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Analysis of the oligopeptide transporter gene family in *Ganoderma lucidum*: structure, phylogeny, and expression patterns

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Abstract: Oligopeptide transporters (OPTs) are believed to transport broad-ranges of substrates across the plasma membrane from the extracellular environment into the cell and are thought to contribute to various biological processes. In the present study, 13 putative OPTs (Gl-OPT1 to Gl-OPT13) were identified through extensive search of *Ganoderma lucidum* genome database. Phylogenetic analysis with OPTs from other fungi and plants indicates that these genes can be further divided into five groups. Motif compositions of OPT members are highly conserved in each group, indicative of functional conservation. Expression profile analysis of the 13 Gl-OPT genes indicated that, with the exception of Gl-OPT7-9, for which no transcripts were detected, all paralogues were differentially expressed, suggesting their potential involvement in stress response and functional development of fungi. Overall, the analyses in this study provide a starting point for elucidating the functions of OPT in *G. lucidum*, and for understanding the complexities of metabolic regulation.

Keywords: Expression patterns; Heavy metal stress; Gene structure

Introduction

Organisms must transport various organic and inorganic molecules across the cell plasma membrane for use as nutrient sources. Intake of these substrates is vital for all living organisms, and several types of transporters are involved in this process. Peptides are transported into cells and hydrolyzed by peptidases into amino acids, which are used for protein synthesis or as alternative sources of nitrogen and carbon. Based on sequence similarity and mechanism of action, three peptide transporter families have been identified in plants: the ABC-type transporter family, the nitrate transporter 1/peptide transporter (NRT1/PTR) family, and the oligopeptide transporter family (OPT) (Rentsch et al. 2007; Zhao et al. 2010; Lran et al. 2014), each of which recognizes and transports different types of peptides and their derivatives across membranes in an energy-dependent manner.

The OPT family, favoring the transport of tetra- and pentapeptides and glutathione, was first characterized in yeast (Lubkowitz et al. 1997). Other *OPT* genes have also been identified in archaea, bacteria (Yen et al. 2001), fungi (Lubkowitz et al. 1998) and plants (Koh et al. 2002), but not found in animals (Lubkowitz 2006). OPT proteins appear to have diverse biological functions. OPT2 from the budding yeast, localized to the peroxisomes, has been reported to associate with glutathione redox homeostasis and drug detoxification (Elbaz-Alon et al. 2014). The coding sequence of AtOPT4 was expressed in a *Saccharomyces cerevisiae* sulfur amino acid auxotrophic mutant strain and the protein conferred growth in the presence of GSH as the sole sulfur source as well as sensitivity to [³⁵S]GSH (Zhang et al. 2015). CaOPT1 has been demonstrated to support the growth on

tetrapeptide KLLG as the sole leucine and lysine source (Lubkowitz et al. 1997). AtOPT3 has been characterized in iron transport and play roles in long-distance iron transport and partitioning (Wintz et al. 2003). Besides above mentioned biological functions, *OPT* genes have been demonstrated to have differential expression profiles upon growth on medium with different nitrogen sources and at different culture period (Hartmann et al. 2011; Quan et al. 2016; Reuss and Morschhauser 2006; Skyba et al. 2016; Xiang et al. 2013). Different biological functions and expression patterns of OPT may contribute to metabolic regulation, growth development, and stress adaptation.

The substrate transfer abilities or expression profiles of several OPT homologs have been partially described in plants and fungi, including *Arabidopsis* (Koh et al. 2002), rice (Liu et al. 2012b; Vasconcelos et al. 2008), *Candida albicans* (Lubkowitz et al. 1997; Reuss and Morschhauser 2006) and *Panerochaete chrysosporium* (Xiang et al. 2013). However, there is limited information on the OPTs of macro-fungi such as *Ganoderma lucidum*. *G. lucidum* produces various bioactive compounds that are involved in multiple therapeutic activities (Boh et al. 2007), making this fungus in high demand in Traditional Chinese medicines. More than 400 such compounds have been identified in *G. lucidum* so far (Shiao 2003). Like other white rot basidiomycetes, *G. lucidum* secretes enzymes that effectively decompose cellulose, hemicellulose and lignin, which is useful for biomass utilization, fiber bleaching, and organo-pollutant degradation (Ko et al. 2001). Owing to its importance in medical and environmental areas, the genome of *G. lucidum* was recently sequenced and annotated (Chen et al. 2012b; Liu et al. 2012a). Moreover, several transcriptomic analyses

of the secondary metabolism and growth development of this fungus have been conducted (Ren et al. 2013; Xu et al. 2012). Despite these studies, the secondary metabolisms of *G. lucidum*, including the ganoderic acids (GAs) biosynthesis pathway (Xu and Zhong 2012), are still poor understood.

Addition of methyl jasmonate induces GAs production in *G. lucidum*, corresponding with the up-regulation of the *OPT* gene (TDF187) (Ren et al. 2013). This suggests that OPTs may be involved in GAs biosynthesis. In this study, we first report the identification and characterization of 13 putative *OPT* genes in *G. lucidum* following a genome-wide search. Next, a detailed analysis of sequence homologies and gene organizations of Gl-*OPT* genes are presented. In addition, conserved motifs and expression patterns under various forms of environmental stress were also investigated. These results provide insights on the molecular basis of peptide transport, which will be useful for further functional investigations, facilitating a better understanding of molecular metabolic mechanisms in *G. lucidum*.

Materials and methods

Identification of *OPT* genes in *Ganoderma lucidum*

Putative members of the *OPT* gene family were identified in the *G. lucidum* genome using BLASTP. A reference OPT protein (SpISP4, Gene ID: 2540364) from *Schizosaccharomyces pombe* was chosen as a query sequence for searching the *G. lucidum* genome database (<http://genome.jgi.doe.gov/Gansp1/Gansp1.home.html>). Protein sequences with an E-value lower than the cutoff of 1E-10 were selected as candidate

proteins. Additional searches were also performed using a keyword query. Obtained sequences were further confirmed using the CDD (Conserved Domain Database) (Marchler-Bauer et al. 2009; Marchler-Bauer et al. 2011) and Pfam databases (Bateman et al. 2004). The physical and chemical parameters of putative GI-OPT proteins were computed using ProtParam in the ExPASy Proteomics Server (<http://www.expasy.org/tools/%20protparam.html>). The prediction of trans-membrane helices was performed with TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) (Krogh et al. 2001). Subcellular localizations were predicted using the WoLFPSORT program (<http://wolfpsort.org/>) (Horton et al. 2007).

Phylogenetic analysis, gene structure, and conserved motif identification

Multiple sequence alignments of amino acid sequences were performed using the program ClustalX (version 2.0) (Larkin et al. 2007). Phylogenetic analysis of the OPT proteins was performed using the neighbor-joining method in MEGA v6 (Tamura et al. 2013). The exon/intron organizations of the *OPT* genes were analyzed online using the Gene Structure Display Server (GSDS; <http://gsds.cbi.pku.edu.cn/>) by alignment of the CDS of each gene with its corresponding genomic DNA sequence (Hu et al. 2014). Structural motif annotation was performed using the MEME program (Bailey et al. 2015).

Strain and culture conditions

The wild domesticated variety of *G. lucidum* strain No.1 Rongbao was used in this study. The fungus was grown on potato dextrose agar plates for 7 days at 30 °C. Three mycelial discs from the actively growing peripheral region were inoculated into 50 mL

basal medium (Fahraeus and Reinhammar 1967) or Potato Dextrose Agar medium (PDA). The cultures were statically incubated at 30 °C in the dark. For nutrient stress studies, 2 g glucose or 0.25 g asparagine was used in basal medium. For heavy metal treatments, 200 mM CdCl₂ or Pb(NO₃)₂ were added to liquid PDA medium. For expression analysis of the *Gl-OPT* genes, total RNA was extracted (see below) from samples after 15 and 30 days of growth. The 30-day-old mycelia cultured by PDA medium were used to analyze the transcript profiles of *Gl-OPTs* in mycelium growth stage. *G. lucidum* fruiting bodies were obtained from Chengdu Rongbao Biological Science and Technology Development Co. Ltd (<http://www.scrongbao.com/>).

RNA extraction and real-time RT-PCR analysis

Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1.5 µg of total RNA using a reverse transcription kit (TaKaRa), and synthesized cDNAs were used as templates in the following PCR reactions.

RT-PCR was performed in a 10 µL reaction volume containing 12.5 ng cDNA, 5µL SsoFast™ EvaGreen® Supermix (Bio-Rad, USA), 400 nM of each primer, and nuclease-free water to a final volume of 10 µL. Primers used in RT-PCR are shown in Table S2. Reactions were carried under the following conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 63 °C for 20 s and 72 °C for 26 s. A melting curve analysis was performed to confirm that a single product was generated by each reaction. A negative control (water) was included in each run. Thermal cycling, fluorescence data collection, and

data analysis were carried out with the iCycler iQ5 thermocycler (Bio-Rad, USA) detection system according to the manufacturer's instructions. The relative expression level of each *Gl-OPT* gene was standardized to the expression level of the *G. lucidum* constitutive gene ribosomal protein L4 (RPL4) (Xu et al. 2014), with the formula $Y = 10^{-(\Delta Ct/3)} \times 100\%$, (Chen et al. 2014; Chen et al. 2012a), where ΔCt is the differences in the cycle threshold value of the target *Gl-OPT* gene and the control RPL4 products. Mean values were obtained from three biological replicates.

Results

Identification of *OPT* genes in *G. lucidum*

In order to identify members of the *OPT* gene family in *G. lucidum*, we searched the genome database of *G. lucidum* (<http://genome.jgi.doe.gov/Gansp1/Gansp1.home.html>) using SpISP4 as a query. Keyword-searches were also conducted. The obtained sequences were further confirmed to be OPTs using the CDD (Conserved Domain Database) (Marchler-Bauer et al. 2009; Marchler-Bauer et al. 2011) and Pfam databases (Bateman et al. 2004). As a result, a total of thirteen putative OPTs were identified in *G. lucidum*. These were given the names *Gl-OPT1*– *Gl-OPT13* (Table 1).

The *Gl-OPT* genes encode highly hydrophobic polypeptides (grand average hydrophobicities of 0.267 to 0.508) ranging from 681 to 819 amino acids in length, with predicted protein isoelectric points ranging from 5.31 to 8.8. These features are similar to those of the OPT proteins of other fungi (Lubkowitz et al. 1998; Reuss and Morschhauser 2006; Xiang et al. 2013). The *Gl-OPT* proteins generally contain 12–16 transmembrane

helices, with molecular weights ranging from 72 to 92 kDa. Protein subcellular localization prediction revealed that, with the exception of Gl-OPT2 and Gl-OPT4, all candidate Gl-OPT proteins exhibited a 100% probability of being localized to the plasma membrane. Gl-OPT2 and Gl-OPT4 are likely localized to membranes such as the endoplasmic reticulum or peroxisome.

Analysis of Gl-OPT phylogeny and gene structures

In order to examine the relationships among *OPT* genes, we created a phylogenetic tree with sequences of Gl-*OPT* genes and those of *OPT* genes from other species. Some fungi and plant *OPT* genes have been found experimentally to transport oligopeptides or metal chelates (Fig. 1). The results show that *OPT* genes can be classified into five groups according to their phylogenetic relationships, and these have been designated Groups 1–5. Two *OPT* proteins from plants (OsOPT1 and AtOPT1) were grouped together (Group 2). Group 3 includes *OPT*s from *C. albicans* and *S. cerevisiae*, which belong to the basidiomycota. Most members in Group 3 have been demonstrated to be involved in peptide transport (Hauser et al. 2000; Lubkowitz et al. 1997; Reuss and Morschhauser 2006). *OPT* proteins in the other three groups are those that originate from ascomycota and basidiomycota.

Comparative analysis of gene structures may provide clues for understanding the evolution of gene families. Thus, exon-intron boundaries for the 13 Gl-*OPT* genes were compared. As shown in Fig. 2, with the exception of Gl-*OPT4*, which contains 33 exons, Gl-*OPT* genes contain fewer than 20 exons. Our phylogenetic analysis indicates that genes

that are grouped together usually possess similar gene structures. For example, the stretch from the third to the fifth exons is identical in *Gl-OPT3* and *Gl-OPT5*, as evidenced by identical exons that are connected by slashes in Fig. 2b. Not only are intron positions shared, but intron phases are also shared between some *Gl-OPT* genes. For example, *Gl-OPT 8* and *Gl-OPT 13* share virtually identical introns positions and phases.

Conserved domains and motifs of OPT proteins

CCD analysis was used to identify structurally conserved domains in OPT proteins. However, this type of analysis is unable to recognize smaller, individual motifs and those with more divergent patterns. Thus, MEME (<http://meme.sdsc.edu>) (Bailey et al. 2015) was used to study the diversification motifs in *Gl-OPT* proteins. As a result, ten distinct motifs were identified in these proteins (Table 2) (Fig. 1). As mentioned above, phylogenetic analysis divided *OPT* genes into five groups. Most of the closely related members in each of these groups share common motif compositions, suggesting functional similarities between group members (Fig. 1). Among the ten motifs, only motifs 1 and 2 are shared by all OPT proteins. Members in Group 5 only possess these two motifs. With the exception of *PcOPT1*, *PcOPT3*, and *PcOPT4*, all members in Group 1 possess all ten motifs. OPT proteins in plants are divided into the PT and YSL subfamilies, each with specific motifs (Cao et al. 2011). It is currently unknown whether the distinct motifs among the diverse groups play unique functional roles. However, the fact that OPT proteins from the same group possess similar conserved motifs provides additional support to the results of the phylogenetic analysis. Moreover, differences in motif composition among different groups

may point to functional diversification of OPT proteins.

Analysis of Gl-*OPT* expression during various developmental stages

In order to better understand the potential roles of *OPT* genes in *G. lucidum*, the expression patterns of each Gl-*OPT* gene during various growth stages, including in the mycelium and fruiting body, were monitored by real time RT-PCR. The transcript levels of *RPL4*, which is expressed at stable levels under various fermentation conditions and in different tissues, was used as internal control (Xu et al. 2014). Genes, whose expression levels exhibited at least a two-fold increase or decrease under various conditions, were considered to be up- or down-regulated in response to stress. Quantitative data revealed that no transcripts were observed for Gl-*OPT6*, Gl-*OPT7*, Gl-*OPT8*, or Gl-*OPT9*, while the transcripts of all other Gl-*OPTs* were detectable in both of the two growth stages. All of these genes, exhibited distinct but partially overlapping expression profiles (Fig. 3). Among the detectable transcripts, Gl-*OPT4* and Gl-*OPT11* were abundantly expressed, while Gl-*OPT10* exhibited a relatively low expression level. Transcript levels of Gl-*OPT1*, Gl-*OPT2*, and Gl-*OPT11* were 1.9, 3.4, and 3.9 times higher, respectively, in the mycelium stage than in the fruiting body. In contrast, the transcript levels of Gl-*OPT3*, Gl-*OPT4*, Gl-*OPT10*, and Gl-*OPT12* were 13.9, 2.5, 3.2, and 5.3 times higher in the fruiting body than in the mycelium. Gl-*OPT5* and Gl-*OPT13* exhibited relatively similar expression levels in both the mycelium and fruiting body stages, suggesting that they may have multiple functions in *G. lucidum* or that they may be involved in housekeeping functions during *G. lucidum* growth and development.

Analysis of *Gl-OPT* expression in response to nutrient stress

In order to gain insight into the transcription of *Gl-OPT* genes under nutrient-stress conditions, transcript levels in the presence of low concentrations of carbon and nitrogen were assessed by RT-PCR. Because fermentation time can also affect metabolic products, the mycelium was sampled at two time points (15 d and 30 d). Except for *Gl-OPT7*, *Gl-OPT8*, and *Gl-OPT9*, for which no transcripts were detected, all other *Gl-OPTs* exhibited altered expression profiles (Fig. 4) under low carbon or nitrogen treatment.

Under low carbon conditions, *Gl-OPT2*, *Gl-OPT3*, and *Gl-OPT10* were up-regulated on day 15, while *Gl-OPT1*, *Gl-OPT6*, and *Gl-OPT11* were down-regulated. These expression changes were most conspicuous in *Gl-OPT1* and *Gl-OPT3*. *Gl-OPT3* was six times more highly expressed, while *Gl-OPT1* expression was reduced by a factor of ten. In contrast to the results from day 15, *Gl-OPT1*, *Gl-OPT2*, *Gl-OPT12*, and *Gl-OPT13* were up-regulated on day 30 under low carbon conditions, while *Gl-OPT4*, *Gl-OPT5*, *Gl-OPT10*, and *Gl-OPT11* were significantly down-regulated to varying degrees. The greatest change in expression occurred in *Gl-OPT10*, which was drastically reduced under limited carbon resources. In low-nitrogen medium, *Gl-OPT6*, *Gl-OPT10*, and *Gl-OPT11* were down-regulated in samples from day 15. Similar to the results of low-carbon stress, four *Gl-OPT* genes (*Gl-OPT1*, *Gl-OPT2*, *Gl-OPT12*, and *Gl-OPT13*) were up-regulated and five (*Gl-OPT3*, *Gl-OPT4*, *Gl-OPT5*, *Gl-OPT10*, and *Gl-OPT11*) were down-regulated in the samples from day 30.

From the above results, it is clear that all detectable *Gl-OPT* genes exhibited

differential expression, under carbon or nitrogen stress. This suggests that *G. lucidum* does indeed employ the OPT family under nutrient stress. In addition, the transcriptional profiles of Gl-OPT genes changed significantly over time, which may be in response to the changing concentrations of nutrients during the course of incubation. As the nutrient supply decreases, relevant transport systems may be up- or down-regulated in order to the environment.

Analysis of Gl-OPT expression in response to heavy metals

OPT genes are suspected to play roles in long-distance heavy metal transport in fungi and plants. To examine whether the expression of Gl-OPT family members is regulated by heavy metal stress, *G. lucidum* hyphae were cultured in the presence of 200 mM CdCl₂ and Pb(NO₃)₂. Among the 13 Gl-OPT genes, ten genes exhibited differential expression patterns under heavy metal stress treatment, while no transcripts were detected for three genes (Gl-OPT7, Gl-OPT8 and Gl-OPT9) (Fig. 5, Table S3).

In response to lead treatment, the expression levels of four genes (Gl-OPT10, Gl-OPT11, Gl-OPT12 and Gl-OPT13) were reduced in samples from day 15. In contrast, with the exception of Gl-OPT10 and Gl-OPT13, lead stress enhanced the transcription of all other detectable Gl-OPT genes in sample from day 30. Gl-OPT13 was down-regulated and Gl-OPT10 expression remained stable under lead treatment in the samples from 30.

In response to cadmium treatment, the expression levels of seven genes (Gl-OPT1, Gl-OPT4, Gl-OPT5, Gl-OPT10, Gl-OPT11, Gl-OPT12 and Gl-OPT13) were decreased and that of one gene (Gl-OPT2) was increased in the samples from day 15. Cadmium stress lead

to enhanced transcription of all Gl-*OPT* genes except Gl-*OPT13* in samples from day 30. Up-regulation of genes was more conspicuous in samples from day 30 than those from day 15, exhibiting increases of 2.2- (Gl-*OPT4*) to 44.4-fold (Gl-*OPT1*) over expression under the normal conditions.

Discussion

A search of the *G. lucidum* genome database identified 13 putative *OPT* genes (Gl-*OPT1*–Gl-*OPT13*). Sixteen and eight putative *OPT* genes have been identified in *C. albicans* (Reuss and Morschhauser 2006) and *P. chrysosporium* (Xiang et al. 2013), respectively. Although multiple mechanisms contribute to the evolution of multigene families, comprehensive analysis of a phylogenetic tree, exon/intron gene structures, and conserved motifs allow us to make certain predictions and generalizations about the possible origin of *OPTs*, as well as the relationships among *OPT*. Phylogenetic analysis suggests that *OPT* proteins from fungi and plants can be divided into five subgroups. *OPT* proteins from plants cluster together and comprise Group 2. Group 3 consists of *OPT* proteins from the ascomycota. The other three groups contain *OPT* proteins from both the basidiomycota and ascomycota. Phylogenetic analyses can reveal evolutionary conservation (Cao et al. 2011). The random distribution of Gl-*OPT* genes across the phylogenetic tree suggests that the gene duplication events that gave rise to the Gl-*OPT* genes occurred before the separation of *C. albicans*, *P. chrysosporium*, and *G. lucidum* from a common ancestor. Genes with the same functions are often closely related (Afoufa-Bastien et al. 2010; Hu et al. 2010). Such a trend is also found in *OPT* genes. For example, Group 3,

includes CaOPT and ScOPT2 proteins, most of which are involved in peptide transport (Hauser et al. 2000; Lubkowitz et al. 1997).

Differences in the exon/intron structures between genes can be used, to some extent, to determine the evolutionary history of gene families (Lecharny et al. 2003). In the present work, comparative analysis of exon/intron boundaries in *G. lucidum* genes revealed that the number and position of introns are highly conserved among some *OPT* genes in *G. lucidum*. Gl-*OPT8* and Gl-*OPT13* share virtually identical exon/intron patterns. Based on analysis of intron positions in orthologous genes from various eukaryotic lineages, it is inferred that the common ancestor of animals and plants possessed an intron-rich genome (Rogozin et al. 2005). Thus, Gl-*OPT4*, which possesses the most introns, may be more closely represent the ancestral *OPT* gene, while intron-loss may have occurred in other Gl-*OPT* genes. However, more information, especially the chromosomal distribution of the *G. lucidum* *OPT* genes, is necessary to more clearly understand the evolutionary history of *G. lucidum* *OPT* genes.

OPT family members have been experimentally characterized in various fungi and plants. *OPT* proteins do not share a common biological function and may be involved in diverse functions such as oligopeptide transport (Reuss and Morschhauser 2006), glutathione transport (Cagnac et al. 2004; Pike et al. 2009), heavy metal detoxification and homeostasis (Lubkowitz et al. 1998; Victoria et al. 2012), long distance nitrogen allocation (Koh et al. 2002). Our expression analysis of the Gl-*OPT* gene family showed that nine genes exhibit transcript accumulation across two growth stages, suggesting that these genes

may be involved in housekeeping functions during *G. lucidum* growth. Transcripts of *Gl-OPT7-9* were not detected under any condition in this study, and more researches should be performed to make sure whether they are pseudogenes. OPT proteins have been shown different profiles of transcriptional response to nutrient or heavy metal stress (Hartmann et al. 2011; Xiang et al. 2013). However, little is known about the role of *Gl-OPT* genes in response to stress. Based on the results of expression analysis, we gleaned some understanding of the role of *Gl-OPT* genes in response to stress. Ten of the 13 *Gl-OPT* genes exhibited differential expression in response to nutrient and heavy metal stress. Transcriptional profiles of most *Gl-OPT* genes were changed under low glucose or asparagines conditions. One possible explanation for this is that *G. lucidum* does employ the function of this family to adapt nutrimental stress. Although *Gl-OPT6* expression was not detected under several normal growth conditions, this gene was specifically up-regulated under cadmium treatment (Fig. 5). Interestingly, a lot of detectable *Gl-OPT* genes were up-regulated under heavy metal treatment after 30 days, with only *Gl-OPT13* being down-regulated. Transcriptional profile of *Gl-OPT* gene family did change greatly during the later growth stage, which might a response to the changing concentrations of the metals during the course of incubation. Although it is premature to conclude that these *OPT* genes are involved in heavy metal stress in *G. lucidum*, the above results imply the action of *Gl-OPT* genes in response to heavy metal stress.

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Table 1 Putative oligopeptide transporter genes identified in the *Ganoderma lucidum* genome

Gene name	Genomic position	CDS	Protein	Mol Wt	pI [†]	GRAVY [§]	TMs	P.L. [‡]
			Length	(kDa) [†]				
Gl-OPT1	scaffold_10:1696394-1699036	2175	724	81.62	6.46	0.412	14	plas: 26
Gl-OPT2	scaffold_1:1298969-1302665	2436	811	91.48	8.68	0.353	15	plas: 22, E.R.: 3
Gl-OPT3	scaffold_11:610059-613598	2298	765	86.25	7.96	0.372	12	plas: 25
Gl-OPT4	scaffold_5:2002661-2007294	2460	819	92.53	7.98	0.373	16	plas: 23, pero: 2
Gl-OPT5	scaffold_11:626013-628899	2337	778	87.67	7.94	0.355	15	plas: 26
Gl-OPT6	scaffold_3:2562431-2565761	2328	775	87.25	7.59	0.369	14	plas: 26
Gl-OPT7	scaffold_3:2359131-2362230	2088	695	74.23	8.93	0.508	13	plas: 26
Gl-OPT8	scaffold_7:834937-838016	2262	753	84.68	5.37	0.267	13	plas: 26
Gl-OPT9	scaffold_5:1721901-1725213	2253	750	83.46	8.36	0.335	16	plas: 26
Gl-OPT10	scaffold_14:542286-546000	2355	784	85.78	6.67	0.328	13	plas: 26

Gl- <i>OPT11</i>	<u>scaffold_12:1358955-1362067</u>	2181	726	77.38	7.10	0.445	13	plas: 27
Gl- <i>OPT12</i>	<u>scaffold_15:144626-147528</u>	2046	681	72.68	5.63	0.492	15	plas: 26
Gl- <i>OPT13</i>	<u>scaffold_9:954967-958114</u>	2286	761	84.93	5.81	0.285	15	plas: 26

[†] Mol Wt: molecular weight; ^{*} pI: protein isoelectric point; [§] GRAVY grand average of hydropathicity; ^{||} Number of transmembrane helices predicted with TMHMM Server; [¶] P.L : PSORT predictions: Plas (plasma membrane), E.R. (endoplasmic reticulum), pero. (peroxysome).

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Table 2 Motif sequences identified by MEME

Motif	E-value	Multilevel consensus sequence
1	1.4e-574	[QT][AS][NT][FM][TL][SQ]D[LF]K[LT]G[HQ]Y[ML]K[IL]PPR[AV][MT]F[YV][VC]Q[LY][IL] [GA]
2	1.8e-666	[VT][LM][SA][TS][QA][LF][IL]G[FY][GS][LI][AG]G[IL][LA]R[RK][FL][LV]Y[YW][PK][PT][SK] [MA][IV][WY]P[SA][NT]L[VP][TV][CV][AT]L[FN][NQ] [TA]LH
3	1.8e-459	W[WF][TK]KYNY[YI]L[SA][AT][AG]L[DN][AG]G[TV][AQ][IV][SM][ST][IF][IL][FS]F[AC][VL]
4	6.7e-392	[LT]NPGP[FY][NT][IV]KE[HQ][VA][LI][IA][TS][IL][MF][AS][SN][VT][GA]
5	4.4e-334	[YE][FW][IV]P[GS][YF]F[PQ][AL]L[ST][GY][FY][NS][WI]VI[CT][WL][IA][VK][PQ][ND] [NS]
6	5.2e-245	[RK][LM]M[KS][KA]Y[PK][EQ]VP[ED]WWY[LA]
7	7.3e-410	[GS][LT]N[VQ][LI][TV][EQ][LM][IV][IX]G[YA][AV][LI]PG[RK]P[IV]A[NM][ML][YM][FI][KS][TA][YW]G[YH]
8	8.5e-419	[SR][PN][YG][PE][EV][VA][RS][AS][AV][VL][SA][NP][TY]DDP[TS][ML]P[VA][NL]T[FL]R[AT]

		[WY][FV][LI]G[LI][IL]
9	4.0e-364	[YW][YF][TS]N[TV][WK][YN][AS][QK][YF][LFM]P[IF][SM][ST][TN][GS][LS][FY][DT][NR][TN] [G[QT][PS]Y[ND][VQ][TS]
10	2.4e-299	A[LI][SA]Y[GL][LI][SA]F[AS][SL][IV][TS]A[TV][IL][TV]H[TV][FI]L[YW][YF][RG]K[QD]I[WM]][VT][QR][AF][RK][RS][SA][LM][SR][ED][QK][PK]D[IV]H

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Figure captions:

Fig. 1 Phylogenetic relationships and motif compositions based on amino acid sequences of *OPT* genes within *G. lucidum* and other species. (a) Phylogenetic tree was generated by Mega6.0 using the neighbor-joining method. The five major groups are designated from Group 1 to 5. Groups with circles and triangles have been functionally demonstrated to transport oligopeptides and metal compounds, respectively. (b) A schematic representation of conserved motifs and P value (obtained by MEME) in *OPT* proteins. Detailed information of *Gl-OPT* genes from *G. lucidum* are given in Table 1, and gi numbers of *OPTs* from other species are given as: *PcOPT1*, gi|430007707|; *PcOPT2*, gi|430007709|; *PcOPT3*, gi|4300077011|; *PcOPT4*, gi|4300077013|; *PcOPT5*, gi|4300077015|; *PcOPT6*, gi|4300077017|; *PcOPT7*, gi|4300077019|; *PcOPT8*, gi|4300077022|; *PcOPT9*, gi|4300077024|; *PcOPT10*, gi|4300077026|; *PcOPT11*, gi|4300077028|; *PcOPT12*, gi|4300077030|; *PcOPT13*, gi|4300077032|; *PcOPT14*, gi|4300077034|; *PcOPT15*, gi|4300077036|; *PcOPT16*, gi|4300077038|; *AtOPT1*, gi|9758213|; *OsOPT1*, gi|1002228577|; *SpISP4*, |19112445|; *ScOPT1*, gi|6322249|; *ScOPT2*, gi|6325452|; *CaOPT1*, gi|51094117|; *CaOPT2*, gi| 87045960|; *CaOPT3*, gi| 87045962|; *CaOPT4*, gi| 87045966|; *CaOPT5*, gi| 87045970|; *CaOPT6*, gi| 87045972|; *CaOPT7*, gi| 87045976|; *CaOPT8*, gi| 87045980|.

Fig. 2 Phylogenetic relationships and gene structure schematic of Gl-*OPT* genes. (a) Phylogenetic tree drawn as described in Figure 1 using *AtOPT1* as an outgroup. (b) Black boxes represent exons and black lines represent introns. Intron phases are shown on top of introns. Identical exons are connected with slashes.

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Fig. 3 Expression profiles of *Gl-OPT* genes during different developmental stages. qRT-PCR analyses were used to assess *Gl-OPT* transcript levels in mycelium (M) and fruiting body (F). The relative expression levels of each *Gl-OPT* gene was indicated as percentage to the expression of internal control, the constitutively expressed *RPL4*. The value represents the expression percentage of *Gl-OPT* gene accounts for the expression of *RPL4*. Data represent means of three replicates and denote relative expression levels.

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Fig. 4 Expression profiles of *Gl-OPT* genes in response to nutrient stress. qRT-PCR analyses were used to assess *Gl-OPT* transcript levels in response to low carbon and nitrogen concentrations. CK-15, LC-15, and LN-15 refer to samples collected after 15 d under normal, low carbon and low nitrogen conditions, respectively, while CK-30, LC-30, and LN-30 refer to samples collected after 30 d under normal, low carbon, and low nitrogen conditions, respectively. The relative expression levels of each *Gl-OPT* gene was indicated as percentage to the expression of internal control, the constitutively expressed *RPL4*. The value represents the expression percentage of *Gl-OPT* gene accounts for the expression of *RPL4*. Data represent means of three replicates and denote relative expression levels.

Fig. 5 Expression profiles of *Gl-OPT* genes in response to cadmium (Cd^{2+}) and lead (Pb^{2+}) treatments. qRT-PCR analyses were used to assess *Gl-OPT* transcript levels in response to Cd^{2+} and Pb treatments. CK-15, Cd^{2+} -15, and Pb^{2+} -15 refer to samples collected after 15 d under normal, cadmium, and lead treatment conditions, respectively, while CK-30, Cd^{2+} -30, and Pb^{2+} -30 refer to samples collected after 30 d under normal, cadmium, and lead treatment conditions, respectively. The relative expression levels of each *Gl-OPT* gene was indicated as percentage to the expression of internal control, the constitutively expressed *RPL4*. The value represents the expression percentage of *Gl-OPT* gene accounts for the expression of *RPL4*. Data represent means of three replicates and denote relative expression levels.

650x300mm (300 x 300 DPI)

400x139mm (300 x 300 DPI)

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253x183mm (300 x 300 DPI)

297x198mm (300 x 300 DPI)

281x175mm (300 x 300 DPI)

Table S1 Identity matrix for the putative *Ganoderma lucidum* OPT genes

	Nucleotide Identity (%)												
	GI-OPT1	GI-OPT2	GI-OPT3	GI-OPT4	GI-OPT5	GI-OPT6	GI-OPT7	GI-OPT8	GI-OPT9	GI-OPT10	GI-OPT11	GI-OPT12	GI-OPT13
Amino acid identity (%)	GI-OPT1	55.97	59.35	53.58	59.12	58.41	42.59	47.22	46.29	46.51	44.54	41.55	48.22
	GI-OPT2	43.98	62.40	55.30	59.40	58.95	44.43	46.58	45.77	45.23	44.63	40.08	45.85
	GI-OPT3	44.85	51.55	64.65	79.34	75.62	46.24	49.14	49.07	49.28	48.42	44.48	49.44
	GI-OPT4	44.59	46.46	62.57	63.50	63.76	42.84	44.24	45.28	44.31	43.74	40.22	44.24
	GI-OPT5	45.07	49.20	77.75	62.58	72.70	45.91	48.22	46.93	47.43	46.87	42.83	48.40
	GI-OPT6	45.28	48.59	75.10	62.17	72.4	44.71	48.39	47.90	47.17	47.10	42.77	47.82
	GI-OPT7	17.89	17.55	14.65	15.14	16.22	16.56	41.09	39.63	42.00	74.21	43.03	42.01
	GI-OPT8	29.26	27.35	28.25	27.49	26.93	27.40	18.71	54.76	52.02	42.19	39.51	79.73
	GI-OPT9	29.31	27.82	27.38	26.69	27.11	27.02	16.59	44.41	49.03	39.80	38.99	53.85
	GI-OPT10	25.62	25.38	27.43	25.17	27.64	28.01	16.19	37.09	35.29	42.06	37.98	50.93
	GI-OPT11	18.14	19.66	16.92	16.72	17.13	17.63	78.76	18.11	16.14	16.10	43.57	42.81
	GI-OPT12	17.37	16.23	16.64	15.02	16.78	15.31	26.44	15.68	17.48	14.48	28.13	40.50
	GI-OPT13	28.97	25.83	28.13	26.33	26.46	25.95	18.23	85.92	43.80	37.90	17.60	15.08

Table S2 Gene-specific primers used for Real-time RT-PCR amplification of *Ganoderma lucidum* *OPT* genes.

Gene name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Gl- <i>OPT1</i>	TGGGCTCGTTCTTCTGGTTC	ATTCCAAGGCCGCCAGATAC
Gl- <i>OPT2</i>	ACCATTTCTTCACT TCGCGC	GTAAAACCAAGCGTGCGGAA
Gl- <i>OPT3</i>	TTCTTCGTCTTCCTCGGCTC	GAAGAGCTGGTTGACGACGA
Gl- <i>OPT4</i>	TGCCCTTTGGTTGTCTGGTT	GCGACAGGAAGCCGATCATA
Gl- <i>OPT5</i>	TTGACAAGGAGCTCGTCGTC	TAGTGTTCAACGGCATGTCC
Gl- <i>OPT6</i>	AACTATGTACTGTCCGCGGC	TAAAGGAGACGGTGTGCCC
Gl- <i>OPT7</i>	TTGGACGCTCTACCGGATTG	GATCGAGCCGCCAAAGAAAC
Gl- <i>OPT8</i>	GCCACAAACACTGCTCGTTT	TGAATGGCCCAGGGTTGATC
Gl- <i>OPT9</i>	TGCTACAAC TTCAGCCTGGG	TGGGTCGTCGATATCTTGCG
Gl- <i>OPT10</i>	TCATCGCTCTTCTCCTTGGC	AAGTAGAGGTTGCAACGGG
Gl- <i>OPT11</i>	GGTGTGATCTTCGACTGGCA	GGAGGCGTTGATACCGGAAA
Gl- <i>OPT12</i>	ATGGTCCTTTTCGCGGTTCT	TTGAGGAACCCAATCGCGAA
Gl- <i>OPT13</i>	AGAAGAAAGCCGTGGATGGG	AGACCTTGATCGCAACCTCG
RPL4	GTCAACAAGGGCGTTCTCTT	GTCAACAAGGGCGTTCTCTT

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Table S3 The relative expression levels of *Gl-OPT* genes under Pb^{2+} and Cd^{2+} treatments after 30 days

Gene name	Relative expression levels			Fold changes	
	30-CK	30- Pb^{2+}	30- Cd^{2+}	Pb^{2+} /CK	Cd^{2+} /CK
<i>Gl-OPT1</i>	0.006	0.261	0.296	39.116	44.379
<i>Gl-OPT2</i>	0.044	0.212	1.509	4.807	34.165
<i>Gl-OPT3</i>	0.008	0.061	0.253	7.499	30.922
<i>Gl-OPT4</i>	2.227	2.964	4.997	1.330	2.2432
<i>Gl-OPT5</i>	0.021	0.173	0.486	8.093	22.703
<i>Gl-OPT6</i>	0.017	0.060	0.544	3.404	30.753
<i>Gl-OPT10</i>	0.009	0.010	0.127	1.082	13.802
<i>Gl-OPT11</i>	0.881	1.970	3.388	2.234	3.8431
<i>Gl-OPT12</i>	0.055	1.127	0.580	20.128	10.363
<i>Gl-OPT13</i>	1.291	0.615	0.339	0.4764	0.2625

Note: up-regulated (fold change > 2) or down-regulated (fold change < 0.5) were respectively shown in red and purple under heavy metal treatments.