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Functional characterization of the manganese transporter smf2 homologue gene, PsMnt, of Phanerochaete sordida YK-624 via homologous overexpression

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One sentence summary: The yeast natural resistance-associated macrophage protein family transporter was identified as the Mn transporter in the white-rot fungus Phanerochaete sordida YK-624.

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ABSTRACT

A homologue gene of the yeast natural resistance-associated macrophage protein (Nramp) family transporter smf2 was identified in the white-rot fungus Phanerochaete sordida YK-624. Relative expression levels of the homologue, designated PsMnt, were roughly equivalent in cultures containing 0 to 1000 μM Mn(II), a concentration non-toxic to the fungus. In the PsMnt-overexpressing mutant, cellular Mn accumulation and manganese peroxidase (MnP) activity increased significantly in 4-day cultures containing 10 μM MnSO4. Compared with the wild-type strain, MnP activity in the overexpressing mutants was higher at lower Mn concentrations (specifically 10–15 times higher). These results suggest that PsMnt is a high-affinity Mn transporter involved in cellular Mn accumulation under Mn-deficient conditions. This is the first report of an smf2 homologue in wood rot fungi.

Keywords: homologous expression; manganese transporter; manganese peroxidase; white-rot fungi

INTRODUCTION

Filamentous white-rot fungi can degrade recalcitrant woody polymer components such as cellulose, hemicellulose and lignin. The name ‘white rot’ derives from the tendency of these fungi to degrade brown lignin prior to white polysaccharides during wood degradation. As other microorganisms generally do not degrade lignin, this fungal group plays a very important role in carbon cycling in forest ecosystems. Lignin, a heterogeneous aromatic biopolymer gives mechanical strength and high resistance for chemical/biological degradation to wood. Lignin forms an amorphous matrix together with hemicellulose in the cell wall to protect cellulose and other polysaccharides by preventing penetration of enzymes. Although white-rot fungi secrete various enzymes for lignin degradation, it is said that ligninolytic enzymes (e.g. lignin peroxidase (LiP), manganese
peroxidase (MnP) and laccase) play the most important part in lignin degradation (Martínez et al. 2005; Dashtian et al. 2010).

It is well known that metal ions are important for lignin degradation as well as polysaccharide decomposition. Manganese (Mn) ions in particular are directly involved in lignin degradation by white-rot fungi. Mn is a well-known inducer of MnP (Bonnarme and Jeffries 1990), and the Mn(I)-chelator is oxidized as a substrate of MnP; the resulting Mn(III)-chelator functions as a radical mediator to oxidize phenolic residues contained in lignin. The Mn(III)-chelator also oxidizes other organic compounds, such as derivatives of unsaturated fatty acids and sulfur compounds, to produce respective reactive radicals that attack the recalcitrant ‘condensed structures’ of lignin (Hofrichter 2002). Addition of Mn at an optimum concentration enhances lignin degradation (Kerem and Hadar 1995). Phanerochaete sordida YK-624, a typical white-rot fungus with excellent lignin-degradation capability, secretes MnP and LiP as major ligninolytic enzymes. (Hirai, Kondo and Sakai 1994; Sugiyura, Hirai and Nishida 2003). Although this fungus usually produces MnP, LiP is produced under Mn-deficient conditions (Hirai et al. 2005). Generally Mn is abundant in trees and thus Mn deficiency in trees is rare. Stone (1968) gives a good summary of the concentrations of Mn contained in various tree species. Mn concentrations in trees had various values depending on tree species, habitat, site and season, but the deficiency level (less than 20 ppm) was hardly observed except in orchard trees. Therefore, Mn is considered a critical micronutrient in lignin degradation by P. sordida YK-624 in the natural environment. Based on these facts and a speculation, it is expected that P. sordida YK-624 retains the efficient Mn uptake systems and MnP expression mechanisms.

Diss et al. (2011) identified multiple candidate Mn transporter genes in fungal genomes. In this report, 11 transporters belonging to several Mn transporter families were identified in the genome of a typical white-rot fungus, Phanerochaete chrysosporium. Some of these candidates are predicted to function in Mn homeostasis based on the results of heterologous expression in yeast, and PcpHO84 (plasma membrane phosphate transporter family) is predicted to be involved in Mn uptake under excess Mn conditions. Yeast phosphate transporter PHO84p is involved in cellular phosphate accumulation and it prefers a divalent metal complex of phosphate as a substrate. PHO84p transports the Mn–phosphate complex under Mn surplus conditions, but the expression level is strongly regulated by phosphate (Reddi, Jensen and Culotta 2009). On the other hand, functional characterization of SMF homologues has been not carried out in white-rot fungi. Saccharomyces cerevisiae SMF1 and SMF2 are members of the Nramp (natural resistance-associated macrophage protein) family of metal transporters that are widely conserved in bacteria, plants, fungi and animals. It is considered that SMFs are the significant important transporters in cellular manganese homeostasis, and are involved in activation of Mn-dependent enzymes and resistance in oxidative stress (Reddi, Jensen and Culotta 2009). These metal–proton symporters act on a wide range of divalent metals (Culotta, Yang and Hall 2005). In yeasts, SMF transporters are localized at the cellular surface or intercellular vesicles where they play a role in Mn uptake or distribution under Mn-starvation conditions. Under high Mn conditions, by contrast, SMF transporters are moved into vacuoles and degraded to prevent toxic effects caused by high Mn concentrations (Reddi, Jensen and Culotta 2009). SMF2 significantly affects Mn accumulation and the activities of numerous Mn-dependent enzymes other than SMF1.

As mentioned above, there is no report on the function of the smf homologue in the white-rot fungi. However, we speculated that smf homologues in white-rot fungi are strongly involved in the uptake of Mn and the expression of the Mn-dependent enzyme including MnP, as with yeast SMF. Hence, we searched for smf homologues retained by P. sordida YK-624, a strong ligninolytic white-rot fungus. In this study, we identified the gene encoding an smf2 homologue in P. sordida (PsMnt). The function of PsMnt was then characterized, particularly with respect to its involvement in MnP activity, using a homologous overexpression strategy.

**MATERIALS AND METHODS**

**Strains and cultivation conditions**

White-rot fungus P. sordida YK-624 (ATCC 90872), the isogenic uracil auxotrophic strain UV-64 and the prototrophic transformant strain U2 were used in this study (Mori et al. 2016a). Strain U2 was used as control strain because it had best ligninolytic properties (ligninolytic activity and selectivity) among all seven prototrophic transformants (Mori et al. 2016a). All strains were maintained on potato dextrose agar (PDA) medium at 4°C. Strains were pre-cultured on PDA medium (9 cm inner diameter) at 30°C for 3 days, and then two mycelial pellets (10 mm i.d.) were inoculated into the liquid medium described below.

**PsMnt sequence determination and PsMnt expression plasmid construction**

Phanerochaete sordida YK-624 genomic DNA and total RNA were obtained from mycelia grown on potato dextrose broth (PDB) medium and Kirk’s low-nitrogen (LN, ligninolytic condition) medium (Fenn and Kirk 1981; Tien and Kirk 1988), respectively. DNA extraction (from PDB culture), RNA extraction (from LN culture) and cDNA synthesis were performed as previously reported (Mori et al. 2016b). Full-length DNA and cDNA sequences of PsMnt were determined using PCR techniques. The primers were designed from a partial cDNA sequence of putative PsMnt obtained from unpublished RNA-seq data (see Supplementary Fig. 1 in the online supplementary material). Downstream of the 3’-sequence of cDNA was determined by the 3’-RACE (rapid amplification of cDNA ends) method and genomic PCR for determination of the full-length PsMnt gene was performed by thermal asymmetric interlaced (TAIL)-PCR using degenerate primers (Liu and Whittier 1995; Yamagishi et al. 2007).

Restriction sites (Kpn I and Xba I) were incorporated into the PsMnt genomic DNA sequence using the following primers: 5’-GGTACCGTGTGGTACCATGCCCCCTGAGCCTC-3’ and 5’-AAGCAGCGAGGATTGTACC-3’. The amplified product was digested with KpnI and XbaI and cloned into KpnI-XbaI-digested pGPDpro (Suzuki et al. 2014), yielding pPsMnt.

**Genetic transformation**

The pPsMnt expression plasmid was transformed into UV-64 protoplasts using a co-transformation method with pPsUR5 (Yamagishi et al. 2007). Regenerated Ura+ prototrophic transformants were screened by genomic PCR for verification of target gene transformation. Mycelia were boiled in 50 mM NaOH solution for 5 min, then neutralized with 1 M Tris-HCl (pH 8.0). The resulting supernatant was used as crude DNA solution. The primer pair 5’-AAGCAGCGAGGATTGTACC-3’ and 5’-CAGACAGAGGAAGGCATAAG-3’ was used to amplify the transformed gene.
Transcriptional analysis

Fungal strains were cultivated in 10 ml of LN medium with 0, 1, 10, 100 or 1000 μM MnSO₄ for 4 days, at 30°C. Mycelia were separated from the culture broth by filtration. The filtrate was used for MnP activity testing, as described below. Total RNA was extracted from mycelia using an RNasey Mini kit (Qiagen, Hilden, Germany) according to the instruction manual; cDNA was synthesized from 200 ng of total RNA using a PrimeScript RT-PCR kit (TaKaRa Bio Inc., Shiga, Japan). Synthesized cDNA was used in quantitative reverse transcription (qRT)-PCR analyses to evaluate the PsMnt expression level (primers: 5′-GACGGGTCCTGATCTTGCG-3′ and 5′-CCTACAGACTCAGCAGGTGAAA-3′; the standard curve is illustrated in Supplementary Fig. 2 in the online supplementary material). Actin was used as a reference gene (primers: 5′-CCCTCAAGCAGATAGGCTCAAG-3′ and 5′-TAGAAGCACCCTGCGTGAC-3′) (Hirabayashi et al. 2015). SYBR Premix Ex Taq II (Tli RNaseH Plus, TaKaRa Bio Inc.) was used as a qRT-PCR reagent following the manufacturer’s protocol and qRT-PCR conditions were as follows: 95°C for 3 min; 44 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s.

MnP activity

Two mycelial discs on PDA medium (i.d. 10 mm) were inoculated into 10 ml of LN medium (containing 10 or 30 μM MnSO₄) or Mn-deficient LN medium. In the case of Mn-deficient cultures, MnSO₄ solution was added (final concentration 0, 1.0, 5.0 or 10 μM) after 3 days incubation, and the cultures were incubated for an additional 24 h. After incubation for a total of 96 h, the culture broth was collected and filtered through a 0.22-μm membrane filter. In the case of Mn-supplemented LN medium, filtrate was collected every 2 days. MnP activity in the filtrate was measured according to a method described previously (Hirai, Kondo and Sakai 1994).

Mn uptake

Fungal strains were cultivated in Mn-deficient (10 μM) LN medium (50 ml) for 4 days at 30°C. Mycelia were collected by centrifugation and washed 3 times with Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), freeze-dried, then weighed. Dried mycelia (15–30 mg) were mineralized with 8% nitric acid and 6% hydrogen peroxide at 120°C for 8 h. After dilution to 10 ml with milli-Q water, the Mn content was determined using inductively coupled plasma optical emission spectrometry on an SP57800 instrument (Seiko Instruments Inc., Chiba, Japan).

Statistical analysis

Data presented are the average of three or more replicates in each experiment. One-way analysis of variance (ANOVA) was carried out to determine the significance of differences in PsMnt expression and MnSO₄ concentration. For other statistical analyses, the Student’s t-test was performed to determine the significance of differences compared with control strain U2. Differences between means at a 5% confidence level (P < 0.05) were considered statistically significant.

RESULTS AND DISCUSSION

We initially sought to determine the DNA and mRNA sequences of the putative P. soridica YK-624 manganese transporter. From RNA-seq analysis (our unpublished result), a partial SMF-like sequence exhibiting low expression levels on beech wood meal culture was obtained. Based on this sequence data, PsMnt full-length DNA and cDNA sequences were determined using PCR techniques. The open reading frame of PsMnt (accession number: LC326251) has 2141 bp and six introns, with an 1824-bp coding sequence. Blast search (Altschul et al. 1997) results indicated that PsMnt homologues are preserved with high homology among Agricomycetes (especially Polyporales) at the amino acid sequence level (Supplementary Table 1 in the online supplementary material). The translated amino acid sequence showed high similarity (identity 32–41%) to the Nram family of divalent ion transporters, according to SmartBlast analysis (Table 1). Therefore, PsMnt was presumed to be an SMF homologue. In yeast, it is thought that SMF2 (for which evidence of cell surface localization is still lacking) is the most important proton-coupled transporter of Mn ions into the cell. Reportedly, Mn homeostasis is significantly affected in smf2∆ deletion yeast, reducing Mn accumulation and the activity of Mn-dependent enzymes (Reddi, Jensen and Culotta 2009; Cyert and Philpott 2013). The relative PsMnt expression level and dry mycelial weight of P. soridica YK-624 in cultures containing various MnSO₄ concentrations are shown in Fig. 1. As no significant difference was observed in mycelial dry weight between cultures at any MnSO₄ concentration, it can be said that MnSO₄ concentrations in the range used in this experiment are physiologic for P. soridica YK-624 (except 0 μM). Because P. soridica YK-624 produces LiP instead of MnP as a
ligninolytic enzyme under Mn(II)-deficient conditions (Hirai et al. 2005), it is likely that a lack of Mn in the culture medium does not critically affect the growth of this fungus. PsMnt was constitutively expressed; no significant difference was observed in relative PsMnt expression level.

A previous review described Mn transporters in S. cerevisiae (Culotta, Yang and Hall 2005). According to this report, transcription of yeast smf1 and smf2 is not regulated by the Mn concentration, Mn starvation just increases the stability of SMF proteins. Portnoy et al. have reported post-translational regulation of SMF1 and SMF2; these transporter protein levels have been repressed under culture conditions of supplemented 10 μM Mn (Portnoy, Liu and Culotta 2000). As other transporters also function in controlling the cellular Mn concentration under physiological conditions, it is difficult to characterize the function of PsMnt from the transcription level. Therefore, we investigated the involvement of PsMnt in Mn(II) uptake using homologous overexpression. By genomic PCR screening, 15 clones (MT-1 to -15) of PsMnt co-transformants were obtained from 180 uracil prototrophic clones. Bonnarme and Jeffries (1990) reported that the production of MnP is regulated by Mn(II) in several white-rot fungi. In addition, MnP activity in P. sordida is reportedly regulated by the Mn(II) concentration in liquid culture (Rüttimann-Johnson, Cullen and Lamar 1994). Therefore, Mn(II) uptake driven by PsMnt was indirectly evaluated by measuring MnP activity. MnP activity in the culture filtrates obtained from 4-day LN cultures (30 μM Mn) incubated with the wild-type strain, control strain U2 and the 15 MT strains was measured. Although 80% of all transformants showed higher MnP activity than the wild type, only two co-transformants (MT-20 and -40) showed significantly higher activity than control strain U2 (Supplementary Fig. 3 in the online supplementary material).

To evaluate the Mn uptake activity in more detail, MnP activity was measured after 24 h of induction by the addition of Mn(II) to a Mn-deficient LN culture. As shown in Fig. 2A, the MnP activity of all strains was correlated with the addition of Mn. However, PsMnt transformants MT-20 and -40 showed significantly higher MnP activity than the control strain U2 (Supplementary Fig. 3 in the online supplementary material).

Figure 2. Enhancement of MnP activity in PsMnt recombinant strains at various MnSO4 concentrations. (A) MnP activity in culture supernatants of wild type, control strain U2 and PsMnt transformants (MT-20 and MT-40) incubated for 4 days. Asterisks indicate significant difference from control strain U2 as determined using the Student’s t-test ($P < 0.05$). (B) Rate of increase in MnP activity in U2, MT-20 and MT-40 at each MnSO4 concentration, compared with the wild-type strain.

Figure 3. Activity of total MnP secreted from control strain U2 and PsMnt recombinant MT-20 over time in cultures containing 30 μM (A) or 10 μM (B) MnSO4. Asterisks indicate significant differences from control strain U2, as determined using the Student’s t-test ($P < 0.05$).

Although control strain U2 showed almost constant ratios in the tested range, the co-transformants showed much higher relative MnP activity at lower Mn(II) concentrations. Time-course analysis of the MnP activity of U2 and MT-20 in cultures containing 30 or 10 μM MnSO4 are shown in Fig. 3A and B, respectively. At both Mn(II) concentrations, both strains secreted MnP 2 days after inoculation and showed maximum activity.
Table 2. Cellular accumulation of Mn, MnP activity and PsMnt expression by co-transformant MT-20 and control strain U2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mn accumulation (ng mg⁻¹)</th>
<th>MnP activity (nkat ml⁻¹)</th>
<th>Relative PsMnt expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2</td>
<td>6.85 ± 0.12</td>
<td>0.35 ± 0.36</td>
<td>205.2 ± 16.4</td>
</tr>
<tr>
<td>MT-20</td>
<td>9.33 ± 0.32*</td>
<td>9.71 ± 0.38*</td>
<td>613.0 ± 38.2*</td>
</tr>
</tbody>
</table>

Dry mycelial weight. *Significant difference from control strain U2, as determined using the Student’s t-test (P < 0.05).

at 4 days of incubation. Although MT-20 consistently showed higher MnP activity (except for day 2), these strains exhibited similar MnP expression patterns in both cultures. From these results, we hypothesized that co-transformants exhibit higher Mn uptake activity and higher resulting intracellular Mn(II) concentrations.

The intracellular Mn concentration was compared in strains MT-20 and U2 cultured in 10 μM Mn(II). Because the amount of Mn accumulated in mycelia obtained after Mn(II) addition to Mn-deficient cultures did not reach the lower detection limit, the cultures were scaled up (10 to 50 ml), and strains were cultivated at constant Mn concentrations. As shown in Table 2, the amount of Mn accumulated in MT-20 mycelia was 1.36 times higher than that in U2 mycelia at 4 days of incubation. At 4 days of culture, the MnP activity of MT-20 was 2.8 and 3.0 times higher per unit volume and per unit mycelium dry weight, respectively, than the activity of U2. The relative level of PsMnt expression in MT-20 was also much higher. These results indicate that overexpression of PsMnt leads to incremental increases in the amount of Mn taken up and a resulting increase in MnP activity. Although the increase in MnP activity for Mn uptake seems reasonable, the values for the increment of the activity and Mn uptake with respect to the fold change in relative PsMnt expression appear to be too small. In S. cerevisiae, most Nramp-type Mn transporters (SMF1 and SMF2) are quickly degraded under physiologic conditions (Reddi, Jensen and Culotta 2005); therefore, it is assumed that the product of PsMnt overexpression is also degraded under these experimental conditions. It was reported that yeast SMFs are regulated by manganese and metal homeostatic protein BSD2. SMF proteins are delivered for the degradation to vacuole through the function of BSD2. On the other hand, there is no knowledge about the mechanism of degradation of smf homologue proteins and the presence of absd2-like gene of white-rot fungi. Additionally, in these questions, the investigation of the function of PsMnt during wood decay will be a future research subject.

CONCLUSION

Several transporter proteins mediate import and distribution of cellular Mn in all organisms, including white-rot fungi. Nevertheless, the expression and function of SMF Nramp family high-affinity Mn transporters in the white-rot fungi had not been explored. In this study, we found an smf2 homologue, PsMnt, in a P. sordida YK-624 cDNA library. Overexpression of PsMnt led to significantly enhanced Mn uptake and activity of MnP (a Mn-dependent enzyme). These results indicate that PsMnt is a high-affinity Mn transporter exhibiting significant Mn uptake activity under Mn-deficient conditions. By clarifying the action of PsMnt on ligninolysis in future research, some useful information will be obtained to elucidate the details of lignin degradation mechanism of white-rot fungi.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

Conflict of interest. None declared.

REFERENCE


