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# OmZnT1 and OmFET, two metal transporters from the metal-tolerant strain Zn of the ericoid mycorrhizal fungus *Oidiodendron maius*, confer zinc tolerance in yeast

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## Abstract

Two full-length cDNAs (*OmZnT1* and *OmFET*) encoding membrane transporters were identified by yeast functional screening in the heavy metal tolerant ericoid mycorrhizal isolate *Oidiodendron maius* Zn. *OmZnT1* belongs to the Zn-Type subfamily of the cation diffusion facilitators, whereas *OmFET* belongs to the family of iron permeases. Their properties were investigated in yeast by functional complementation of mutants affected in metal uptake and metal tolerance. Heterologous expression of *OmZnT1* and *OmFET* in a Zn-sensitive yeast mutant restored the wild-type phenotype. Additionally, *OmZnT1* expression also restored cobalt tolerance in a Co-sensitive mutant. A GFP fusion protein revealed that *OmZnT1* was targeted to the endoplasmic reticulum membrane, a result consistent with a function for *OmZnT1* in metal sequestration. Similarly to other iron permeases, *OmFET*-GFP was localized on the plasma membrane. *OmFET* restored the growth of uptake-defective strains for iron and zinc. Zinc-sensitive yeast mutants expressing *OmFET* specifically accumulated magnesium, as compared to cells transformed with the empty vector. We suggest that *OmFET* may counteract zinc toxicity by increasing entry of magnesium into the cell.

## 1. Introduction

Transition metal ions such as iron, copper, manganese and zinc are essential micronutrients that play pivotal roles in several biochemical processes (Waldron et al., 2009). Zinc, in particular, has both a catalytic and a structural function in more than 300 enzymes belonging to the six major functional enzyme classes (Vallee and Auld, 1990). Although an essential nutrient, zinc can become toxic if accumulated in excess. Competition for catalytic sites and for transporter proteins has been reported as being potentially responsible for the toxicity caused by zinc excess (Eide, 2003). To maintain concentrations of this metal ion within physiological thresholds, organisms have evolved metal homeostasis mechanisms to regulate zinc uptake, distribution, and storage (Eide, 2006). In eukaryotes, zinc homeostasis is largely attributed to the coordinated action of two transporter families: the ZIP (ZRT-IRT-like Protein) and the CDF (Cation Diffusion Facilitator) families (Eide, 2006). A number of ZIP and CDF transporters have been characterized in various organisms (mammals, yeast, nematode, plants) and many candidate genes have been identified with the increasing number of genome sequencing projects (Diffels et al., 2006, Bolchi et al., 2011, Diss et al., 2011, Eide, 2006, Kambe et al., 2006, Kiranmayi and Mohan, 2006, Migeon et al., 2010 and Montanini et al., 2007).

The name of the ZIP transporters family derives from the first two members that were described: the Zrt1 zinc transporter from *Saccharomyces cerevisiae* (Zhao and Eide, 1996a and Zhao and Eide, 1996b) and the Irt1 iron transporter from *Arabidopsis thaliana* (Eide et al., 1996). A key feature of the ZIP family is that these proteins transport zinc and/or other metal ions from the extracellular space (or organellar lumen) into the cytosol (Eide, 2006). CDF transporters were first described in *S. cerevisiae* (*Zrc1p*) (Kamizono et al., 1989) and in the Gram-negative bacteria *Ralstonia metallidurans* (*CzcD*) (Nies, 1992), but they were later also found in plants and animals (see in Montanini et al., 2007). The key feature of the CDF transporters is

that they export zinc and/or other metal ions out of the cells, or they sequester them into intracellular compartments ( Eide, 2006, Montanini et al., 2007 and Peiter et al., 2007), thus reducing cytosolic metal concentrations ( Anton et al., 1999 and MacDiarmid et al., 2002).

Much of the current knowledge on zinc homeostasis in fungi comes from studies in the yeast *S. cerevisiae*, which was also used as model organism for the identification of several zinc transporters from plants and animals. Four transporters involved in zinc uptake have been reported for *S. cerevisiae*: (a) Zrt1p, a high affinity transporter highly expressed at low external zinc concentrations ( Zhao and Eide, 1996a); (b) Zrt2p, a low affinity transporter involved in zinc acquisition under much higher zinc concentrations (Zhao and Eide, 1996b); (c) Fet4p, a transporter involved in the low affinity uptake of iron, copper and zinc ( Waters and Eide, 2002); (d) Pho84p, a high affinity phosphate transporter also likely involved in zinc and manganese uptake ( Bun-Ya et al., 1991 and Jensen et al., 2003). The iron permease Fet4p is a low-affinity system and was responsible for the increase of intracellular zinc in iron-depleted yeast cultures ( Li and Kaplan, 1998). Similarly, FETD, the FET4 ortholog of *Aspergillus fumigatus*, was involved in the accumulation of zinc during iron starvation ( Yasmin et al., 2009). The CDF transporters Zrc1p and Cot1p are the major determinants of zinc tolerance in yeast (Gaither and Eide, 2001), and they are responsible for the vacuolar delivery and sequestration of zinc ( MacDiarmid et al., 2000 and MacDiarmid et al., 2002). Two other CDF members, Msc2p and Zrg17p, form a heteromeric zinc transport complex in the ER membranes, and have been implicated in the homeostatic maintenance of ER function (Ellis et al., 2004 and Ellis et al., 2005). The yeast transcription factor Zap1p regulates zinc homeostasis at the transcriptional level, by controlling the expression of both ZIP (e.g. *ZRT1*, *ZRT2*, *ZRT3*) and CDF transporters (e.g. *ZRC1*) under low zinc concentration ( MacDiarmid et al., 2003).

Despite the importance of zinc both as an essential micronutrient and a potential toxicant, the mechanisms of zinc homeostasis and tolerance in fungi others than yeast are poorly understood. Although CDF genes have been identified in several fungi other than the budding yeast, only three CDF proteins have been characterized to date. The Zhf1 protein from the fission yeast *Schizosaccharomyces pombe* is involved in zinc transport into the ER ( Clemens et al., 2002). Similarly, the HcZnT1 protein from the ectomycorrhizal fungus *Hebeloma cylindrosporum* was localized on the ER membrane and conferred zinc tolerance when expressed in yeast ( Blaudez and Chalot, 2011). The GiZnT1 protein may be involved in zinc homeostasis in the endomycorrhizal fungus *Glomus intraradices* ( González-Guerrero et al., 2005) but its role in zinc tolerance was not demonstrated.

In the present work we isolated and characterized two transporters belonging to the CDF and to the iron permease families from the metal-tolerant strain Zn of the ericoid fungus *Oidiodendron maius* ( Martino et al., 2000 and Martino et al., 2003) and we demonstrated, for the first time in an endomycorrhizal fungus, their involvement in zinc tolerance. *O. maius* ( Barron, 1962) is an ascomycete (class Leotiomycetes) that can establish endomycorrhizal symbioses with the roots of ericaceous plants ( Perotto et al., 1996, Hambleton et al., 1998 and Chambers et al., 2000). Among fungi, mycorrhizal species represent an important community in metal polluted soils, as they form mutualistic symbioses which can alleviate the effects of heavy metal toxicity to their host plant ( Adriaensen et al., 2005 and Adriaensen et al., 2006) and provide a more balanced access to mineral elements, either by improving supply of essential elements or by reducing uptake of toxic elements ( Bellion et al., 2006 and Colpaert et al., 2011). *O. maius* strain Zn was isolated in the Niepolomice forest (Poland) from the roots of *Vaccinium myrtillus* plants growing on heavy metal contaminated plots ( Turnau, 1988). The early colonization of these plots by ericoid mycorrhizal plants suggests that *O. maius* can confer to the host plant the ability to survive in metal polluted environments.

To identify fungal transporters involved in zinc tolerance, a functional screening of an *O. maius* cDNA library was performed in the zinc sensitive  $\Delta zrc1$  mutant of *S. cerevisiae*. Here, we describe the identification and characterization of two *O. maius* genes (*OmZnT1* and *OmFET*) coding for membrane transporters and conferring zinc tolerance to yeast. We provide evidence that *OmZnT1* functions as a zinc transporter responsible for relocating cytoplasmic zinc into the endoplasmic reticulum, whereas *OmFET* is a plasma membrane iron permease that seems to counteract zinc toxicity by increasing magnesium entry into fungal cells.

## 2. Materials and methods

### 2.1. Yeast strains and growth media

Yeast strains used in this study are listed in Table 1. Growth was either in yeast peptone dextrose (YPD) medium or in synthetic defined medium without uracil (SD-ura) containing 1.7% (w/v) yeast nitrogen base without amino acids and ammonium, 2% (w/v) glucose or galactose, 5% (w/v) ammonium sulfate and 1.92% (w/v) yeast synthetic drop-out medium supplement without uracil (Sigma). pH was adjusted to 5.8.

Table 1.  
Yeast strains used in this study.

Strain	Function of the deleted gene(s)	Genotype	Phenotype	Reference
BY4741	–	<i>MAT<math>\alpha</math>; his3; leu2; met15; ura3</i>	Wild-type	Euroscarf
$\Delta ccc1$	Vacuolar Fe and Mn transporter	<i>MAT<math>\alpha</math>; his3; leu2; met15; ura3; CCC1::kanMX4</i>	Fe sensitive	Euroscarf
$\Delta cot1$	Vacuolar Zn and Co transporter	<i>MAT<math>\alpha</math>; his3; leu2; met15; ura3; COT1::kanMX4</i>	Co and Zn sensitive	Euroscarf
$\Delta pmr1$	Golgi Mn- and Ca-ATPase	<i>MAT<math>\alpha</math>; his3; leu2; met15; ura3; PMR1::kanMX4</i>	Mn sensitive	Euroscarf
$\Delta yap1$	bZIP transcription factor	<i>MAT<math>\alpha</math>; his3; leu2; met15; ura3; YAP1::kanMX4</i>	Cd sensitive	Euroscarf
$\Delta zrc1$	Vacuolar Zn transporter	<i>MAT<math>\alpha</math>; his3; leu2; met15; ura3; ZRC1::kanMX4</i>	Zn sensitive	Euroscarf
$\Delta ctr1/$ $\Delta ctr3$ (MPY17)	Plasmalemma high-affinity Cu transporters	<i>MAT<math>\alpha</math>; ura3; his3; trp1; gal1; CTR1::URA3::Knr CTR3::TRP1</i>	Cu-uptake deficient	Peña et al., 1998
$\Delta fet3/$ $\Delta fet4$	Plasmalemma components of the high/low affinity Fe-uptake systems	<i>MAT<math>\alpha</math>; his3; leu2; met15; ura3; YMR058w::kanMX4; YMR319c::kanMX4</i>	Fe-uptake deficient	Euroscarf
$\Delta zrt1/$ $\Delta zrt2$ (ZHY3)	Plasmalemma high/low-affinity Zn transporters	<i>MAT<math>\alpha</math>; ade6; can1; his3; leu2; trp1; ura3; ZRT1::LEU2 ZRT2::HIS3</i>	Zn-uptake deficient	Zhao and Eide, 1996b

### 2.2. Yeast transformation

Yeast transformation was performed using the lithium acetate-based method described by Gietz et al. (1992). Briefly, yeast strains were inoculated in 50 ml YPD medium and incubated overnight at 30 °C on an orbital shaker. Yeast cultures were harvested at  $OD_{600nm} = 0.6$  and centrifuged at 4000 rpm for 5 min. The cells were washed twice with 1 ml sterile water and twice with 1 ml LiAc/TE solution (10 mM Tris-HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5). For each transformation experiment, the final yeast cell suspension (50  $\mu$ l) was mixed with 1  $\mu$ g plasmid DNA, 50  $\mu$ g single strand salmon sperm carrier DNA, and 300  $\mu$ l sterile LiAc/TE solution containing 50% (w/v) PEG 4000. The mix was first incubated at 30 °C for

30 min, and then at 42 °C for 15 min. Cells were centrifuged at 4000 rpm for 5 min, washed with 1 ml sterile water and plated on SD-ura medium.

### 2.3. Library screening

To identify cDNAs involved in zinc tolerance, an *O. maius* cDNA library (Abbà et al., 2011) was used to transform the zinc-sensitive  $\Delta zrc1$  yeast mutant. The cDNAs were cloned into the yeast expression plasmid pFL61 (Minet et al., 1992). Transformation of  $\Delta zrc1$  yeast cells with the cDNA library followed the same procedure described above except that, after the 42 °C shock, cells were washed with 1 ml of sterile water and incubated in liquid YPD medium for 4 h. Cells were plated on SD-ura medium containing 17.5 mM  $ZnCl_2$ . About 200 resistant colonies were picked and further tested on 12.8 mM 5-fluoroacetic acid (FOA) to eliminate false positives. Plasmids extracted from positive colonies with the Zymoprep yeast plasmid minipreparation kit (Zymo Research) were subsequently amplified in *Escherichia coli*. As a definitive confirmation of the plasmid-associated zinc resistance, plasmids were extracted from *E. coli* (minipreparations, Promega) and used to transform the  $\Delta zrc1$  yeast mutant. Only plasmids that conferred zinc tolerance were sequenced.

### 2.4. Sequence analyses

Analyses of similarity were performed using the program BLASTX at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence translations were performed using Expasy (<http://www.expasy.ch/>). Full-length amino acid sequences of orthologs were obtained from both the Broad Institute website (<http://www.broadinstitute.org/>) and the NCBI server (<http://www.ncbi.nlm.nih.gov/>). For phylogenetic tree construction, sequences were aligned by CLUSTALW and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 5.05 (Tamura et al., 2011). Phylogenetic analyses were conducted using the neighbor-joining (NJ) method implemented in MEGA, with the complete deletion option, and a Poisson correction model for distance computation. Bootstrap tests were conducted using 1000 replicates. Branch lengths are proportional to phylogenetic distances. Sequences were also aligned and edited by the multiple sequence alignment editor and shading utility GeneDoc (version 2.6.003) (<http://www.psc.edu/biomed/genedoc>). Prediction of the hydropathy profiles was generated with the topology prediction programme TMHMM version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>).

### 2.5. Phenotyping tests

A set of *S. cerevisiae* mutants was used to investigate metal tolerance specificity of the proteins encoded by the identified cDNAs (Table 1). Wild-type (BY4741) and mutant strains, carrying the empty vector (pFL61), pFL61-*OmFET*, or pFL61-*OmZnT1*, were grown on control SD-ura medium, and on the same medium supplemented with either 20 mM  $ZnCl_2$  (for  $\Delta zrc1$ ), 5.25 mM  $FeCl_3$  (for  $\Delta ccc1$ ), 2 mM  $CoCl_2$  (for  $\Delta cot1$ ), 50  $\mu M$   $CdCl_2$  (for  $\Delta yap1$ ), or 2 mM  $MnCl_2$  (for  $\Delta pmr1$ ).

To test metal uptake properties of OmFET, an additional set of yeast mutants was also used (Table 1). The ZHY3 ( $\Delta zrt1/\Delta zrt2$ ) strain was grown on YPD medium or on SD-ura medium supplemented with 1 mM EDTA and containing either no extra Zn or 0.5, 1 or 1.5 mM  $ZnCl_2$ . The double mutant  $\Delta fet3/\Delta fet4$  was grown on SD-ura medium supplemented with 0, 50, 100, 200  $\mu M$   $FeCl_3$ , or on YPD medium supplemented with 200  $\mu M$   $FeCl_3$ . MPY17 ( $\Delta ctr1/\Delta ctr3$ ) was grown on SD-ura medium with the addition of different concentrations (0, 50, 100, 200  $\mu M$ ) of the copper chelator Bathophenanthrolinedisulfonic acid (BPS), or on

YPD medium. *AtIRT1*, an *A. thaliana*-metal ion transporter, was used as a positive control (Vert et al., 2002).

## 2.6. Metal content analysis

The  $\Delta zrc1$  yeast strain, transformed either with the empty vector pFL61, pFL61-*OmFET*, or pFL61-*OmZnT1*, and the WT strain (BY4741), transformed with the empty vector pFL61, were grown in 50 ml of the control SD-ura medium, and in the same medium supplemented either with 500  $\mu$ M ZnCl<sub>2</sub> or with 500  $\mu$ M ZnCl<sub>2</sub> and 200  $\mu$ M FeCl<sub>3</sub>. Three different clones were used for each transformant. Yeast cultures were harvested when OD<sub>600nm</sub> was 0.75–0.8 and were centrifuged at 4000 rpm for 5 min. Cells were washed three times with 10 mM EDTA in 50 mM Tris–HCl buffer (pH 6.5), and with milli-Q water. Finally, samples were dried at 60 °C for 2 days, and subsequently mineralized with 5 ml HNO<sub>3</sub> 70% (v/w) in a microwave oven. After dilution to a final concentration of 3.5% HNO<sub>3</sub>, the metal content was finally determined using Induced Coupled Plasma (ICP-OES Vista MPX, Varian). Controls made up of ultrapure water and nitric acid were also analyzed. Quality control was based on the use of internal control samples and certified samples (Astasol-Mix from Analytica Ltd., Prague, Czech Rep.).

## 2.7. Construction of GFP fusion proteins for expression in yeast and confocal microscopy

To construct *OmZnT1::GFP* and *OmFET::GFP*, the open reading frames (ORFs) of *OmZnT1* and *OmFET* were amplified by PCR using the pFL61-*OmZnT1* and pFL61-*OmFET* plasmids previously isolated as templates. Both forward primers contained a *KpnI* site (*KpnOmZnT1f* 5'-ATCAGGTACCATGGCGTGGTCAAAATCGAC-3' and *KpnOmfet4f* 5'-ATCAGGTACCATGCAGCGAATAATCAGAGC-3'), while reverse primers contained a *NotI* site (*NotOmZnT1r* 5'-ATATGCGGCCGCTGAACGTGGTTCGTTGTCATC-3' and *NotOmfet4r* 5'-ATATGCGGCCGCGCCACGGGTAGCAATTGACG-3'). The PCR products were *NotI*- and *KpnI*-digested and inserted into the *KpnI*-*NotI*-digested pYES2-GFP plasmid (Blaudez et al., 2003). Fluorescence emission from yeast cells expressing *OmZnT1::GFP* or *OmFET::GFP* was observed with an epifluorescence microscope (objective 100 $\times$ ) and a Nikon D1 digital camera was used to document results. To reveal the nuclear position within cells, a 10  $\mu$ g/ml DAPI (4',6-diamidino-2-phenylindole) solution was added to yeast cells just prior to observations. Yeast vacuolar membranes were stained with FM4-64 (Molecular Probes, Heyden, The Netherlands) as previously described (Blaudez et al., 2003). For both FM4-64 and GFP-deriving fluorescence, a filter was used to select the wavelength of the excitation light to the 475–500 nm band, and the fluorescence emission was recorded above 520 nm. In the case of DAPI, a UV filter was used (excitation band: 300–400 nm, emission band >420 nm).

## 3. Results

### 3.1. Identification of two metal transporters belonging to the CDF and the iron permease families by yeast functional screening

*ZRC1* encodes a transporter that sequesters zinc into the vacuole, and its deletion makes the yeast  $\Delta zrc1$  mutant highly sensitive to zinc (Li and Kaplan, 1998). This mutant was used for the functional screening of the *O. maius* cDNA library. Approximately 1% of the  $\Delta zrc1$  transformants were able to grow at 17.5 mM ZnCl<sub>2</sub>, but after the final screening step (removal of false positives) only ten yeast clones were obtained. Amplification and sequencing of the inserted genes revealed that five tolerant clones harbored sequences coding for metal transporters.

In one out of the five tolerant transformants, the *in silico* translation of the full-length cDNA (1833 bp) identified an open reading frame of 1626 bp encoding a 542 amino acid polypeptide with a predicted molecular weight of 58.7 kDa. Comparisons with protein sequence databases from NCBI showed that it was related to members of the CDF family, and a close relative of putative CDF proteins of *Sclerotinia sclerotiorum* (gi|156054901) and *Botryotinia fuckeliana* (gi|150847745) (61% and 60% of protein identity, respectively). The analysis also showed 55% and 50% identity with the previously characterized Zrc1p and Cot1p from *S. cerevisiae*, respectively (Li and Kaplan, 1998 and Lyons et al., 2000), and 55% identity with ZHF1 from the fission yeast *S. pombe*. The clone was therefore named *OmZnT1* (accession number JX243000). The hydropathy profile generated with the topology prediction programme TMHMM predicted six transmembrane domains in the deduced amino acid sequence ( Fig. 1A). This feature is typical of the majority of the previously described CDFs ( Montanini et al., 2007). A histidine-rich motif (HX)<sub>8</sub> was also found in the cytoplasmic loop located between helices IV and V. As indicated in the neighbor-joining tree in Fig. 2, *OmZnT1* belongs to the CDF sub-family I. This sub-family groups CDF proteins transporting zinc as the major substrate ( Montanini et al., 2007). Zrc1p, Cot1p, and ZHF1, but not Msc2p, also grouped in the same cluster ( Fig. 2). Contrary to members of the Msc2-like cluster, members of the Zrc1-like cluster are usually involved in Zn tolerance in eukaryotes ( Montanini et al., 2007).



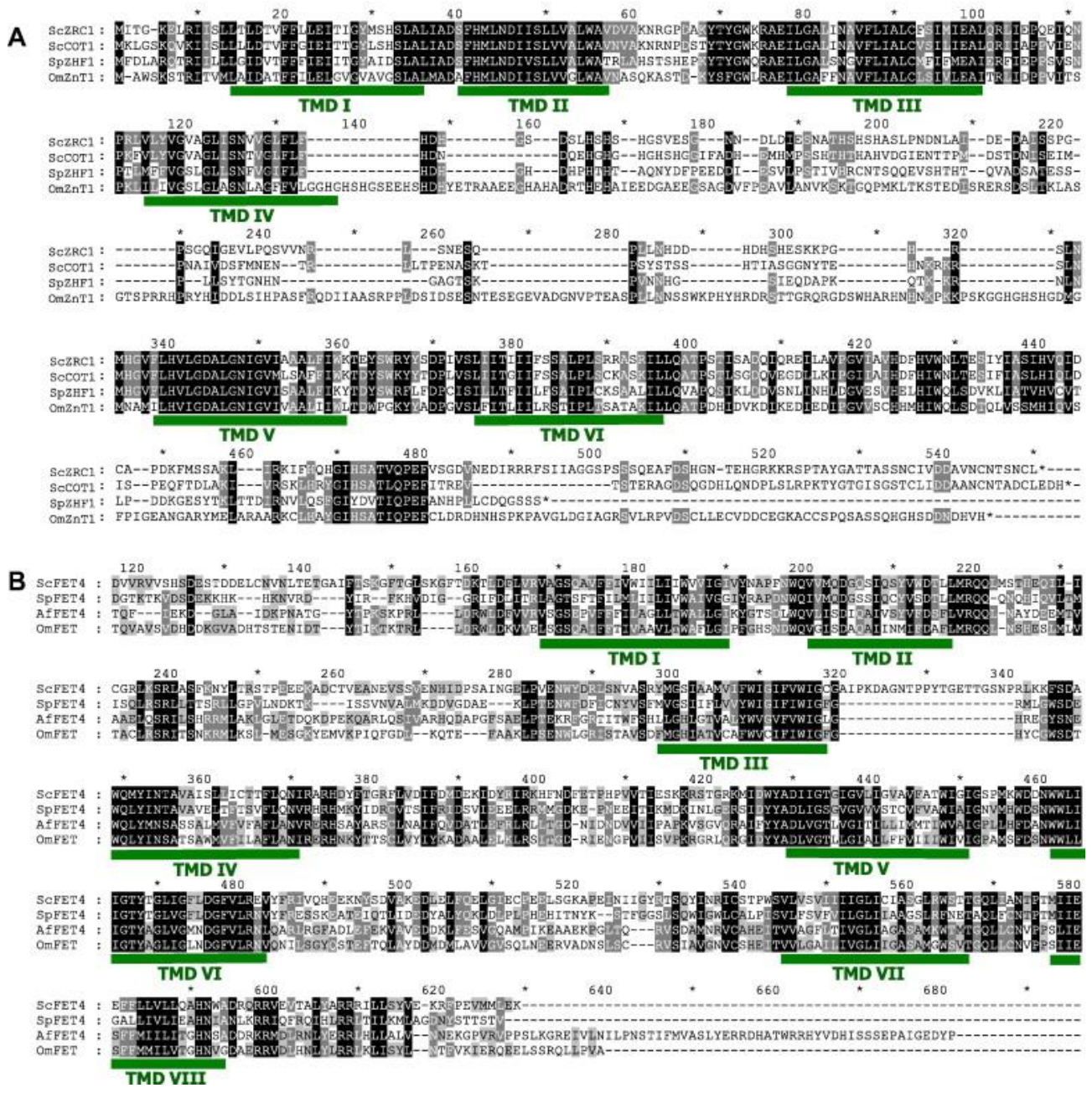


Fig. 1. Protein sequence comparison of OmZnT1 (A) and OmFET (B) with ascomycetous orthologs. Amino acid residues that are conserved in all four sequences, or in at least two of them, are shown on a black or gray background, respectively. The positions of predicted transmembrane domains (TMDs) are underlined in green. The reference proteins and their corresponding accession numbers used were: SpZHF1 (gi|2330778), ScZRC1 = Zrc1p (gi|736309), ScCOT1 = Cot1p (gi|1420694), ScFET4 = Fet4p (gi|984692), SpFET4 (gi|74624693), and AfFET4 = FETD (gi|70993042).



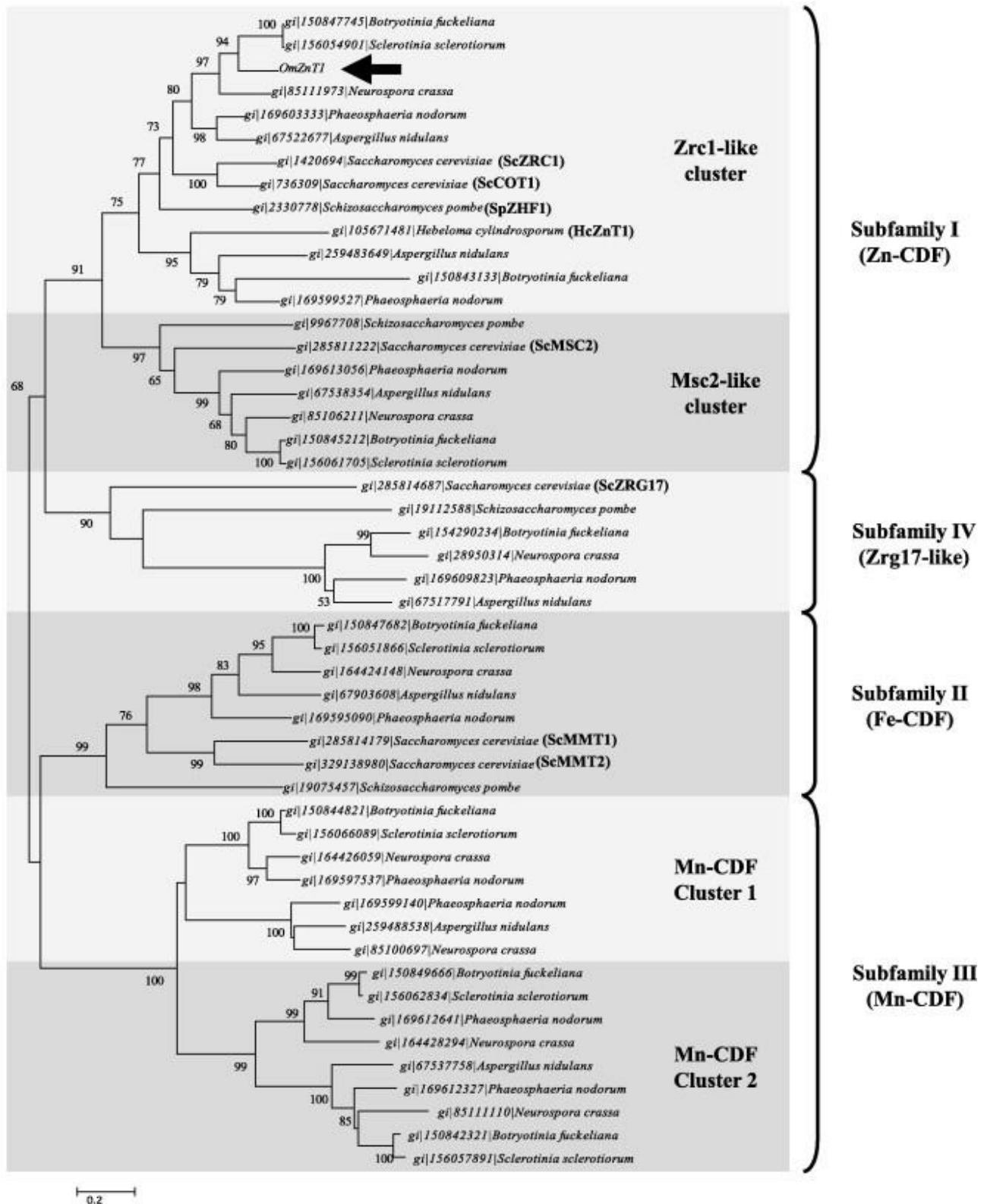


Fig. 2.

An unrooted, neighbor-joining (NJ)-based tree of the Cation Diffusion Facilitator (CDF) family from selected fungi. The arrow highlights the position of OmZnT1 in the tree. Full-length amino acid sequences were obtained from the Broad Institute website (<http://www.broadinstitute.org/>). The tree was generated using MEGA version 5.05 after sequence alignment. Bootstrap values >50% (1000 replicates) are indicated. Branch lengths are proportional to phylogenetic distances.

The other four zinc tolerant yeast clones selected from the cDNA library screening all harbored an identical sequence, consisting of a 1622 bp cDNA fragment. *In silico* translation of this sequence revealed an ORF of 1512 bp and a corresponding polypeptide of 504 aminoacids, with a predicted molecular weight of 56.3 kDa. BLAST searches against protein databases showed that the deduced protein is related to members of the iron permease family. The best identified hit corresponded to the low affinity Fe<sup>2+</sup> transporter FET4 of *Aspergillus* (53% of similarity) and subsequent hits also corresponded to putative or characterized FET4 orthologs from ascomycetes. The clone was therefore named *OmFET* (accession number JX242999), and the neighbor-joining tree ( Fig. 3) confirmed that *OmFET* cluster with FET4 proteins from filamentous fungi. The hydropathy profile generated with TMHMM predicted eight transmembrane domains ( Fig. 1B).

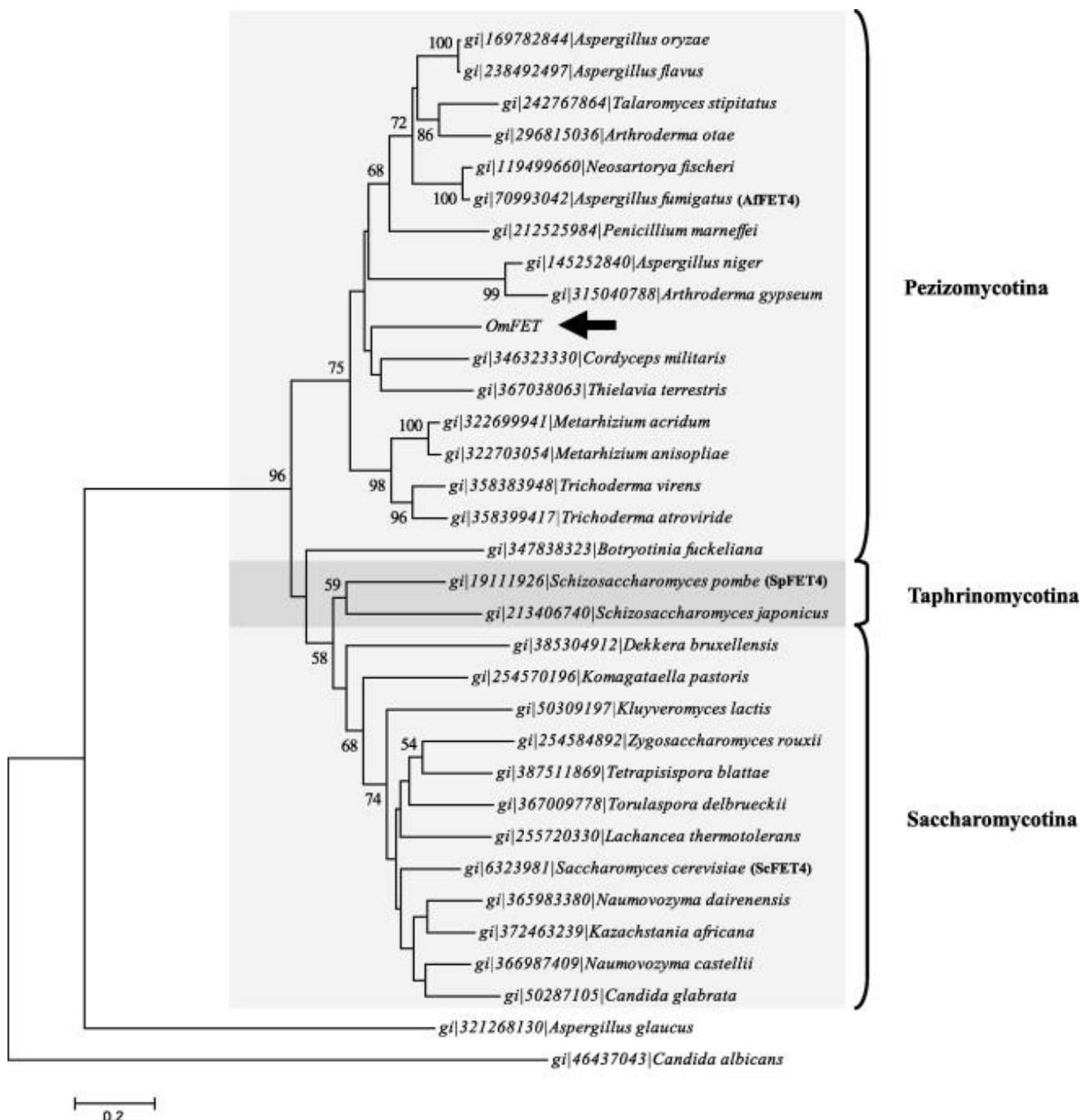


Fig. 3.

An unrooted, neighbor-joining (NJ)-based tree of the iron permease family in different eukaryotes. The arrow highlights the position of OmFET in the tree. Full-length amino acid sequences were obtained from the NCBI server (<http://www.ncbi.nlm.nih.gov/>). The tree was generated using MEGA version 5.05 after sequence alignment. Bootstrap values >50% (1000 replicates) are indicated. Branch lengths are proportional to phylogenetic distances. Zip members from *Candida albicans* (gi46437043) and *Aspergillus glaucus* (gi321268130) were used as out-groups.

### 3.2. Metal tolerance of *OmZnT1* and *OmFET* expressing yeast mutants

*OmZnT1* and *OmFET* were isolated on high zinc-containing medium by functional complementation of the zinc hypersensitive  $\Delta zrc1$  mutant, suggesting that they are both involved in zinc tolerance. To further investigate the role of *OmZnT1* and *OmFET* in metal tolerance, cDNAs were expressed in *S. cerevisiae* mutants unable to grow at high concentrations of various metals, and growth was monitored on control and metal-supplemented media. The mutants used are listed in Table 1.

As expected, the zinc-sensitive phenotype of the  $\Delta zrc1$  mutant was totally complemented when overexpressing *OmZnT1* or *OmFET* ( Fig. 4A). Similar results were found when expressing *OmZnT1* or *OmFET* in the zinc-sensitive  $\Delta cot1$  mutant (data not shown). Both *OmZnT1* and *OmFET*-expressing  $\Delta zrc1$  strains were as tolerant as the wild-type strain at 20 mM Zn ( Fig. 4A) and grew even better at higher concentrations (data not shown). Growth of the  $\Delta ccc1$  mutant at 5.25 mM Fe was partially restored by *OmZnT1* or *OmFET* ( Fig. 4B), but the difference with the strain transformed with the empty vector was not significant. Interestingly, the cobalt sensitive phenotype of the  $\Delta cot1$  mutant was partially complemented by *OmZnT1*, but not by *OmFET* ( Fig. 4C). Expression of *OmZnT1* and *OmFET* did not restore cell growth of the  $\Delta yap1$  mutant on 50  $\mu$ M Cd ( Fig. 4D), and the same was true for the  $\Delta pmr1$  mutant on 2 mM Mn ( Fig. 4E). The results obtained with the five yeast mutants thus confirmed that *OmZnT1* and *OmFET* confer tolerance to zinc, and indicate that *OmZnT1* also confer tolerance to cobalt. No significantly improved tolerance could be found towards iron, manganese or cadmium.

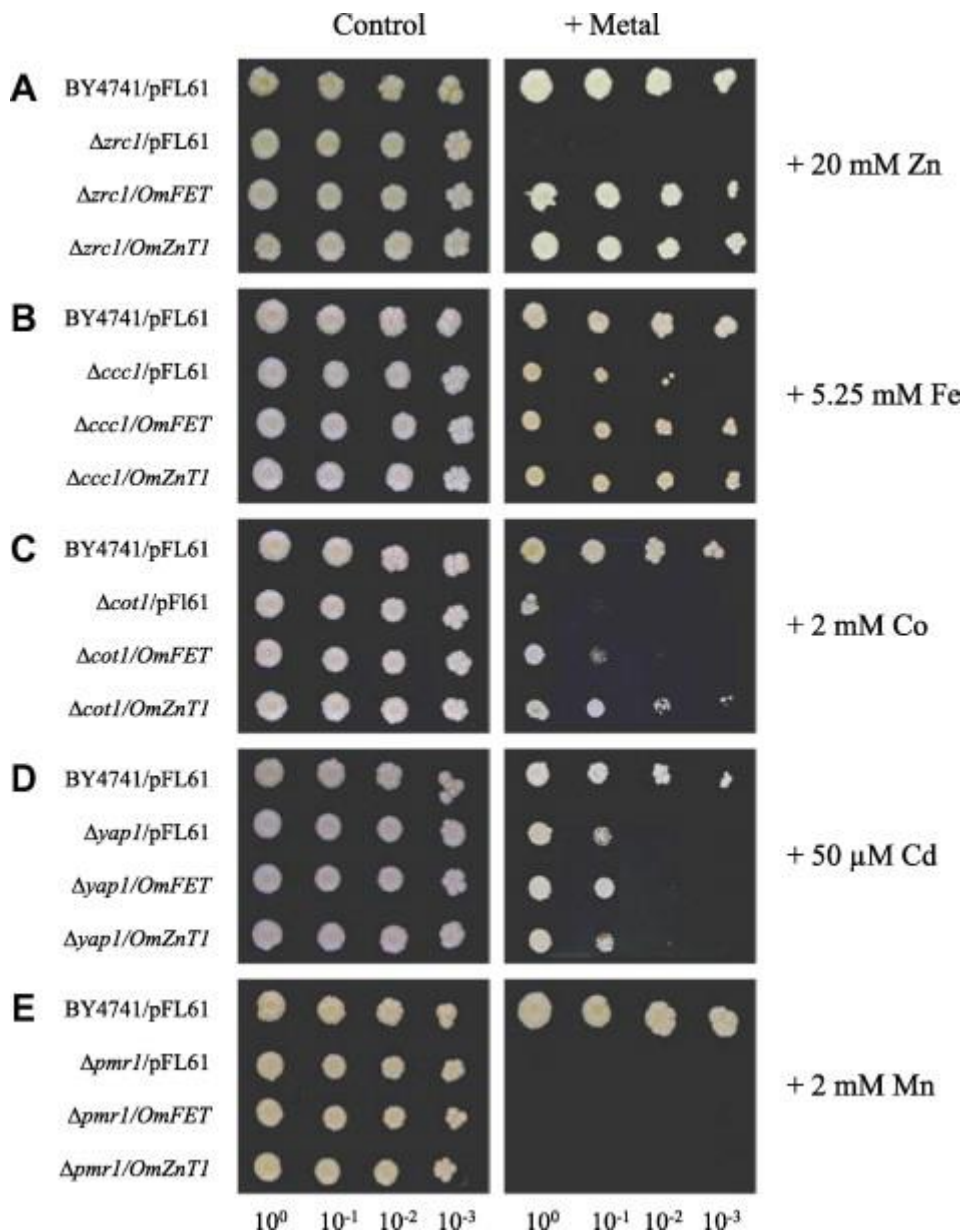


Fig. 4.

Functional complementation of metal-sensitive yeast strains by *OmFET* and *OmZnT1*. Cultures of WT (BY4741) and mutant strains were tenfold serial diluted and spotted on either control or metal-supplemented SD-ura medium. The WT strain (BY4741) was transformed with the empty vector pFL61. The mutant strains were transformed either with the empty vector pFL61, pFL61-*OmFET*, or pFL61-*OmZnT1*. Pictures were taken after 3 days of growth and are representative of two separate experiments.

### 3.3. *OmFET* is targeted to the plasma membrane, whereas *OmZnT1* is localized on the endoplasmic reticulum membrane

The *OmFET::GFP* and *OmZnT1::GFP* fusion proteins were constructed to determine the sub-cellular localization of *OmFET* and *OmZnT1* in yeast. At the standard image acquisition settings used for GFP visualization, cells transformed with the untagged genes did not show autofluorescence (data not shown), so all detectable fluorescence in the transformants was GFP-specific. Fig. 5B shows that exponentially growing *OmFET::GFP* transformed yeast displayed a fluorescent ring surrounding the cells, reflecting a localization on the plasma membrane ( Fig. 5A). The location of the vacuolar membranes as revealed by

FM4-64 staining unambiguously confirmed the plasma membrane localization of OmFET::GFP. By contrast, GFP fluorescence of *OmZnT1::GFP* transformed cells showed a pattern that suggests a localization at the endoplasmic reticulum membrane ( Fig. 5D) and rule out a localization on the vacuolar membrane as evidenced by FM4-64 counterstaining. The fluorescence was noticeably detected around the nuclei, as shown by DAPI counterstaining ( Fig. 5F), and resembled the pattern reported by Blaudez and Chalot (2011) for a fungal ER-localized CDF transporter expressed in yeast. *OmZnT1::GFP* fusion protein was still functional since it complemented the Zn-sensitive growth phenotype of the  $\Delta zrc1$  strain (data not shown).

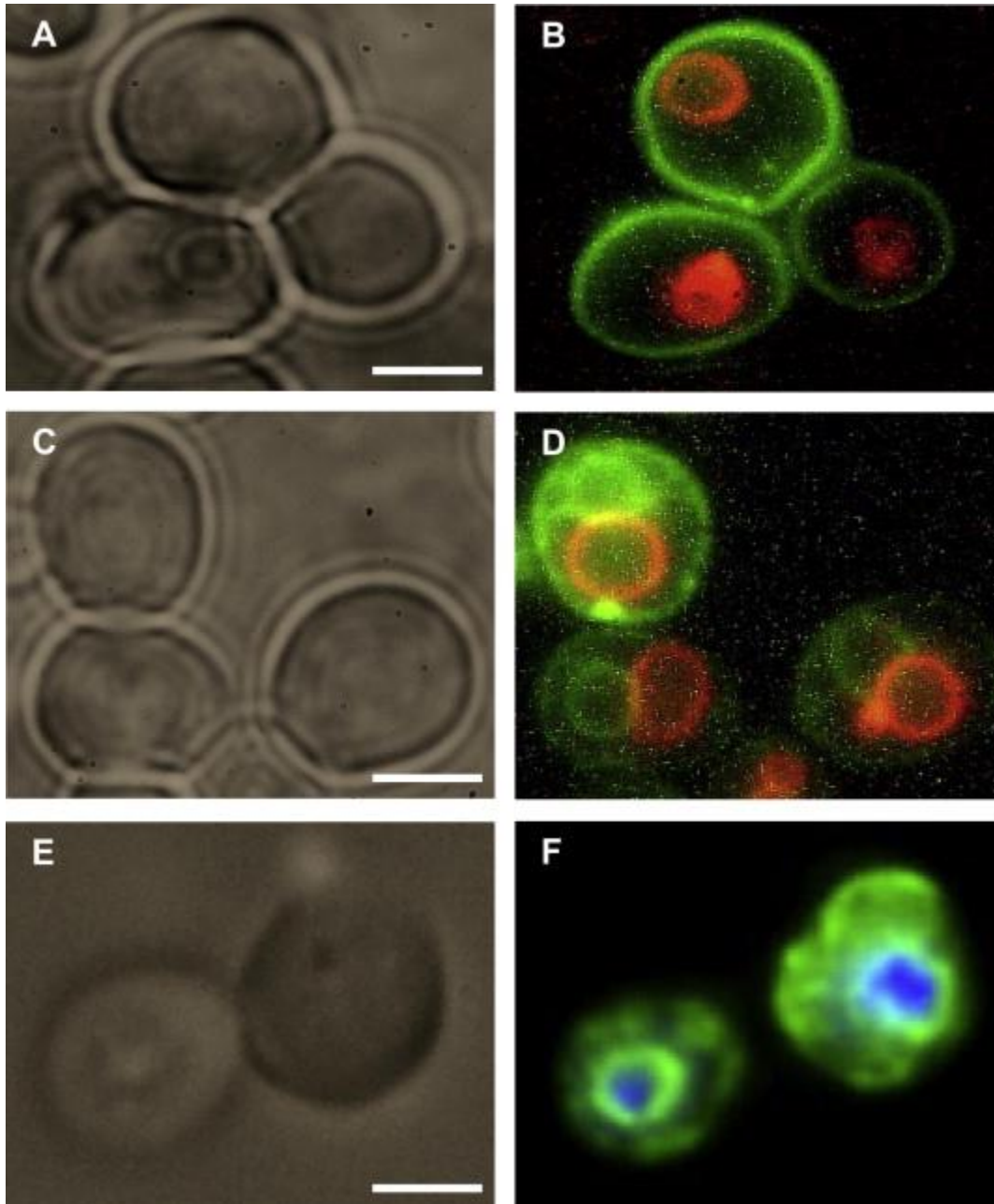


Fig. 5.

Sub-cellular localization of OmFET::GFP and OmZnT1::GFP fusion proteins in yeast by epifluorescence microscopy. Cells were grown in SD-ura medium. Different images from the same cells are shown: bright field (A, C, and E), and GFP fluorescence (B, D, and F). Cells were incubated with FM4-64 (B and D) or DAPI (F). Bars = 5  $\mu$ m.

### 3.4. OmFET rescued growth of different metal uptake-deficient yeast strains

Neighbor-joining analysis (Fig. 3) and GFP localization (Fig. 5) indicate that OmFET is a plasma membrane protein homologous to other fungal low affinity iron permeases (FET4 family). The *S. cerevisiae* FET4 transporter is primarily involved in iron uptake, but it also transports other metals including zinc, manganese and cadmium. In order to investigate the ability of OmFET to take up metals into the cytosol, we expressed *OmFET* in several metal uptake-defective yeast mutants, listed in Table I. Growth was monitored on control medium as well as on metal-limiting conditions ( Fig. 6). The double mutants  $\Delta fet3/\Delta fet4$ ,  $\Delta ctr1/\Delta ctr3$ , and  $\Delta zrt1/\Delta zrt2$  are not able to grow on Fe, Cu, and Zn-deficient conditions, respectively ( Fig. 6). In these functional complementation experiments, *AtIRT1* was used as a positive control as it encodes the main high-affinity iron transporter in *Arabidopsis* roots ( Vert et al., 2002), and is reported to also transport zinc, cobalt, manganese, and cadmium when expressed in yeast ( Rogers et al., 2000 and Connolly et al., 2002).

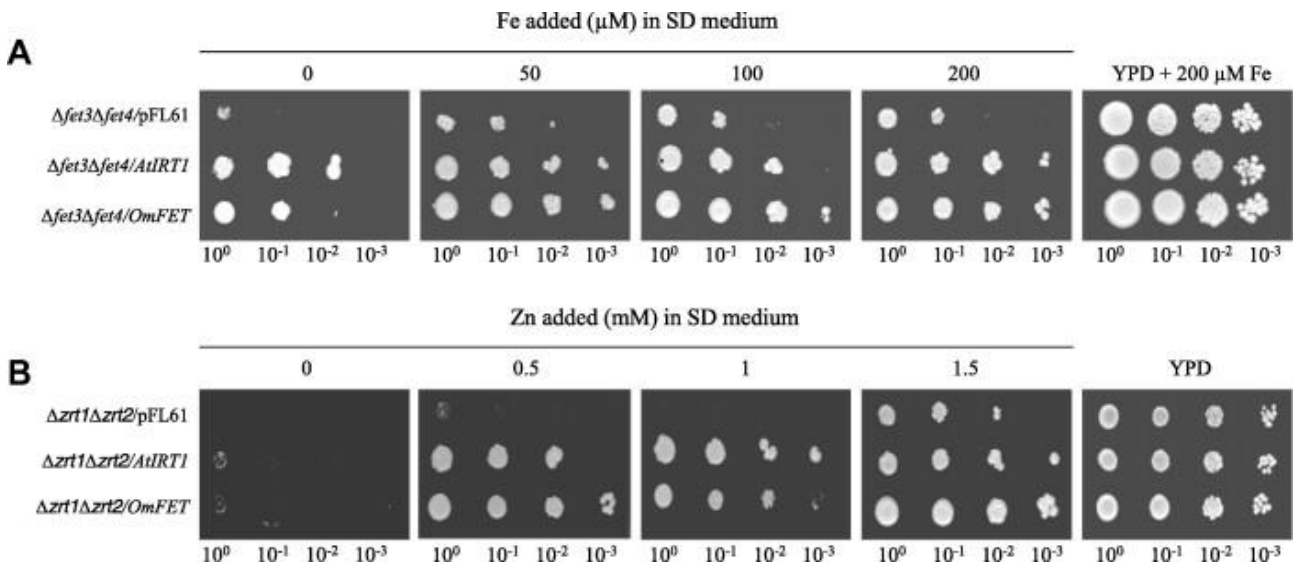


Fig. 6.

Functional complementation of metal uptake-deficient yeast strains by *OmFET*. Cultures of strains were tenfold serial diluted and spotted on YPD medium, or on either control or metal-limiting SD-ura medium. The SD-medium is naturally a Fe-limiting medium since the Fe uptake-deficient  $\Delta fet3/\Delta fet4$  strain could not grow. Low Zn medium was obtained by the addition of 1 mM EDTA in SD medium. The mutant strains were transformed either with the empty vector pFL61, pFL61-*AtIRT1*, or pFL61-*OmFET*. *AtIRT1* was used as a positive control. Pictures were taken after 3 days of growth and are representative of two separate experiments.

As expected, *OmFET* could complement the Fe-uptake defective  $\Delta fet3/\Delta fet4$  strain at the same level as *AtIRT1* ( Fig. 6A). Overexpression of *OmFET* also restored the ability of the  $\Delta zrt1/\Delta zrt2$  strain to grow on a Zn-deficient medium (by the addition of EDTA) at the same level as *AtIRT1* transformed cells ( Fig. 6B). Finally, *OmFET* did not restore cell growth of  $\Delta ctr1/\Delta ctr3$ , a Cu-uptake deficient strain ( Peña et al., 1998) under copper limiting conditions (data not shown). Altogether, these data suggest that OmFET is an uptake system that could transport iron, zinc, but not copper.

### 3.5. Metal content of $\Delta zrc1$ yeast mutant expressing the *O. maius* transporters

To better understand the mechanisms of zinc tolerance conferred by OmZnT1 and OmFET when expressed in the  $\Delta zrc1$  yeast mutant, metal content was measured in yeast cells exposed to various conditions. When exposed to 500  $\mu\text{M}$  zinc (as  $\text{ZnCl}_2$ ),  $\Delta zrc1$  yeast cells transformed with pFL61-*OmZnT1* showed the same zinc content as control cells transformed with the empty vector ( Fig. 7B), a result consistent with a role of this CDF transporter in intracellular sequestration.

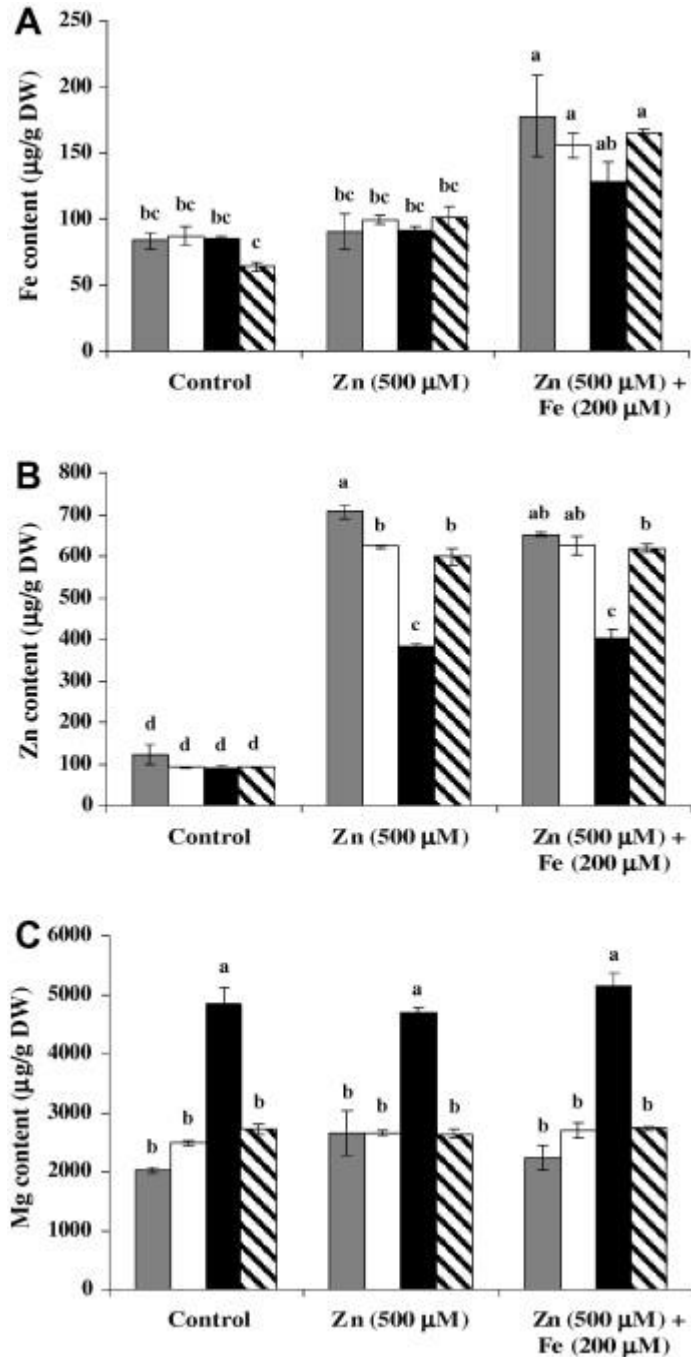


Fig. 7.

Iron (A), zinc (B), and magnesium (C) contents in the wild-type yeast transformed with the empty vector pFL61 (shaded bars) and in the  $\Delta zrc1$  yeast strain transformed either with the empty vector pFL61 (open bars), pFL61-*OmFET* (closed bars), or pFL61-*OmZnT1* (hatched bars). Growth was on control SD-ura medium, and on the same



medium supplemented with 500  $\mu\text{M}$   $\text{ZnCl}_2$  with or without 200  $\mu\text{M}$   $\text{FeCl}_3$ . Data are the means ( $\pm\text{SE}$ ) of three independent clones, and statistically significant differences ( $P = 0.05$ , ANOVA followed by Tukey's test) are indicated by different letters above the bars.

The mechanism of the increased tolerance conferred by *OmFET* to the  $\Delta\text{zrc1}$  yeast mutant is less clear, given the localization of this protein on the plasma membrane ( Fig. 5) and its role in zinc and iron uptake ( Fig. 6). Fig. 7B shows that yeast transformed with pFL61-*OmFET* accumulated significantly less zinc than cells transformed with the pFL61 empty vector when exposed to  $\text{ZnCl}_2$  in SD medium. This likely explains the positive selection of these transformants during the library screening. As the phenotyping experiments showed that *OmFET* confers resistance to zinc and could transport iron into the cytoplasm, we speculated that the lower zinc content could derive from the ability of *OmFET* to increase cytosolic iron concentrations under zinc stress, thus counteracting zinc toxicity. However, no significant differences were observed in the iron content of yeast cells harboring the *OmFET*-expressing vector as compared to the control cells (Fig. 7A). An increase in the iron concentration in the medium did not lead to an increase in iron cell content in *OmFET* expressing yeast ( Fig. 7A), neither to a decrease in the cell zinc content ( Fig. 7B). Thus, zinc tolerance conferred by *OmFET* is not apparently due to increased iron uptake. Interestingly, *OmFET*-expressing yeast cells showed a significant accumulation of magnesium when compared to control cells transformed with the empty vector, irrespectively of the presence of Zn in the medium ( Fig. 7C). This result was specific of *OmFET*, and was not observed in the *OmZnT1* transformed yeast ( Fig. 7C). A significant increase in magnesium content was observed in *OmFET*-expressing yeast under different concentrations of magnesium, iron and zinc (data not shown).

#### 4. Discussion

Zinc is an essential metal fundamental for the structure and/or function of many prokaryotic and eukaryotic proteins, but, although oxidatively non-reactive, its excess is deleterious to cells. All organisms have therefore developed homeostatic mechanisms aimed to ensure the minimal zinc levels required for normal metabolism but also to avoid zinc intoxication (Pagani et al., 2007). *O. maius* Zn is a metal tolerant ericoid mycorrhizal isolate derived from roots of *V. myrtillus* growing in a heavy metal polluted site where zinc was the most abundant contaminant ( Martino et al., 2000). This strain was found to be highly zinc-tolerant ( Martino et al., 2000, Martino et al., 2002 and Martino et al., 2003), but the cellular and molecular bases of zinc tolerance have not been fully understood. Functional complementation of a Zn-sensitive yeast mutant with an *O. maius* cDNA library has allowed us to identify *OmZnT1* and *OmFET*, two genes encoding transporters that confer zinc tolerance to the *Zrc1* yeast mutant.

*OmZnT1* encodes a protein which has been assigned to the CDF family of zinc transporters based on its sequence and general structural features. The CDF proteins described to date confer tolerance to zinc, cadmium, cobalt, manganese and nickel, and many are located on internal membranes ( Paulsen and Saier, 1997). Most CDF family members are predicted to have six transmembrane domains and two cytoplasmic loop regions ( Gaither and Eide, 2001). The same characteristics were found in *OmZnT1*, together with a close sequence similarity with other characterized Zn-CDF members from ascomycetes. Expression of the *OmZnT1* protein restored the growth of several metal-sensitive yeast mutants. In particular, *OmZnT1* was able to restore the growth of Zn- and Co-sensitive mutants lacking vacuolar transporters. Localization of the GFP-tagged *OmZnT1* protein on the ER membrane suggests that it could mediate zinc delivery into this compartment. Zn-specific CDF members have a range of different sub-cellular localizations, including most intracellular membranes and the plasma membrane, and many have been found to locate on the ER membranes. The ER-resident CDF protein ZHF1 from the yeast *S. pombe* has been demonstrated to be the

major determinant of zinc tolerance in this organism (Clemens et al., 2002). Contrary to *S. cerevisiae*, where vacuoles ensure zinc storage and detoxification, zinc is mainly stored in the ER in *S. pombe*, suggesting different storage compartments involved in zinc homeostasis/tolerance in the two yeast model systems.

Another Zn-CDF transporter localized on the ER membrane is HcZnT1, identified in the basidiomycete *H. cylindrosporum* (Blaudez and Chalot, 2011). When expressed in various metal sensitive yeast mutants, HcZnT1 was able to specifically confer zinc tolerance (Blaudez and Chalot, 2011). These two ER-resident CDF proteins (SpZHF1 “gi|2330778” and HcZnT1 “gi|105671481”) cluster together with OmZnT1 within the same cluster of the Zn-CDF subfamily, but also with tonoplast-resident CDF members (Zrc1p, Cot1p) (Fig. 2). In *S. cerevisiae*, two other CDF family members, Msc2p (gi|285811222) and Zrg17p (gi|285814687), form a heteromeric zinc transport complex in the ER membranes, and they have been implicated in the homeostatic maintenance of ER function (Ellis et al., 2004 and Gaither and Eide, 2001). However, Msc2p and Zrg17p are not orthologs of OmZnT1 (Fig. 2).

OmZnT1 is the first transporter involved in zinc tolerance described in a filamentous ascomycete. Our results would indicate a role of OmZnT1 in zinc transport into the ER and a contribution in zinc detoxification, as observed in *S. pombe* (Clemens et al., 2002). However, whereas ZHF1 is the only Zn-CDF gene from the Zrc1-like cluster found in the genome of *S. pombe*, the recent release of *O. maius* sequenced genome (<http://genome.jgi.doe.gov/>) revealed the presence of two other putative Zn-CDF transporters in this organism. Further studies will be necessary to characterize these proteins.

The second clone isolated from the *O. maius* library screening encodes the OmFET protein. The BLAST search and phylogenetic analysis indicate that OmFET is a low affinity iron transporter. Close orthologs of OmFET comprise the FET4 proteins from *S. cerevisiae* (gi|984692) and *S. pombe* (gi|74624693), and FETD from *A. fumigatus*(gi|70993042). Fungi take up iron by a variety of high and low affinity mechanisms that have been studied mainly in yeast (Kosman, 2003 and Kwok and Kosman, 2006). Extracellular ferric iron is reduced to ferrous iron and subsequently used as substrate by the high affinity transport system composed of the FET3 multicopper oxidase and the FTR1 permease (Waters and Eide, 2002). In addition to this high affinity system, yeast presents a low affinity ferrous iron uptake system encoded by the FET4 gene (Dix et al., 1994 and Dix et al., 1997). This gene has also been found in filamentous fungi, but despite the increasing number of sequenced genomes is revealing new orthologs, this transporter has been thoroughly characterized only in *S. cerevisiae* (Kosman, 2003).

The *S. cerevisiae* Fet4p protein was originally identified as being a low-affinity ferrous iron transporter (Dix et al., 1994), but this protein has a relatively low substrate specificity and it can transport other metals such as zinc (Waters and Eide, 2002), copper (Hassett et al., 2000), cobalt, manganese (Li and Kaplan, 1998) and stannous (Viau et al., 2012). Similarly, Dainty et al. (2008) reported that the FET4 protein encoded by the ortholog of *S. pombe* can also transport zinc. FET4 plays therefore a central role in the uptake of a number of metal ions.

Similarly to the Fet4p characterized in yeast (Waters and Eide, 2002), OmFET has eight predicted membrane spanning regions and is localized on the plasma membrane. Successful complementation of the iron uptake deficient yeast mutant  $\Delta fet3/\Delta fet4$  with OmFET indicates that OmFET is a transporter delivering ferrous iron to the cytoplasm. In addition, it could also take up zinc (as shown by OmFET expression in the  $\Delta zrt1/\Delta zrt2$  mutant), but not copper.

Our experiments indicate that OmFET is clearly involved in the uptake of some essential elements under metal depleted conditions. However, its role in zinc tolerance under excess of this metal, and the

mechanisms involved, are not clear. Several observations suggest that the uptake and homeostasis of iron and zinc, both essential elements but toxic at high cellular concentrations, are intertwined. Pagani et al. (2007) reported the disruption of Fe homeostasis by high Zn. For instance, it was shown that Zn stress could induce the expression of several components of iron uptake (Fet3, Ftr1). However the results of our analyses of the metal contents in cells overexpressing OmFET are intriguing.

The standard SD medium used for the *O. maius* library screening and for most of the experiments is not iron depleted ( $\approx 1.23 \mu\text{M FeCl}_3$ ). Under these conditions, the addition of  $500 \mu\text{M ZnCl}_2$  resulted in a cellular content of zinc significantly lower in yeast transformants expressing *OmFET* than in cells harboring the empty vector. This phenotype would explain the positive selection of *OmFET* in the screening of the *O. maius* cDNA library.

Given the interplay between iron and zinc metabolism (Yasmin et al., 2009), we hypothesized that zinc tolerance could derive from the ability of OmFET to increase the accumulation of iron under zinc excess, consequently counteracting zinc toxicity. However, no significant iron accumulation was recorded in the *OmFET* expressing yeast, as compared to control cells, even when iron in the medium was increased up to a concentration of 1.25 mM. Thus, the mechanism of zinc tolerance conferred by OmFET does not involve an increase in iron uptake.

By contrast, magnesium content was found to be always significantly higher in *OmFET* expressing cells than in control cells. The effect of intracellular and extracellular magnesium concentrations on heavy metal toxicity has been described in several studies, although the mechanisms have not been elucidated. A general protective effect of magnesium against heavy-metal cations such as manganese, copper, nickel, cadmium and cobalt in yeast (Blackwell et al., 1998, Karamushka and Gadd, 1994, Joho et al., 1991, Kessels et al., 1985 and Norris and Kelly, 1979), and zinc and cadmium in plants (Pedler et al., 2004 and Kupper and Kochian, 2010) has been reported. In particular, the uptake of divalent cations in yeast was reduced by the addition of extracellular magnesium (Pisat et al., 2009, Blackwell et al., 1998 and Bianchi et al., 1981).

Based on our results, we suggest that OmFET could enhance zinc tolerance in yeast by increasing the cellular content of magnesium. Interestingly, preliminary results showed also an interplay between zinc and magnesium in *O. maius* strain Zn (data not shown). The increase of magnesium content in yeast may reflect competition/inhibition with zinc uptake, and could protect cells by interfering with the mechanisms of zinc toxicity, as observed in plants (Pedler et al., 2004). Further experiments, for example with yeast mutants for iron and magnesium uptake, and with *O. maius* mutants, will help to elucidate this suggested role.

*O. maius* strain Zn was isolated in the Niepolomice forest (Poland) from the roots of *V. myrtillus* plants growing on heavy metal contaminated plots (Turnau, 1988). The colonization of these plots by ericoid mycorrhizal plants suggests that *O. maius* can confer to the host plant the ability to survive in metal polluted environments. The understanding of zinc homeostasis and tolerance in *O. maius* Zn could help to explain the role played by this fungus in the tolerant behavior of the host plant.

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