic aconitase when iron is plentiful, and this level of activity can be replicated in Mn^{2+}-replete and Fe^{2+}-deplete conditions. Mn^{2+} has also been reported to generate cytotoxity through the inactivation of mitochondrial Fe–S-containing enzymes, namely aconitase, NADH-ubiquinone reductase (complex I) and succinic dehydrogenase (SDH, complex II) (Chen et al. 2001). The transcriptional enhancement of TDF CsMn41, the putative *nbp35* homolog, may be due to the need of Fe–S clusters for the sensing of Mn^{2+} and triggering of mechanisms needed to achieve Mn^{2+} homeostasis. Under stationary Mn^{2+} conditions at 160 μM Mn^{2+} and also after a 90-min time course, the expression profile reveals a twofold increase, compared to the 5 μM Mn^{2+} control (see Fig. 1). This expression profile of this fragment at 160 μM Mn^{2+} suggests a constant requirement for the synthesis of this protein under Mn^{2+}-replete conditions. With respect to the homolog to acyl-CoA DH, we suggest that if this protein participates in the catabolism of fatty acids through *β*-oxidation, this may be due to two reasons: (1) a need for extra energy to achieve Mn^{2+} homeostasis after supplementation and (2) a need for acetyl-CoA as a substrate for the de novo synthesis of phospholipids. Culotta et al (2005) suggested that in case of high biosynthesis of phospholipids, Pmr1p would pump the excess of Mn^{2+} into the Golgi and to excrete them via secretory vesicles back into the extracellular environment in the presence of toxic Mn^{2+} concentrations.

Eide et al. (2005) characterized the yeast ionome through a genome-wide analysis of 4,000 mutants in *S. cerevisiae* to screen for genes involved in trace element homeostasis. About 80% of the mutations that had an effect on intracellular Mn^{2+} produced an increase of its concentration. This clearly indicates that Mn^{2+} homeostasis is achieved through an active excretion processes. Identification of the mutated Mn^{2+}-responsive genes that led to an increased cellular Mn^{2+} concentration reinforced the importance of genes related to vacuolar biogenesis. Our transmission electron microscopy (TEM) images showing a clear and surprising increase in vesicle-like shapes in response to

Mn^{2+} in the cultures with constant Mn^{2+} concentration are in strong agreement with the hypothesis of Culotta et al. (2005) and the conclusions of Eide et al. (2005) (see Fig. 2). In this figure, the merging of vesicles with the plasma membrane seems to account for a secretory detoxifying mechanism. The expression of TDF CsMn36 is induced by the Mn^{2+} supplementation; however, it does not change significantly in response to increasing constant Mn^{2+} concentrations in 14 days cultures (see Fig. 1). This lends support to our hypothesis of a de novo membrane biosynthesis after the Mn^{2+} supplementation stress, since over a longer period of time (constant Mn^{2+} concentration scenario) a membrane/phospholipid recycling pathway may be operating.

Among the proteins we expected to find were the MnPs, whose regulation and expression we had previously studied. However, to our surprise, under all assayed conditions, none of the upregulated TDFs corresponded to any of the manganese peroxidases we had characterized. This result might be explained by the fact that we isolated only those TDFs that showed a substantial change in their expression levels. On the other hand, it cannot be ruled out that one or more of the 14 differentially expressed TDFs with an irregular expression pattern that was not further studied corresponded to a manganese peroxidase gene. In this vein, the observed results should not be so unexpected, since *mnp1* and *mnp3* are not expressed in high Mn^{2+} and *mnp2* exhibits only a relatively modest increase upon incubation with this metal (Manubens et al. 2003).

Contrary to the above behavior, TDFs CsMn01 and CsMn26 are downregulated by Mn^{2+}. The former is a homolog to PAL. This enzyme catalyzes the first step of the phenylpropanoid pathway converting L-phenylalanine to cinnamic acid, which is further metabolized to a variety of different phenolic compounds through this pathway (Cochrane et al. 2004). Although this enzyme has been historically linked to plant secondary metabolism, it has also been described in fungi where a symbiotic function has been suggested (Nehls et al. 1999). A previous study aimed to determine the biochemical response to high Mn^{2+} concentrations in *Populus cathayana* revealed that the amount of intracellular L-phenylalanine increased three- to fivefold. The presence of high Mn^{2+} concentrations also increased the levels of polyamines and other free amino acids, especially proline and histidine (Lei et al. 2007). These data favor the hypothesis of a higher translational rate in response to Mn^{2+} treatment, as possibly occurs in our model. In the stationary Mn^{2+} scenario, TDF CsMn01 shows no evident response to Mn^{2+} (see Table 1). In turn, CsMn26 is a homolog to EF3. This factor has been characterized in *S. cerevisiae* [yeast elongation factor 3 (YEF3)] where it has been described as a protein necessary for growth and in vivo translation that has been classified among the "traffic ATPases" family of proteins (Chakraburtty 2001). EF3 has also been associated with bivalent cation homeostasis, as it was found to be downregulated by zinc; there was a 50-fold repression on the activity of a reporter construct when yeast cells were grown in 100 μM Zn^{2+} compared to that when grown on 0.1 μM Zn^{2+} (Yuan 2000). In 1997, Lussier et al. (1997) conducted a large-scale identification of genes involved in cell surface biosynthesis and architecture in *S. cerevisiae*. EF3 was identified as a gene not previously related to the cell surface. In Mn^{2+}-depleted cells, *pmr1* and *YEF3* yeast strain might all share a defect in protein N- and O-linked glycosylation, which would strongly suggest a novel role for EF3 in Mn^{2+} homeostasis (Dürr et al. 1998). Additionally, affinity capture-mass spectroscopy experiments showed that YEF3 forms a protein complex with Hrk1, a protein kinase implicated in activation of the plasma membrane H^+-ATPase Pma1p, which plays a role in Mn^{2+} homeostasis (Gavin et al. 2006; Goossens et al. 2000). Although the repression of EF3 transcription may be evidence for a reduced translational rate after Mn^{2+} supplementation, EF3 might also be linked to other Mn^{2+}-dependent cellular processes. There may be a basal level of EF3 needed for efficient translation elongation, and under manganese-/zinc-depleted scenarios, this ATPase would somehow help to overcome defects on post-translational modifications of cell wall proteins.

Although this protein has been characterized as an essential translation elongation factor, there is evidence linking it to metal homeostasis. EF3 is involved in the biogenesis and architecture of the cell wall, and also forms a complex with a kinase related to the activation of a plasma membrane transporter (Hrk1), which participates in Mn^{2+} homeostasis (Gavin et al. 2006). These potential regulators could act reciprocally to counteract the effects of a rise (SSD1) or drop (EF3) in the extracellular concentration of Mn^{2+}.

It was not surprising to find that Mn^{2+} altered previously unlinked bivalent cation-related processes such as Fe–S biosynthesis, high Ca^{2+} concentration tolerance and Zn^{2+}-dependent hydrolysis/oxidoreduction. Protein–cation interactions may not be specific for each metal, and we believe that this flexibility plays an important role in metal homeostasis.

The current knowledge of Mn^{2+} homeostasis, together with the finding of genes not previously known to be related to metal metabolism reported in this work, opens new lines of research into this fascinating and complex topic. Among all transcripts studied, TDFs CsMn04, CsMn09, CsMn21 and CsMn36 showed the greatest increase in relative expression over time. Further study of the upstream regions of these TDFs could give insights on hypothetical Mn^{+2}- or metal-responsive regulatory elements. Eventually, these upstream regions might drive ectopic Mn^{2+}-dependent gene expression and become a

genetic tool for the study of the biology of *C. subvermispora*, just as copper-inducible promoters have for other fungi (Gebhart et al. 2006).

In conclusion, we hereby present the results of an unbiased and systematic search for Mn^{2+}-related genes in an organism that has not been sequenced. The isolated TDFs suggest a picture of the complexity of Mn^{2+} homeostasis. Cells apparently maintain a robust machinery that deals with this metal, possibly through the excretion of Mn^{2+} excess encoding proteins related to diverse cellular functions, including protein sorting, protein storage, as well as organelle and cell wall biosynthesis. Several of these putatively identified genes are upregulated and some are downregulated in response to manganese.

The analysis of the promoter sequences of genes with similar expression patterns can yield information of consensus motifs that might identify common functional regulatory elements that have not yet been defined. Systematic studies of regulatory elements of this kind have been performed in mammalian genomes (Xie et al. 2005). It will also be interesting to determine if in silico modeling shows common elements that might correspond to metal- or manganese-responsive sequences (Rodionov et al. 2006). We are presently trying to characterize promoter sequences of upregulated TDFs to answer these fascinating questions.

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