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Identification and characterization of *CcCTR1*, a copper uptake transporter-like gene, in *Coprinopsis cinerea*

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Summary

Copper (Cu) is an essential element for the physiological function of organisms. In basidiomycetes, Cu is necessary for the production of phenol oxidase enzymes such as laccase and tyrosinase. We isolated and characterized two genes, *CcCTR1* and *-2*, from the model basidiomycete *Coprinopsis cinerea*. *CcCTR1* and *-2* showed similarity to the Cu transporter *CTR1* in *Saccharomyces cerevisiae*. Both CcCTRs had a MLxxM motif that is conserved in other CTR homologs. The addition of Cu to a liquid culture of *C. cinerea* decreased the mRNA accumulation of *CcCTR1* and *-2*. Heterologous expression of CcCTR1 in *S. cerevisiae* increased Cu sensitivity, suggesting that CcCTR1 is a Cu uptake transporter. Together, these results suggest that CcCTR1 plays an important role in Cu accumulation in *C. cinerea*.

Introduction

The micronutrient Cu is an essential cofactor for numerous enzymes that participate in redox reactions. These include proteins involved in the detoxification of oxygen radicals, such as the cytoplasmic Cu/Zinc superoxide dismutase (SOD); electron-transport proteins, including mitochondrial cytochrome c oxidase; and proteins with oxidase activity, including fungal laccase, secreted phenol oxidase, and plant ascorbate oxidase. Some of these enzymes belong to the blue copper oxidase family, whose members contain 4 Cu atoms per molecule (Riva 2006).

Cu can be toxic even at low concentrations. Cu(I) and Cu(II) ions may bind to inappropriate sites of non-copper-containing proteins with high affinity (Predki and Sarkar 1992), resulting in the generation of oxygen radicals and thus catalyzing the

Abbreviations: Cu, copper; CTR, Cu transporter; SOD, superoxide dismutase; MCO, multicopper oxidase; CuSE, copper-signaling element.

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auto-oxidation of biomolecules such as lipids, proteins, and nucleic acids (Halliwell and Gutteridge 1984). Organisms possess several mechanisms to maintain intracellular Cu concentrations at appropriate levels. These include various homeostasis factors, including transporters, which control the uptake, distribution, and sequestration of Cu inside the cell. *Saccharomyces cerevisiae*, a model organism for investigating Cu transporter mechanisms, has three Cu transporters (CTR): two high-affinity transporters (CTR1, -3; Knight et al. 1996; Dancis et al. 1994) and one vacuolar localized transporter (CTR2; Kampfenkel et al. 1995). CTR1 and CTR3 are regulated by the Cu sensor MAC1p, which is a regulatory protein related to Cu-dependent transcription factors (Peña et al. 1998; Jungmann et al. 1993). CTR homologs exist broadly in animal, plant, and fungal genomes, but have not been identified in prokaryotes (Dumay et al. 2006). Almost all CTRs have structurally conserved domains such as three transmembrane regions (Dumay et al. 2006); clustered methionine residues in the hydrophilic extracellular domain and an MxxxM motif in the second transmembrane domain, which are important for Cu uptake (Puig et al. 2002); and a number of functionally important charged amino acids at the C-terminal end (Peñas et al. 2005). These conserved characteristics in CTRs suggest that CTRs play a common and essential role in eukaryotes.

Basidiomycetes have many secreted enzymes that contain Cu in their active sites. Among these are the multicopper oxidases (MCOs), which include the laccase type, with three domains, and the ceruloplasmin type, with six domains (Nakamura and Go 2005). Metal affects the expression of some MCOs. For instance, laccase mRNA levels in the fungi *Trametes versicolor* (Collins and Dobson 1997) and *Pleurotus ostreatus* (Palmieri et al. 2000) are stimulated by the addition of Cu(II) to the culture medium. The addition of Cu increases the productivity of laccase in *Trametes multicolor* (Hess et al. 2002) and *P. ostreatus* (Baldrian and Gabriel 2002; Baldrian et al. 2005). Uptake of Cu from outside of the cells is necessary for the synthesis of Cu-containing proteins such as laccases and for the maintenance of Cu levels in cells. Thus, laccase function seems to be controlled at the transcriptional level or through localization, but Cu homeostasis in basidiomycetes remains largely unexplored. Uldschmid et al. (2002, 2003) reported two P-type ATPases involved in Cu trafficking in *T. versicolor*, and *ctr1* was isolated from *P. ostreatus* by Peñas et al. (2005), but there have not been any other reports of CTRs in basidiomycetes.

In this study, we found genes that contribute to Cu homeostasis in *Coprinopsis cenerea*, a model basidiomycete. We isolated and characterized two *CTR*s from the cDNA of *C. cinerea* to investigate Cu homeostasis in basidiomycetes. CcCTR1 is a homolog of high-affinity CTR1 from *S. cerevisiae*, which is thought to play major roles in Cu uptake (Dancis et al. 1994). We also isolated and analyzed a homolog of CTR2 from *S. cerevisiae* in *C. cenerea*, and we will discuss the roles of both CTRs.

Materials and methods

Organisms and culture conditions

C. cinerea, strain Okayama7, was used throughout this study. Mycelia were maintained on 1.5% agar plates with 0.25 × MYPG medium, as in Nagai et al. (2003). For liquid culture, we used the 0.25 × MYPG medium without agar. For Cu treatment, 100 mM $CuSO_4$ solution was diluted in the medium to various concentrations.

Phylogenetic analysis

A similarity search was carried out using the amino acid sequence of ctr1 from *Pleurotus* sp. 'Florida' (AJ705045, Peñas et al. 2005) and CTR1–3 from *S. cerevisiae* as a query. The amino acid sequences of the CTRs were obtained using the BLASTP programs at the Broad Institute's *Coprinus cinereus* Database (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html) first version and databases for *Laccaria bicolor* (Martin et al. 2008; Joint Genome Institute, http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html), *Phanerochaete chrysosporium* (Joint Genome Institute, http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Phchr1&advanced=1), *Ustilago maydis* (Broad Institute, http://www.broad.mit.edu/annotation/genome/ustilago_maydis/Home.html), and *Cryptococcus neoformans* (Broad Institute, http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/Home.html). ScCTR1–3 from *S. cerevisiae*, SpCTR4–6 from *Schizosaccharomyces pombe* (Puig et al. 2002) and CaCTR1 from *Candida albicans* (Marvin et al. 2003) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Locus numbers were also obtained from the Broad Institute, the Joint Genome Institute, and the NCBI database. Multiple alignment was performed with ClustalW, available at DDBJ (http://clustalw.ddbj.nig.ac.jp/top-j.html), and Parallel PRRN (http://prrn.ims.u-tokyo.ac.jp/). A Neighbor-Joining tree was constructed with PAUP*4.0b10 (Swofford 2002), using the mean character difference