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An oligopeptide transporter gene family in *Phanerochaete chrysosporium*

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ABSTRACT

Oligopeptide transporters are believed to translocate their substrates across the membrane from the extracellular environment into cell. In the present study, sixteen oligopeptide transporter (OPT) orthologs (*PcOPT1* to *PcOPT16*) were identified in *Phanerochaete chrysosporium* strain BKMF1767. They were predicted to encode integral membrane proteins with 13 to 16 transmembrane domains. The cDNA and genomic sequences of these genes were confirmed by clone sequencing and Transcriptome Sequencing. Based on sequence similarities, the *PcOPT* genes can be grouped into three different subfamilies. Comprehensive analysis revealed correlations between gene groupings and gene structures. The *PcOPT* genes showed differential expression patterns at different metabolic stages or in various stress conditions, suggesting that they may play different functional roles. PcOPT14 was able to mediate the uptake of tetrapeptide KLGL when heterologously expressed in *Saccharomyces cerevisiae*. This study provides some basic genomic information for *OPT* genes in *P. chrysosporium* and will be useful for elucidating their roles in growth, stress response and survival of this organism. Moreover, the results can help us to better understand the complexity of metabolism of *P. chrysosporium*.

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1. Introduction

Peptide transport has been observed in all organisms from archaea, bacteria to plants and animals. The transport of extracellular peptides (2 to 6 amino acids in length) into cells, being an energy-dependent process, is accomplished by transporter systems located in the membrane. Transported peptides are hydrolyzed by peptidases into amino acids (aa), which are used for protein synthesis or as alternative sources of nitrogen and carbon (Perry et al., 1994; Steiner et al., 1995). Besides nutritional roles, peptide transport systems are also involved in some other cellular processes, such as bacterial quorum sensing, yeast mating and mammalian immune response (Stacey et al., 2002b). In addition, peptide transport systems can take up physiologically active peptide derivatives. For example, peptide transport systems in animals are pharmacologically important for the uptake of peptide-derived antibiotics (Okano et al., 1986a, 1986b), anticancer compounds (Inui et al., 1992) and mediating intracellular uptake of bacterial products that can induce inflammation and NF-κB activation (Zucchelli et al., 2009).

Based on sequence similarities and mechanisms of action, three peptide transport families, namely the ATP-binding cassette superfamily, the peptide transporter (PTR) family and the oligopeptide transporter (OPT) family were classified in plants. The plant ATP-binding cassette proteins utilize the energy from ATP hydrolysis to drive the transport of substrates such as peptides, metal chelates or glutathione conjugates (Oppedisano et al., 2002; Rea, 2007). However, the PTR and OPT families are energized by the proton-motive force (Hauser et al., 2001). Unlike the peptide transport system which transports nitrate and di/tripeptides (Chiang et al., 2004; Wang et al., 1998), the oligopeptide transport system highly favors the transport of tetra/pentapeptides and glutathione (Lubkowitz et al., 1997; Stacey et al., 2002a; Zhang et al., 2004). Longer peptides up to eight amino acids in length could also be transported by oligopeptide transporter system (Reuß and Morschhäuser, 2006). OPT, firstly described in the pathogenic yeast *Candida albicans* (Lubkowitz et al., 1997), has been found in bacteria (Yen et al., 2001), fungi (Lubkowitz et al., 1998) and plants (Koh et al., 2002; Zhang et al., 2004) by database comparisons. The OPT proteins may be involved in four different processes: long-distance metal distribution, nitrogen mobilization, heavy metal sequestration and glutathione transport (Cao et al., 2011). These different processes may play roles in metabolism, growth development, etc. Structurally, the OPT proteins are predicted to contain about 16 trans-membrane segments (TMS). It is documented that the 16-TMS proteins might have arisen from a 2-TMS precursor-encoding genetic element which was subject to three sequential duplication events (Gomolplitinant and Saier, 2011). Since OPTs are supposed to be functional in peptide uptake, the expansion or fusion of the TMS might make excellent physiological sense in evolution (Cao et al., 2011).

Abbreviations: OPT, oligopeptide transporter; PcOPT, oligopeptide transporter from *Phanerochaete chrysosporium*; PTR, peptide transporter; TMS, trans-membrane segments; *Ka*, nonsynonymous substitution rate; *Ks*, synonymous substitution rate; RT-PCR, semi-quantitative reverse transcription-PCR.

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OPTs have been further studied both in plants, like *Arabidopsis* (Koh et al., 2002) and rice (Vasconcelos et al., 2008), and fungi like *C. albicans* (Lubkowitz et al., 1997; Reuß and Morschhäuser, 2006). However, there is limited information about OPTs in white rot fungi such as *Phanerochaete chrysosporium*, which makes essential contributions to global carbon cycling by efficiently degrading the recalcitrant aromatic biopolymer lignin by lignin peroxidases and other enzymes. As a model microorganism for studying the physiology and genetics of lignin degradation (Cullen and Kersten, 2004), the genome of *P. chrysosporium* has been sequenced and annotated (Martinez et al., 2004; Wymelenberg et al., 2006). Since then, proteomic work has been carried out on this intensively researched white rot fungus (Fernandez-Fueyo et al., 2012; Vanden Wymelenberg et al., 2009, 2010). The lignin peroxidases are extracellular and produced during secondary metabolism, caused by nutrient starvation (Tien and Kirk, 1988b). However, the yield of lignin peroxidases from *P. chrysosporium* is poor and instable, which becomes a major obstacle in using this fungus for enzyme production. This is mainly due to the poor understanding of the regulatory mechanisms of *P. chrysosporium* responding to different nutrient sources in the culture media, such as carbon or nitrogen sources, trace elements, lignin materials, etc. Recently, eight *OPT* genes were identified to be involved in the nitrogen metabolism (Vanden Wymelenberg et al., 2009) and two *OPT* genes (Expressed Sequence Tags: SSH445 and SSH621) were shown to be significantly upregulated in the secondary metabolism when comparing to the primary metabolism in *P. chrysosporium* (Jiang et al., 2009). Thereafter, our group reported two putative *OPT* genes (Protein ID: 4874 and 25929) using suppression subtractive hybridization (SSH) and dot hybridization (Wu and Zhang, 2010). One of them (Protein ID: 4874) was detected in the period of secondary metabolism, which was found involved in nitrogen metabolism and identified as a homolog of sexual differentiation process protein ISP4 of *Schizosaccharomyces pombe* (Sato et al., 1994), and the other one (Protein ID: 25929) was identified in the period of metabolic switching phase. The different expression patterns suggested that oligopeptide transport genes might play an important role in metabolism process.

In this study, we firstly reported the identification and characterization of 16 *OPT* genes in *P. chrysosporium* by genome-wide approach. The detailed analysis on the sequence homologies and gene organizations of *PcOPT* genes were presented. In addition, adaptive evolution, expression patterns to various forms of environmental stress and functions in peptide uptake were also investigated. These results provide important information on the molecular basis of peptide transport for further functional investigations and facilitate a better understanding of the molecular mechanisms of the lignin metabolism in *P. chrysosporium*.

2. Materials and methods

2.1. Strains, vectors and culture conditions

The strain *P. chrysosporium* BKM-F-1767 (ATCC 24725) was used for DNA and RNA extractions in this study. The fungus was grown on potato dextrose agar plates for 5 days at 37 °C. The conidia were harvested from the plates by passing them through a cheese cloth as described previously (Feng and Zhang, 1999; Wu and Zhang, 2010). The concentration of conidium suspension was adjusted to OD_{600} ~ 1.0. Three milliliters of the suspension were inoculated into 50 mL regular Kirk low nitrogen medium (LN medium) with 0.2 g/L ammonium bitartrate, 1% glucose, 1 mg/L thiamine and some other components (Tien and Kirk, 1988a). The cultures were statically incubated at 39 °C in 500-mL Erlenmeyer flasks with rubber stopper. As to environmental stresses, different concentrations of ammonium bitartrate, glucose or thiamine were added to the LN medium, and variable oxygen supplies or culture temperatures were set to incubate the cells (Supplementary Table S1 for details). The mycelia were harvested at two different time intervals, 2 days (2d, representing metabolic switching phase) and 6 days (6d, representing secondary metabolism phase), for RNA extractions. The RNA was used for semi-quantitative RT-PCR to investigate the expression patterns of *PcOPT* genes.

Saccharomyces cerevisiae PB1X-9B (*MATa ura3-52 leu2-3,112 lys1-1 his4-38 ptr2-2*) (Perry et al., 1994), being deficient in di/tripeptide transporter genes, was used to investigate the functions of heterogenous transporter genes as described previously (Lubkowitz et al., 1997). Briefly, *S. cerevisiae* PB1X-9B cells were maintained on YPD medium. The minimal medium, which was made by adding 10 mL 10× filter sterilized YNB (Yeast Nitrogen Base; Difco) without amino acids to 90 mL sterile water containing 2 g glucose, 0.5 g ammonium sulfate and other auxotrophic supplements (Sherman et al., 1986), was used for yeast transformant selection and functional test. For those experiments where proline was used as low nitrogen source, YNB without amino acids and without ammonium sulfate was supplemented with 0.1% proline instead of ammonium sulfate. The mutant strain *S. cerevisiae* PB1X-9B was grown in Synthetic Complete medium (SC), which consisted of minimal medium with histidine, uracil, lysine and leucine. *S. cerevisiae* PB1X-9B transformed with p426GPD and its derivatives were grown on SC lacking uracil (SC-Ura).

Vector pMD18-T (Takara, Dalian, China) was used for the T/A cloning of PCR products. The plasmid p426GPD (ATCC 87361) (Mumberg et al., 1995), which was kindly provided by Dr. Melinda Hauser and Dr. Steve Minkin, University of Tennessee, was used for functional analysis of peptide uptake.

2.2. Searching for OPT family genes in P. chrysosporium

The searches of *OPT* genes in *P. chrysosporium* were keyword-based. Since the first identified *OPT* gene in *S. pombe* was designated as "Sexual Differentiation Process Protein" (Sato et al., 1994), the keywords "Sexual Differentiation Process Protein" and "Oligopeptide Transporter" were used to search against the genome database of *P. chrysosporium* (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html), and only those annotated as oligopeptide transporters were considered as putative *OPT* genes in *P. chrysosporium*.

2.3. Cloning of cDNA and genomic DNA sequences of PcOPT

Frozen mycelia were ground in liquid nitrogen and total RNAs were extracted using TRIzol reagent (Invitrogen, CA). Genomic DNAs were removed from RNA samples by DNaseI (Takara, Dalian, China) digestion. Complementary DNAs (cDNAs) were synthesized by RLM-RACE (for full-length, RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends, Invitrogen, CA) method according to the manufacturer's protocol. The cDNAs of *PcOPT* genes were amplified by specific primers designed on putative coding region sequences of *OPT* genes of *P. chrysosporium* RP78 (gene model: e_gww2.16.107.1, *P. chrysosporium*v2.0) (Martinez et al., 2004). The amplified products were ligated to pMD18-T vector and transformed into *Escherichia coli* (*E. coli*) DH5α. Genomic sequences of *PcOPT* genes were amplified with the same above primers using genomic DNAs as templates. All sequences of *PcOPT* gene were verified by sequencing. The full length sequences of some cDNA which failed to be amplified were obtained by segmental amplification or assembled by Transcriptome Sequencing.

2.4. Sequence analysis

Multiple sequence alignment for OPT protein sequences was generated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Thompson et al., 2002). Phylogenetic tree was constructed using the MEGA 3.1 program by neighbor-joining method (www.megasoftware.net) (Kumar et al., 2004). Gene structure organizations were analyzed

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