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Characterization of the ER-located zinc transporter ZnT1 and identification of a vesicular zinc storage compartment in *Hebeloma cylindrosporum*

Damien Blaudez, Michel Chalot

UMR INRA/UHP 1136 "Tree-microbe Interactions", Faculty of Sciences and Technology, Nancy-University, BP 70239, F-54506 Vandoeuvre-les-Nancy, France

Abstract

Metal tolerance of filamentous fungi is a poorly understood mechanism. In order to unravel the molecular basis of zinc (Zn) tolerance in the ectomycorrhizal fungal model *Hebeloma cylindrosporum*, we carried out a functional screening of an *H. cylindrosporum* cDNA library in the *zrc1D* mutant strain of *Saccharomyces cerevisiae* to search for genes conferring Zn tolerance to yeast cells. This strategy allowed the isolation of HcZnT1, a gene belonging to the cation diffusion facilitator family, which induced tolerance to Zn, but not to other metals. HcZnT1 was constitutively expressed in *Hebeloma* cells, whatever the Zn status of the medium and the fungal cell type (mycelia, sporocarps, mycorrhizas). A HcZnT1:GFP fusion protein was expressed in yeast and the corresponding fluorescence was recorded on endoplasmic reticulum membranes. Taken together, these different findings suggest a dual role of HcZnT1 in Zn homeostasis of fungal cells, by supplying requested Zn ions for the functioning of the endoplasmic reticulum as well as by detoxifying the cytosol under Zn stress. Zn pools were also investigated by using the Zn-specific fluorophore zinquin in *H. cylindrosporum* cells. Zinquin labeling revealed compartmentalization in intracellular vesicles interspersed throughout the cytoplasm that do not correspond to vacuolar compartments. Altogether the present data represent the first steps into the understanding of Zn homeostasis and tolerance in *Hebeloma*.

1. Introduction

Several studies have focused on benefits of symbioses for the plant hosts growing on trace elements (TE) contaminated sites, with the aim of possible biotechnological applications such as phytoremediation (Gadd, 2010; Kozdroj et al., 2007; Krupa and Kozdroj, 2004, 2007). It is now widely accepted that rhizospheric microorganisms may actively participate to phytoremediation processes, and may be useful in extending the application of phytoremediation to additional trace element contaminated sites (Kuffner et al., 2008). Hosting different mycorrhizal types might be of functional importance for plant nutrition (Smith and Read, 2008) and has been shown to contribute to metal tolerance of host plants as fungi can reduce the metal uptake by the plant by sequestration, extracellular precipitation and biosorption to the cell walls (Bellion et al., 2006; Blaudez et al., 2000a; Hildebrandt et al., 2007). Mechanisms have been detailed in different mycorrhizal fungi, including induction of metallothionein (Bellion et al., 2007; Ramesh et al., 2009), superoxide dismutase (Jacob et al., 2001; Vallino et al., 2009) or thiol compounds (Courbot et al., 2004). Using radiotracer flux analyses, the significant accumulation of Cd found in the vacuolar compartment has been suggested as an essential Cd detoxification mechanism in the ectomycorrhizal fungus *Paxillus involutus* (Blaudez et al., 2000b). Indeed, a number of recent data support the hypothesis that mycorrhizal fungi play a filtering/sequestering role on plant roots (Arriagada et al., 2010; Bissonnette et al., 2009; Carvalho et al., 2006; Christie et al., 2004; Cobbett and Goldsbrough, 2002; Colpaert et al., 2004; Lee et al., 2003; Mathur et al., 2007), enhancing root to shoot metal ratio and increasing survival rate in harsh conditions (Leung et al., 2007).

However, there are only few reports on transport mechanisms in mycorrhizal fungi, and even less information on TE transporters. Specific transporters, encoded by multigenic families, are responsible for the uptake and secretion of metal ions, and for their sequestration into organelles (Gaither and Eide, 2001; Hall and Williams, 2003; Kambe et al., 2004; Nies, 2003). Cation diffusion facilitator (CDF, TC 2.A.4) transporters, first identified in bacteria (Nies and Silver, 1995), are ubiquitous, spanning all three kingdoms of life: Archaea, Eubacteria and Eukaryotes (Montanini et al., 2007). The majority of CDF proteins possess six putative transmembrane domains (TMDs), with the N and C termini cytoplasmic, as experimentally demonstrated for bacterial members (Anton et al., 1999; Wei and Fu, 2005). A signature sequence between TMDs I and II was proposed in 1997 (Paulsen and Saier, 1997) and modified in 2007 (Montanini et al., 2007). A characteristic C-terminal cation efflux domain is present in all members (Maser et al., 2001). Most CDF transporters also contain a histidine-rich region, either between TMDs IV and V, or at the N and C termini. Such regions are predicted to be cytoplasmic, cis to metal uptake, and could function as potential metal (Zn^{2+} , Co^{2+} , and/or Cd^{2+}) binding domains. However the ER-localized Zrg17, a Zn^{2+} transporter recently characterized in yeast, displays a histidine-rich loop predicted to reside between TMDs III and IV, toward the ER lumen (Ellis et al., 2005). CDF transporters are postulated to be involved exclusively in transport of metals into intracellular vesicles or out of the cell across the plasma membrane (Palmiter and Huang, 2004). The *Saccharomyces cerevisiae* Zrc1 (MacDiarmid et al., 2000) and Cot1 (Conklin et al., 1992) are located on vacuolar membranes, whereas the *Schizosaccharomyces pombe* Zhf1 (Clemens et al., 2002) and *S. cerevisiae* Zrg17 (Ellis et al., 2005) are located on ER membranes.

CDF-like sequences in mycorrhizal fungi have been obtained in the course of expressed sequence tag projects (Stommel et al., 2001) and a gene coding for a putative CDF has been isolated and characterized from the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* (Gonzalez-Guerrero et al., 2005). Here we report the isolation and functional characterization of the HcZnT1 gene coding for a CDF Zn transporter in *Hebeloma cylindrosporum*, as well as the visualization of zinc pools by fluorescent tracer.

2. Materials and methods

2.1. Yeast strains, fungal materials and media

The *S. cerevisiae* strains used for heterologous expression of HcZnT1 were BY4741 (MATa his3D1 leu2D0 met15D0 ura3D0), *zrc1D* (MATa his3D1 leu2D0 met15D0 ura3D0 ZRC1::kanMX4), *cot1D* (MATa his3D1 leu2D0 met15D0 ura3D0 COT1::kanMX4), *ycf1D* (MATa his3D1 leu2D0 met15D0 ura3D0 YCF1::kanMX4) and *pmr1D* (MATa his3D1 leu2D0 met15D0 ura3D0 PMR1::kanMX4). The four mutants used derived from the parent strain BY4741 and they were all obtained from Euroscarf (<http://www.uni->

frankfurt.de/fb15/mikro/euroscarf/). Synthetic yeast medium without uracil (yeast nitrogen base (Sigma Aldrich, Lyon, France) 6,7 g l⁻¹, 2% glucose, drop out (Sigma) 1.92 g l⁻¹) was used. For metal tolerance assays, yeast was grown in liquid SD until OD_{600nm} = 1. These cultures were subsequently used to inoculate SD control plates (no extra metal added) or SD plates containing toxic concentrations of zinc, cadmium, manganese, or cobalt. Yeast cells expressing HcZnT1::GFP under the control of the Gal1-inducible promoter were grown in liquid SD medium containing 2% (w/v) galactose instead of glucose.

The ectomycorrhizal fungus *H. cylindrosporum* Romagnesi (haploid strain h1) was grown on cellophane-covered agar medium as previously described (Javelle et al., 2003). *H. cylindrosporum*-*Pinus pinaster* mycorrhizas and sporocarps were collected from plants, inoculated with the dikaryotic strain D2, growing in a greenhouse. Material was frozen in liquid nitrogen and stored at -80 °C until use.

2.2. Library screening

To identify ectomycorrhizal fungal cDNAs able to increase zinc tolerance, the zrc1D yeast strain was transformed with a *H. cylindrosporum* cDNA library kindly provided by H. Sentenac (UMR 5004 Agro-M-CNRS-INRA-UM2, Montpellier, France). The library was constructed in the pFL61 yeast expression vector under the control of the strong constitutive PGK promoter (Lambilliotte et al., 2004). Transformations were performed by the standard lithium acetate method (Gietz et al., 1992). Zn²⁺-tolerant Ura⁺ transformants were selected after plating the transformants on SD

medium supplemented with the appropriate amino acids and containing 20 mM ZnCl₂. Recombinant pFL61 plasmids that conferred zinc tolerance were isolated by the Zymoprep Yeast Plasmid Mini-prep kit (Proteogene, Saint Marcel, France) by following the manufacturer's recommendations. Plasmids were subsequently used in retransformation experiments of zrc1D to confirm the functional tolerance before sequencing. The amino acid sequence of HcZnT1 was deduced from the corresponding cDNA sequence and was used in BLAST searches to find the closest fungal orthologs in databases. Amino acid sequences of previously studied fungal orthologs of the same family were also retrieved from databases and were aligned and edited by the multiple sequence alignment editor and shading utility GeneDoc software (version 2.6.003) (<http://www.psc.edu/biomed/genedoc>).

2.3. Construction of GFP fusions for expression in yeast

To construct HcZnT1::GFP, the full-coding sequence of HcZnT1 was amplified from the plasmid pFL61-HcZnT1 with the primers HcZnT1f (5' -CCCCGGATCCATGAAGAATACAACCAAGATA-3') and HcZnT1r (5' -CCCCGAATTCAGCTGCATCGCCGACCTTTAT-3'), introducing a BamHI site and a EcoRI site at the 5' and 3' end, respectively (sites underlined), and cloned into the BamHI-EcoRI-digested pYES2-GFP plasmid (Blaudez et al., 2003). A similar procedure was used to produce the HcAMT1::GFP fusion. HcAMT1 was sub-cloned into the HindIII-BamHI-digested pYES2-GFP plasmid after PCR amplification and HindIII/BamHI digestion. The primers used for HcAMT1 amplification were HcAMT1f (5' -CCCAAGCTTATGGTCAACG TCACATACGAT-3') and HcAMT1r (5' -CCCCGGATCCCCTGCTGATTTCTC TTCTACGCT-3').

2.4. Fluorescence microscopy

Yeast vacuolar membranes were stained with FM4-64 (Molecular Probes, Heyden, The Netherlands) as previously described (Blaudez et al., 2003). Small aliquot (5 l) of the resulting cell suspension was mounted on glass slides and viewed with a fluorescence microscope. A Nikon Optiphot-2 fluorescence microscope equipped with a Nikon D1 camera was used to document results. 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. The staining of yeast nuclei was performed by incubating fungal cells with 1 µg/ml DAPI for 10 min and in this case a UV filter was used. For Zn staining, *H. cylindrosporum* was grown for 1 week on solid NP medium (Javelle et al., 2003) enriched with 200 µM ZnCl₂. Preliminary experiments revealed that this concentration was a sub-toxic condition for *H. cylindrosporum* growth. Mycelia were collected and incubated for 30 min in NP medium containing 18 µM zinquin (Biotium, Inc., Hayward, CA), a zinc-specific fluorescent marker (Mahadevan et al., 1996). Cells were briefly rinsed in NP medium to remove medium-derived fluorescence and cells were finally analysed by fluorescence microscopy by using a UV filter (excitation band: 300–400 nm, emission band >420 nm). The fluorescence pattern was identical in most cells, indicating a uniform staining of the mycelia by the probe. For staining the lumen of the fungal vacuoles, mycelia were incubated in NP medium for 20 min with 10 µM carboxy-DCFDA (Molecular Probes, Heyden, The Netherlands), rinsed and viewed with a fluorescent microscope and with the same filter as used for GFP observation.

2.5. HcZnT1 expression

Total RNAs from free-living fungus, mycorrhizas and sporocarps were extracted by using the Qiagen RNA plant extraction kit with addition of RNase-free DNase (Promega, Madison, WI) to avoid genomic DNA contamination. To study the effect of external Zn concentrations on HcZnT1 expression, mycelia were also exposed for 18 h to a range of Zn (0.3; 3; 30; 300 and 3000 µM ZnSO₄). Reverse transcription (RT) reactions were performed with 0.5 µg total RNA and with the enzyme Omniscript (Qiagen, Hilden, Germany) as recommended by the manufacturer. RT reactions were performed for 1 h at 37 °C. Each PCR reaction contained 0.15 U/l Taq polymerase (Biolabs), 1× buffer, 0.2 mM dNTP, 25 ng of RT products, and 200 nM of adequate primers. The PCR primers used were the following: HcZnT1RTf (5' -ATTGGCTTGGGATTGAATATCGTCA-3') and HcZnT1RTr (5' -TCACTTTCGCGAGGTCGAGATGTAT-3') for HcZnT1 amplification, HcGSf (5' -GGCACCCCGAACAGGACCAA-3') and HcGSr (5' -GTAGTAGGGTCTTGGGGAC-3') for HcGS amplification, and HcUbqf (5' -AGATCCAAGACAAAGAGGGCAT-3') and HcUbqr (5' -GG GATTCTTCTTATCTTGAA-3') for HcUbq amplification. HcGS (accession number: AF411820) and HcUbq (accession number: BU964130) encode the glutamine synthetase and

a ubiquitin from *H. cylindrosporum*, respectively and were used as reference genes. The products were amplified by PCR with a Mastercycle (Eppendorf, Le Pecq, France) under the following conditions: 94 °C for 1 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s and 72 °C for 30 s. PCR samples were taken at successive cycles and analysed by agarose gel electrophoresis for quantification, checking that amplification did not reach saturation. Final quantitative analyses were realized after 27 (HcGS and HcUbq) or 32 (HcZnT1) PCR cycles, respectively. Three independent experiments were conducted and gave similar results.

3. Results

3.1. Isolation of *H. cylindrosporum* ZnT1 cDNA

A *H. cylindrosporum* cDNA library was screened in yeast *zrc1D* for Zn^{2+} tolerance on agar medium containing 20 mM $ZnCl_2$. Deletion of the *Zrc1* gene, which encodes a transporter responsible to sequester Zn into the vacuole, increased the mutant sensitivity towards Zn (Kamizono et al., 1989; Li and Kaplan, 1998). Colonies were selected for their capability to grow vigorously on the Zn^{2+} -toxic medium. Plasmids were isolated and transformed into the *zrc1D* strain and re-screened on Zn^{2+} -toxic medium in order to confirm that expression of fungal cDNAs is responsible for the Zn^{2+} tolerance phenotype. Among 15 clones, restriction digestion and sequencing showed that all the cDNAs were identical and encoded a protein with features similar to the CDF (cation diffusion facilitator) transporter family. BLASTP and BLASTX searches in public databases of the *H. cylindrosporum* protein showed a strong similarity with putative CDF genes/proteins from the basidiomycete fungi *Laccaria bicolor*, *Coprinopsis cinerea*, *Lentinula edodes*, and *Schizophyllum commune*. However the function of these orthologs remains putative since none of the corresponding genes have yet been functionally characterized. Conversely, GintZnT1 (Accession No. CAE00445) from *G. intraradices*, SpZhf1 (Accession No. NP_593645) from *S. pombe*, ScZrc1 (Accession No. CAA88653), and ScCot1 (Accession No. CAA99636) from *S. cerevisiae*, encoding for already characterized Zn-CDF subfamily proteins (Montanini et al., 2007). The protein sequences of these four characterized CDF were therefore used for sequence comparison with HcZnT1 (Fig. 1A and B). The HcZnT1 cDNA encodes a protein of 369 amino acids with a predicted molecular weight of 40 kDa. Analysis of the *H. cylindrosporum* sequence shows that it contains the complete N-terminal signature sequence for the CDF family and also has a histidine-rich region (Fig. 1A) that is commonly found in the subgroup III members of the CDF family (Blaudez et al., 2003; Montanini et al., 2007). The HcZnT1 sequence also possesses a cation efflux domain indicative of the CDF family as identified by the Pfam protein domain database (<http://pfam.wustl.edu>). Furthermore, prediction of TMDs by the TMMOD programme (<http://liao.cis.udel.edu/website/servers/TMMOD/scripts/frame.php?p=submit>) identified a model for HcZnT1 consisting of 6 membrane spanning regions (Fig. 1A). HcZnT1 also contains the typical HxxxD motif on TMD I and TMD V (Fig. 1A) that revealed its zinc specificity (Montanini et al., 2007). The *H. cylindrosporum* HcZnT1 sequence was deposited in GenBank database with the accession number DQ515992.

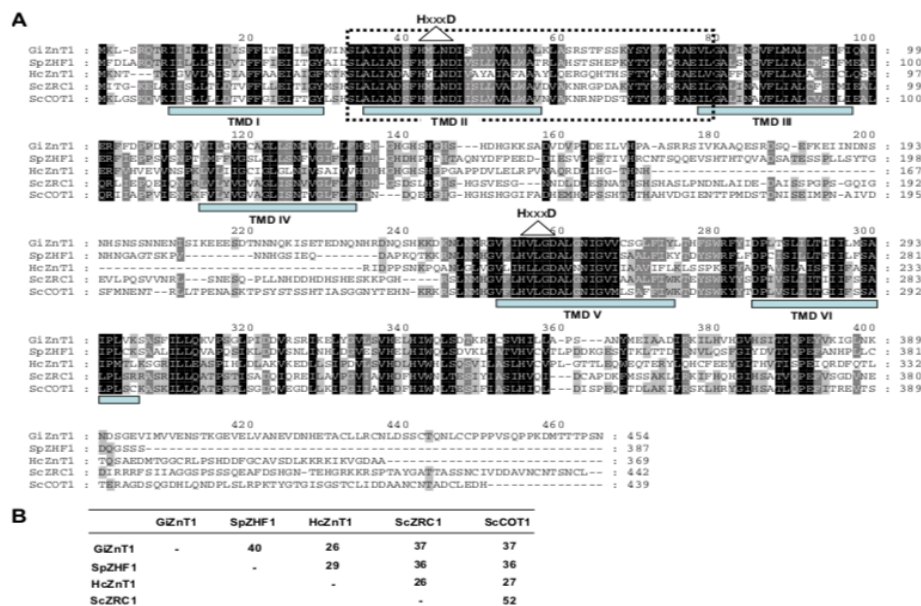


Fig. 1. Sequence comparison of functionally characterized Zn-CDF transporters from various fungal species. (A) Alignment of HcZnT1 with GintZnT1 (Accession No. CAE00445), SpZhf1 (Accession No. NP_593645), ScZrc1 (Accession No. CAA88653), and ScCot1 (Accession No. CAA99636) protein sequences. Amino acid residues that are conserved in all five 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 blue. Location of the CDF signature is shown by a dotted box. (B) Percent similarity between Zn-CDF transporters from the fungal species described in A.

3.2. Metal tolerance in yeast expressing HcZnT1 cDNA

HcZnT1 cDNA was expressed in *S. cerevisiae* mutant strains that are unable to grow on high concentration of various metals, and growth was monitored on either control or metal-supplemented medium. The wild-type strain was able to grow at high Zn concentration (Fig. 2A and B), whereas deletion of either the *Cot1* or the *Zrc1* gene, which encode transporters that sequesters Zn into the vacuole, rendered both mutants highly sensitive to Zn (Kamizono et al., 1989; Li and Kaplan, 1998). The Zn-sensitive phenotype of both *Cot1D* and *Zrc1D* mutants was fully complemented by HcZnT1 on 15 mM Zn (Fig. 2A and B). *Cot1D* mutant is also sensitive to high Co concentrations (Conklin et al., 1992), however HcZnT1 did not complement this phenotype on 2mM Co (Fig. 2A). We further

expressed HcZnT1 in the *pmr1D* and *ycf1D* mutant strains, which are unable to grow on Mn and Cd, respectively (Li and Kaplan, 1997 ; Ton et al., 2002). Transformation of the mutant strains with HcZnT1 did not restore growth of *pmr1D* on 3 mM Mn (Fig. 2C) nor that of *ycf1D* on 0.1 mM Cd (Fig. 2D). The data obtained with the different mutant strains suggest that HcZnT1 specifically transports Zn but not Co, Mn, nor Cd.

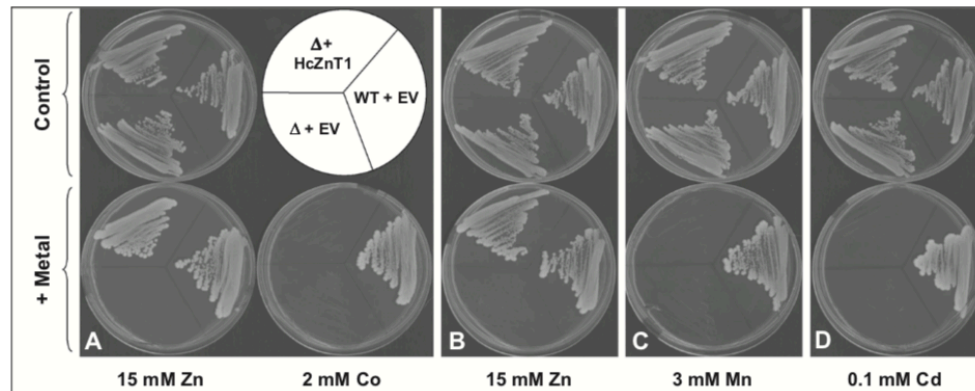


Fig. 2. Complementation of yeast mutants on selective media. Plates with (lower panel) or without (upper panel) added metals were streaked with the wild-type *S. cerevisiae* strain BY4741 transformed with pFL61 empty vector (WT + EV), with mutant strains transformed with pFL61 empty vector (D + EV) or with pFL61 containing HcZnT1 (D + HcZnT1). The mutant strains used are *cot1D* (A), *zrc1D* (B), *pmr1D* (C) and *ycf1D* (D). Plates were incubated at 30 °C for 3 days. A representative plate (out of four) is shown and the experiment was carried out twice with similar results.

3.3. HcZnT1 localization in yeast

Considering that HcZnT1 suppressed the *zrc1D* phenotype and that it possesses numerous predicted transmembrane domains, HcZnT1 acts as a Zn efflux system from the cytosol, either by extruding Zn out of the cell or by transporting Zn into intracellular compartments. We therefore constructed a HcZnT1:GFP fusion protein to determine the intracellular localization of HcZnT1 more precisely. Because the HcZnT1:GFP fusion gene complemented the zinc sensitivity of the *zrc1D* mutant (data not shown), the fusion protein was confirmed to retain its original function. GFP fluorescence was found at the periphery of the cell, as well as forming a central ring-like pattern (Fig. 3A). This pattern is typical of endoplasmic reticulum (ER) localization in yeast. The central ring pattern indeed corresponds to ER structures surrounding the nucleus, whereas the fluorescence at the cell periphery represents strands (Fig. 3A and E, arrows) connecting the nucleus to peripheral ER structures. This ER localization was confirmed after nuclei staining with DAPI which indeed revealed that GFP surrounded the nuclei (Fig. 3G). Undoubtedly, GFP was not present on the vacuolar membrane as demonstrated by the lack of co-localization between GFP (green) and FM4-64 (red) fluorescence patterns. The lipophilic dye FM4-64 is used in yeast to specifically stain the vacuolar membrane (Vida and Emr, 1995). Considering the discontinuous GFP pattern at the cell periphery, the localization at the plasmalemma can also be ruled out. Instead, HcAMT1 (an ammonium transporter from *H. cylindrosporum*; Javelle et al., 2003) was used as a control to illustrate typical plasma membrane staining (Fig. 3C). From these experiments, HcZnT1 therefore appears to be localized on ER membranes.

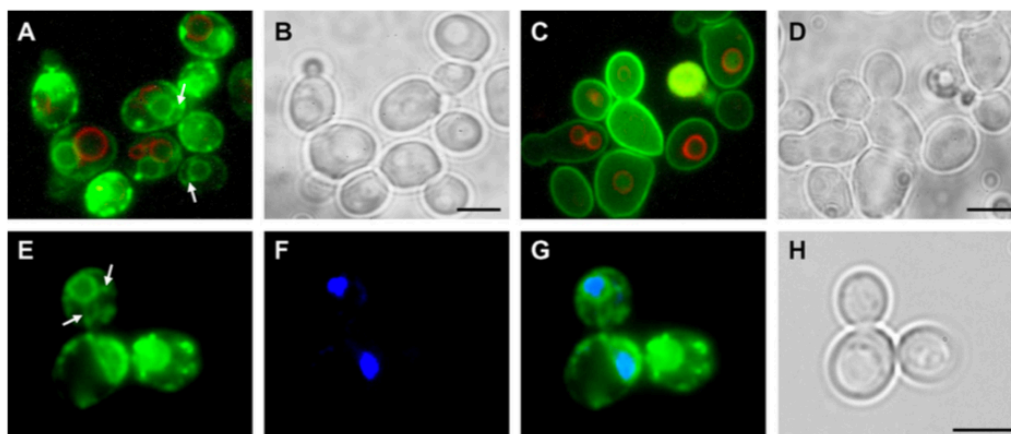


Fig. 3. ER membrane visualization of the HcZnT1:GFP fusion protein in yeast by epifluorescence microscopy. Cells were grown in SD medium and stained with FM4-64 (A and C) or DAPI (F). Different images from the same cells are shown: GFP fluorescence (A, C, and E), and bright field (B, D, and H) images from HcZnT1:GFP (A, B and E-H) or HcAMT1:GFP (C and D) expressing strain. Fig. 3G is a GFP + DAPI merged image from 3E and 3F. White arrows indicate GFP-labeled cytoplasmic strands connecting the nucleus to peripheral ER structures. Bars = 5 μ m.

3.4. Expression patterns of HcZnT1

Transcript levels were monitored by RT-PCR on *H. cylindrosporum* mycelia grown at increasing concentrations of Zn or in different types of mycelia (Fig. 4). Increasing the level of Zn in the growth medium did not significantly change HcZnT1 transcript levels (Fig.

4A, upper panel). A similar pattern was obtained with the control gene HcGS encoding the glutamine synthetase (Fig. 4A, lower panel). HcZnT1 transcripts were also detected in mycorrhiza-associated mycelium and in sporocarps (Fig. 4B), indicating that HcZnT1 probably plays a crucial role in Zn homeostasis, being expressed in various fungal structures.

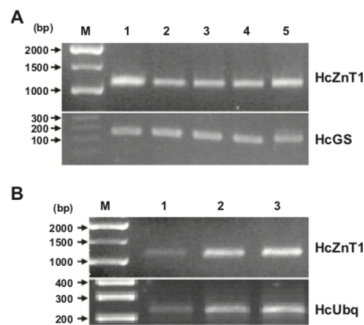


Fig. 4. Expression levels of HcZnT1 in *H. cylindrosporum*. Transcript levels at increasing Zn concentration (A) or in different fungal tissues (B) were estimated by RT-PCR. Expression level of HcZnT1 was compared to that of *H. cylindrosporum* glutamine synthetase (HcGS) or ubiquitin (HcUbq). In A, Zn concentrations are 0.3 (1), 3 (2), 30 (3), 300 (4) and 3000 (5) μ M. In B, RT-PCR reactions were performed on mycorrhizal roots (1), sporocarps (2) and pure culture mycelia (3). Experiments were repeated three times and representative gels are shown.

3.5. Fluorescence detection of zinc localization using zinquin

Accumulation of zinc by *H. cylindrosporum* was further investigated by using fluorescence detection microscopy. The Zn concentration used here (200 μ M) was chosen from preliminary experiments showing no effects on fungal growth. Zinquin labeled numerous small punctuate vesicles in *H. cylindrosporum* mycelia. This fluorescence pattern was identical whatever the age/position of the cell in the hypha (Fig. 5B and F). Consequently it seems very unlikely that this fluorescence pattern coincide with vacuoles. In order to verify this statement, we analysed vacuoles using a staining procedure with carboxy-DCFDA, a specific marker of the vacuolar lumen, at different positions in the hyphae of *H. cylindrosporum*. As previously reported by Fricker et al. (2008), who identified the vacuolar system of *Phanerochaete velutina* hyphae with the same procedure, the structure of the vacuole develops from a reticulum of fine tubes interspersed with small spherical vacuoles at the hyphal tip (Fig. 5A) to a series of small and larger spherical vacuoles in the older parts of the hypha (Fig. 5E). In both cases, the zinquin-derived fluorescence did not co-localize with that of the carboxy-DCFDA labeled vacuoles (Fig. 5D and H), demonstrating that zinquin-bound Zn pools are not localized into vacuoles.

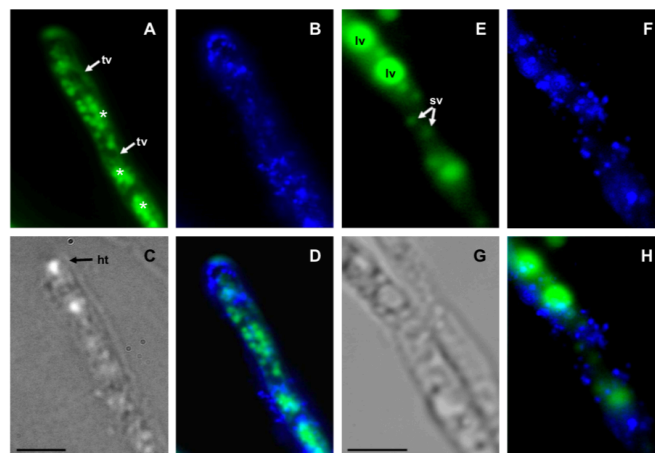


Fig. 5. Fluorescence microscopy images showing Zn localization in *H. cylindrosporum* cells exposed to 200 μ M ZnCl₂. Two representative parts [the apical region (A–D) and the proximal region (E–H)] of an individual hypha are shown. Zinquin fluorescent marker for zinc localization is shown in cyan (B and F). Control staining of vacuoles with the fluorescent marker carboxy-DCFDA is shown in green (A and E). Fig. 5D and 5H are zinquin + carboxy-DCFDA merged pictures from 5A and 5B, and 5E and 5F, respectively. tv: fine tubular vacuoles, sv: small spherical vacuoles, lv: large spherical vacuoles, ht: hyphal tip. The asterisks denote patches of small spherical vacuoles. Bars = 5 μ m.

Table 1
Characterized fungal members of the CDF family that at least transport zinc.

Gene	Substrate	Organism	Localization	Reference
HcZnT1	Zn	<i>H. cylindrosporum</i>	ER	Present study
Cis4	Zn	<i>S. pombe</i>	Golgi	Fang et al. (2008)
GiZnT1	(Zn)	<i>G. intraradices</i>	-	Gonzalez-Guerrero et al. (2005)
Zhf1	Zn (Co, Cd)	<i>S. pombe</i>	ER	Clemens et al. (2002)
Mcs2	Zn	<i>S. cerevisiae</i>	ER/nucleus	Ellis et al. (2004), Li and Kaplan (2001)
Zrg17	Zn	<i>S. cerevisiae</i>	ER/Golgi	Ellis et al. (2005), Yuan (2000)
Zrc1	Zn (Cd)	<i>S. cerevisiae</i>	Vacuole	Kamizono et al. (1989), MacDiarmid et al. (2000)
Cot1	Co, Zn	<i>S. cerevisiae</i>	Vacuole	Conklin et al. (1992), MacDiarmid et al. (2000)

4. Discussion

Ectomycorrhizal fungi, including *Hebeloma* species, have received considerable attention for use as efficient symbionts to improve mineral nutrition (Muller et al., 2007; Smith and Read, 2008) and to alleviate TE toxicity of their host plants (Adriaensen et al., 2006; Krpata et al., 2008; Krupa and Kozdroj, 2007; Krznicaric et al., 2009; Meharg and Cairney, 2000; Roy et al., 2007). Less information is available about the capability of ectomycorrhizal fungi to actively accumulate TE.

In the present study, we identified a transporter gene involved in zinc homeostasis, HcZnT1, which falls into the zinc-CDF group according to the CDF classification (Montanini et al., 2007). This group includes the Zrc1-like cluster enclosing only fungal CDF originating from Ascomycetes, Basidiomycetes, and Zygomycetes. The present work confirms that this CDF group, referred to as the Zn-CDF group, contained characterized members that at least transport zinc (Table 1). Members of the CDF family typically contain conserved histidine-rich regions in the intracellular loop of the protein between transmembrane domains 4 and 5 (Montanini et al., 2007). These are postulated to facilitate Zn²⁺ binding (Bloss et al., 2002; Montanini et al., 2007). Consistent with this observation, we found a total of 17 histidine residues, 7 of which were conserved. Nine of these were located between transmembrane domains 4 and 5. CDF transporters are postulated to be involved exclusively in transport of metals into intracellular vesicles/compartments or out of the cell across the plasma membrane (Palmiter and Huang, 2004). The HxxxD motif typical for Zn-specific CDF members (Montanini et al., 2007) were also detected in HcZnT1, confirming its putative specificity towards zinc. The zinc specificity was confirmed by complementation tests using a range of yeast mutants. Data obtained with the different mutant strains suggest that HcZnT1 specifically transports Zn but not Co, Mn, nor Cd.

HcZnT1 was constitutively expressed in the mycelium whatever the Zn concentration applied to the medium. Conversely, GintZnT1 was slightly up-regulated in the extraradical mycelium of *G. intraradices* by 75 μM Zn after 6 or 12 h of incubation (Gonzalez-Guerrero et al., 2005). Although also in our study was used a short incubation time (18 h) any changes in HcZnT1 were observed (Fig. 4). A shorter time of incubation (6 h) did neither result in a change of expression (data not shown). However, fungal and plant CDFs of the Zn group are usually not regulated by high Zn concentrations (Blaudez et al., 2003). Similarly, Zrc1 was not regulated by high Zn (MacDiarmid et al., 2003) suggesting that the protein is primarily involved in Zn homeostasis under normal conditions and may act as a crucial response element against Zn toxicity when cells are exposed to high concentrations of this element. Zrc1 was yet regulated during Zn-shock after Zn-deficiency (MacDiarmid et al., 2003).

We tried to increase the expression level of HcZnT1 in *H. cylindrosporum* in order to possibly obtain a phenotype with an increased Zn tolerance. For this purpose, we cloned the HcZnT1 cDNA in the pFAT3 binary vector and transformed *Agrobacterium tumefaciens* cells that were subsequently used in cocultures with *Hebeloma* mycelia, according to a method described earlier (Bellion et al., 2007). *Hebeloma* transformants expressed this supplemental copy of HcZnT1 as revealed by RT-PCR but were not significantly more tolerant to Zn when compared to control transformants (colonies transformed with the empty vector) or wild-type colonies (data not shown). Reduction of HcZnT1 expression level would be the reverse strategy to get additional information on the function of this gene; however the production of targeted KO mutants in ectomycorrhizal fungi is not yet feasible due to the strong prevalence of illegitimate recombination over homologous recombination in these organisms. Similarly, RNAi or antisense technologies have not yet been successfully applied to *Hebeloma* and other mycorrhizal fungi, except for *L. bicolor* (Kemppainen et al., 2009).

When compared to previously characterized CDF members (Fig. 1 and Table 1), HcZnT1 was mostly similar to the vacuolar membrane located transporters Zrc1 and Cot1 from *S. cerevisiae* that clustered together with the ER localized Zhf1 from *S. pombe* (Montanini et al., 2007) and to the *G. intraradices* sequence (Gonzalez-Guerrero et al., 2005). Apart from yeast, no full characterization of CDF members was reported in other fungal subphyla. The Glomeromycete GintZnT1 protein was proposed to be involved in Zn homeostasis (Gonzalez-Guerrero et al., 2005). However, it did not confer Zn tolerance when expressed in yeast mutants and its localization was not reported. To gain further information on the role of HcZnT1 in Zn homeostasis/tolerance, we investigated its cellular localization when expressed in yeast. HcZnT1::GFP fluorescent pattern was typical of ER targeting (Fig. 3A, E, and G), with ER structures surrounding the nucleus (as confirmed by DAPI staining of nuclei, Fig. 3G) and cytoplasmic strands connecting the nucleus to peripheral ER structures.

It is thus tempting to speculate that HcZnT1 is located on ER membranes, as shown for the *S. pombe* Zhf1 (Clemens et al., 2002), and participate to Zn loading in this compartment. From this line of evidence, two hypotheses arise concerning the role of HcZnT1. First HcZnT1 may have a general role in Zn homeostasis under normal conditions by providing Zn to zinc-requiring proteins that are processed in the ER. Second HcZnT1 could also be involved in Zn tolerance under Zn stress conditions by loading Zn ions into this subcellular compartment and consequently by contributing to the detoxification of the cytosol. This mechanism could resemble that of *S. pombe* in which the ER-located zinc transporter Zhf1 plays an important role in Zn tolerance and the ER is serving as a major site for Zn storage (Clemens et al., 2002). Interestingly, when investigating the genome sequence of this organism, we found that Zhf1 is the only member of the Zn-CDF gene subfamily (Blaudez, unpublished data). This ER-mediated strategy in transition metal cell biology in *S. pombe* differed from that employed by *S. cerevisiae* where the vacuole is the main site of Zn storage (Simm et al., 2007) and where the two vacuolar Zn-CDF transporters Zrc1p and Cot1p play essential role in Zn sequestration and tolerance (Eide, 2006). The next step will be to develop tools such as KO or RNAi mutant lines in *Hebeloma*, tools necessary to gain more comprehensive details on the role of HcZnT1 in Zn homeostasis/tolerance. The coming sequencing of the *H. cylindrosporum* genome should also reveal other putative key determinants of Zn homeostasis/tolerance.

As another step forward into the understanding of Zn homeostasis in *Hebeloma*, we also investigated Zn pools in hyphae by using zinquin, a Zn-specific fluorescent probe. When grown at a sub-lethal Zn concentration, *H. cylindrosporum* hyphae after zinquin staining showed intracellular zinc pools located in small punctuate cytoplasmic vesicles, but not into vacuolar compartments. These findings are in agreement with previous studies based on microscopy analyses and quantitative measurements by X-ray microanalysis, showing that extracellular binding and cytosolic sequestration of Zn occurred in *Hebeloma* crustuliniforme cells, whereas vacuoles were presumably not a significant Zn storage compartment in this fungus (Frey et al., 2000).

The vesicles that we identified in *H. cylindrosporum* hyphae are similar to the zincosomes described in mammalian cells (Wellenreuther et al., 2009) and to cytoplasmic vesicles reported in yeast (Li and Kaplan, 2001; Devirgiliis et al., 2004), although the identity of these zincosomes is still unknown. Colocalization studies showed that these vesicles do not correspond to vacuoles, prevacuolar compartments, nor to Golgi structures (Eide, 2006). The present study similarly ruled out the vacuolar nature of these Zn-containing vesicles in the filamentous fungus *H. cylindrosporum*. Indeed the zinquin-derived fluorescence pattern of these vesicles did not colocalize with the carboxy-DCFDA-derived fluorescence pattern identifying vacuoles. The putative functions of these zincosomes have been discussed by Eide (2006), who hypothesized that they could serve to buffer cytosolic Zn from transient perturbations much like the vacuole in yeast or to act as a storage compartment for Zn that can be later remobilized under Zn deficiency.

However, the lack of zinquin fluorescence in vacuoles has to be interpreted with caution since zinquin binding is impaired in the vacuole by the acidic pH that is actively maintained inside this compartment. Similarly, vacuolar Zn-ligand complexes could also impair zinquin binding to Zn²⁺. However, the present study clearly identifies a vesicular compartment of free Zn that does not correspond to vacuoles and that may represent the main pool of free Zn ions in *H. cylindrosporum*. Further microscopic observations will have to be performed to identify other potential pools of Zn in *Hebeloma* and in other mycorrhizal fungi.

The approaches used in the present paper highlight the putative function of intracellular compartments other than vacuoles in zinc tolerance/homeostasis in *H. cylindrosporum* cells. In order to know whether the vesicular compartment (or zincosome) evidenced by zinquin is linked to the ER compartment (hosting HcZnT1) or not, future lines of research should focus on the determination of the exact nature of these Zn-containing vesicles.

Mycorrhizal fungi are important for host protection against the toxic effects of trace elements (Baum et al., 2006; Bellion et al., 2006; Kozdroj et al., 2007; Krznanic et al., 2009). Particularly, metal tolerance mechanisms would play a crucial role in the TE biofiltering capacity of fungal cells. Therefore on TE-contaminated soils, the mycorrhizal symbiosis would favor both the establishment of trees but also the production of woody biomass with a reduced TE content, that could subsequently be used in different bioenergy pathways (Khasa et al., 2002; Sell et al., 2005).

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