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Intracellular sequestration of zinc, cadmium and silver in *Hebeloma mesophaeum* and characterization of its metallothionein genes

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ABSTRACT

Sequestration of intracellular heavy metals in eukaryotes involves compartmentalization and binding with cytosolic, cysteine-rich metallothionein (MT) peptides. We examined the roles of these processes in handling of zinc (Zn), cadmium (Cd) and silver (Ag) in sporocarps and a metal-exposed extraradical mycelium of *Hebeloma mesophaeum*, the Zn-accumulating ectomycorrhizal (EM) species frequently associated with metal disturbed sites. Size exclusion chromatography revealed that the majority of Zn and Cd in the sporocarps and mycelium was contained in a low molecular mass fraction attributable to compartmentalized metal. The staining of hyphal cells with the Zn-specific Zinquin and Cd-specific Leadmium fluorescent tracers labeled Zn and Cd in small, punctuated vesicles and vacuoles, respectively. By contrast, the sporocarp and mycelium Ag was associated with cysteine-rich, 5-kDa peptides. The peptides of the same size were also identified in minor Zn and Cd complexes from the metal-exposed mycelium. We have further isolated and characterized Hm*MT1*, Hm*MT2* and Hm*MT3* genes coding for different 5-kDa MTs of *H. mesophaeum* collected at a lead smelter site. Heterologous complementation assays in metal-sensitive yeast mutants indicated that Hm*MT*s encode functional, metal-specific peptides: only Hm*MT1* was able to complement sensitivity to Zn; Hm*MT1* conferred higher tolerance to Cd and Cu than Hm*MT2* or Hm*MT3*; and both Hm*MT2* and Hm*MT3*, but not Hm*MT1*, conferred increased tolerance to Ag. The presence of Hm*MT1* and Hm*MT3*, but not Hm*MT2*, was also confirmed in a *H. mesophaeum* isolate from an unpolluted site. Gene expression analysis in the extraradical mycelium of this isolate revealed that the transcription of Hm*MT1* was preferentially induced in the presence of Zn and Cd, while Ag was a stronger inducer of Hm*MT3*. Altogether, these results improve our understanding of the handling of intracellular Zn, Cd and Ag in *Hebeloma* and represent the first evidence suggesting involvement of MTs in sequestration of Zn in EM fungi.

1. Introduction

The natural capacity of ectomycorrhizal (EM) fungi to accumulate heavy metals from a large volume of colonized soil has been reported since the 1970s (Falandysz and Borovička, 2013; Urban, 2011). While many metals (e.g. Zn, Cu, Mn, Ni, Co) are essential micronutrients, they can be detrimental to organisms when present in excess. Other metals (e.g. Cd, Pb, Hg or Ag) have no known biological function and may exert toxic effects when accumulated even at minute levels (Nordberg et al., 2007). There is evidence that the association of plant roots with a metal tolerant EM fungus can enhance fitness of the host plant in a metalliferrous environment by improving the nutrition, reducing the transfer of the toxic metals into the mycorrhizal root and stimulating the plant response to abiotic stress (Adriaensen et al., 2005, 2006; Jourand et al., 2010; Kozdrój et al., 2007; Krznaric et al., 2009, 2010; Schützendübel and Polle, 2002; Urban, 2011). The metal barrier function is achieved by extracellular precipitation or chelation, biosorption, exclusion, and by cellular uptake of metals (Bellion et al., 2006; Gadd et al., 2012). Like in other organisms, the cellular mechanisms conferring higher metal tolerance upon EM fungi may involve: induction of the oxidative stress response enzymes and elevation of the antioxidant glutathione (GSH) levels (Jacob et al., 2001, 2004; Ott et al., 2002), metal efflux systems (Colpaert et al., 2011; Ruytinx et al., 2013), and the sequestration of excess metal by intracellular complexation or compartmentalization. The three major classes of intracellular peptides chelating thiophilic metal ions comprise phytochelatins (PCs), metallothioneins (MTs) and GSH.

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In most plants and some yeasts, the synthesis of PCs of a general structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$ (PCn; $n = 2–11$) represents the major detoxification mechanism for Cd, As and Hg ions (Clemens and Simm, 2003; Schat et al., 2002). PCs can also function as important chelators of Zn ions (Tennstedt et al., 2009). In EM fungi, the sequestration of Cd by PC2 and PC3 has been reported in *Boletus edulis* (Collin-Hansen et al., 2007) and the gene encoding a PC synthase has been recently isolated and characterized from *Tuber melanosporum* (Bolchi et al., 2011). MTs are cytosolic, cysteine-rich peptides of distinct sizes involved in the heavy metal tolerance of eukaryotes and some prokaryotes (Capdevila and Atrian, 2011), including EM fungi. In *Paxillus involutus*, expression of the Pi*MT1* gene is activated upon Cu and Cd, but not Zn exposure (Bellion et al., 2007; Courbot et al., 2004). Ramesh et al. (2009) characterized two MT genes from *H. cylindrosporum*: Hc*MT1* that is upregulated by Cu and Hc*MT2* that is upregulated by Cu and Cd. In the Ag-hyperaccumulating *Amanita strobiliformis*, the Ag-inducible genes As*MT1a*, *1b,* and *1c* encode isomorphic AsMT1s sequestering virtually all the Ag accumulated by the mycelium and wild-grown sporocarps (Osobová et al., 2011).

Among fungi, the role of compartmentalization in the biology of excess heavy metal ions has been detailed in *Saccharomyces cerevisiae*. The detoxification of Cd in this yeast relies largely upon the *ycf1*-encoded ABC-type transporter involved in the vacuolar sequestration of Cd (Li et al., 1997). The transport of Zn into vacuoles has in *S. cerevisiae* a dominant role in the buffering of cytosolic Zn levels and is mediated by the ZRC1 and COT1 transporters of the cation diffusion facilitator (CDF) family (MacDiarmid et al., 2000, 2002, 2003). The storage of Zn in vacuoles has been reported also in EM *Suillus bovinus* (Ruytinx et al., 2013). Independently of ZRC1 and COT1, *S. cerevisiae* can store Zn in small cytoplasmic vesicles that appear when the cells are challenged with high Zn concentrations (Devirgiliis et al., 2004; Ezaki and Nakakuhara, 2012). Similar Zn-containing vesicles, so-called zincosomes of yet unknown origin, are implicated in zinc storage and detoxification in various mammalian cell types (Eide, 2006; Wellenreuther et al., 2009). Sequestration of Zn in the endoplasmic reticulum (ER) via a CDF transporter ZHF1 has been shown to confer Zn tolerance to *Schizosaccharomyces pombe* (Borrelly et al., 2002; Clemens et al., 2002). Recently, Blaudez and Chalot (2011) characterized the Hc*ZnT1* gene coding for a, presumably ER-located, CDF Zn transporter of *H. cylindrosporum*; they also demonstrated that under Zn replete conditions the excess metal is targeted into zincosome-like vesicles.

In this study, we report on Zn, Cd and Ag sequestration in the sporocarps and cultured extraradical mycelium of Zn-accumulating *Hebeloma mesophaeum*, a conspicuous and important fungal species in pristine as well as metal disturbed environments (Hrynkiewicz et al., 2008; Krpata et al., 2008; Vesterholt, 2005). With the aim of gaining insights into the biology of excess metals, we analyzed intracellular metal species in fungal tissue, isolated and characterized three cDNAs coding for different MTs, and identified intracellular metal sinks by fluorescent labeling.

2. Materials and methods

2.1. Sporocarps and mycelium

Young sporocarps of various *Hebeloma* spp. (Table 1) were collected from their natural habitats in the Czech Republic. The harvested samples were cleared of substrate debris and washed with distilled water. The portions to be used for the metal speciation analysis and molecular analysis were stored at −80 °C and fixed by freeze-drying, respectively. For the total metal content measurements, the sporocarps were dried to constant weight at 65 °C and pulverized in a blender. The metal concentrations in the homogenized samples were determined by instrumental neutron activation analysis (INAA) according to Řanda and Kučera (2004). The species were identified according to Vesterholt (2005) and, when necessary, confirmed by a sequence analysis of their ITS rDNAs. Fungal DNA was isolated from the frozen tissue by using a NucleoSpinR Plant II Extraction Kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. The ITS rDNA regions were amplified by PCR employing primer pairs and reaction conditions as described previously (Borovička et al., 2011) and subjected to a custom DNA sequencing (Macrogen Inc., Seoul, Korea). The acquired sequences were edited using the BioEdit editor (Hall, 1999) and aligned to the sequences retrieved from GenBank and the UNITE database (Abarenkov et al., 2010). Classification was adjusted to recent phylogenetic studies (Boyle et al., 2006; Eberhardt et al., 2009; Eberhardt and Beker, 2010). The ITS rDNA sequences of the *Hebeloma* spp. collections were deposited in the EMBL Database (Table 1). A part of each representative collection was deposited in the herbarium of the Mycological Department, National Museum, Prague (PRM).

The dicaryotic mycelium culture of *H. mesophaeum* (PRM 899241) was obtained from axenic explants (30 mm^3) from a freshly broken flesh of the pileus, as described previously with *A. strobiliformis* (Osobová et al., 2011). The identity of the mycelium was confirmed by sequencing of the rDNA ITS regions as described above. The mycelium was routinely maintained at 25 °C on 50% potato dextrose (PD) agar containing 2 g l^{-1} potato extract (Sigma–Aldrich, Steinheim, Germany) and 10 g l^{-1} glucose. As determined by atomic absorption spectrometry (AAS; model Spectr AA300, Varian, Inc., Palo Alto, CA, USA), the fresh PD medium contained 0.68 μM Zn; Cd and Ag concentrations were below the detection limit of 44 and 91 nM, respectively. To study metal speciation and MT gene expression, 21-day-old colonies were transferred to 50% PD medium and a pure culture was grown for additional 6 weeks. The mycelium (0.5–1 g of fresh weight) was then transferred to 200 ml of fresh PD medium supplemented with $ZnCl_2$, $CdCl_2$ or $AgNO_3$, or without any additional metals (the control), T incubated at 25 °C, upon harvest washed with 0.1% $MgSO_4$, and stored at −80 °C (72-h exposure for metal speciation analysis) or freeze-dried (24-h exposure for MT induction analysis). Mycelium for fluorescence microscopy was obtained from 6-week-old colonies grown on PD agar plates with or without $ZnCl_2$ or $CdCl_2$ supplement.

2.2. Speciation analysis of intracellular metal

The fungal tissue extracts were obtained from 3 g of sporocarps (stipes and caps in natural proportion) or 1 g of the metal-exposed mycelium. These were ground in liquid N_2 with a mortar and pestle and the disintegrated tissue was extracted with 2.5 ml of 50 mM HEPES (pH 7.0). Tissue debris was removed by centrifugation at 20,000*g* and 4 °C for 10 min. To fractionate the tissue extract by size exclusion chromatography (SEC), 2 ml of the extract (6–8 mg of the total protein as determined using BCA Protein Assay Kit from Pierce and BSA and lysozyme as standards) was loaded onto Superdex Peptide 10/300 GL column (GE-Healthcare, Uppsala, Sweden). The fractionation was performed with a BioLogic DuoFlow FPLC system (BioRad, Herkules, CA, USA) and 50 mM HEPES, 25 mM KNO_3 (pH 7.0) as a mobile phase at a flow rate of 0.5 mL min^{-1}. Ribonuclease A (GE Healthcare), ubiquitin (Sigma–Aldrich), a synthetic 2.1-kDa peptide and glutathione (GSH; Merck, Dortmund, Germany) were used as molecular mass standards. The metal contents in each of the SEC fractions were determined by AAS. The low molecular mass fractions were pooled, freeze-dried, dissolved in 140 μl of distilled water (final volume of 160 ± 5 μl) and stored at −80 °C. The ⩾6-kDa metal species were pooled and brought to a final volume of 60 ± 5 μl by ultrafiltration with Microcon