Metallothioneins mediated intracellular copper homeostasis in ectomycorrhizal fungus *Suillus indicus*

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**ABSTRACT**

Ectomycorrhizal fungi (ECM) are known to protect the host plant from heavy metal stress. But the information on molecular mechanisms involved in this process is still ambiguous. The present study intends on providing insight into the Cu detoxification mechanism in ECM fungus *Suillus indicus*, a new species isolated from north western Himalayas. Two metallothionein genes SuiMT1 and SuiMT2, were isolated from the S. indicus cDNA and characterized for their potential role in Cu-detoxification and homeostasis. The response of these genes to the extracellular concentrations of copper was studied by qPCR analysis. Both genes were actively induced under exogenous Cu stress, thus can be classified as Cu-thioneins. Further, the functional complementation of these genes in the Cu sensitive yeast mutant *cups1*, successfully restored their wild type phenotype of Cu tolerance. This shows that both SuiMT1 and SuiMT2 plays an important role in Cu detoxification and homeostasis in ECM fungus *S. indicus*.

**KEYWORDS:** Ectomycorrhizal fungi, *Suillus indicus*, metallothionein, copper, metal homeostasis, yeast complementation, qPCR

**INTRODUCTION**

Ectomycorrhizal fungi (ECM) forms a mutualistic association with the plant roots thus providing them with various nutrients and protecting them from various biotic and abiotic stresses. It is very well known that these ECM fungi protect plants from heavy metals, drought, salinity, pests and pathogens and extreme environments, but the exact mechanisms involved are still inexplicit. There are different mechanisms in ECM fungi to protect itself from heavy metals like, cell wall binding (Bano et al., 2018), extracellular chelation, metal efflux (Ruytinx et al., 2013; Sacký et al., 2016; Benes et al., 2018) or their intracellular chelation through various metal binding ligands like metallothioneins and glutathione (Osobova et al., 2011; Reddy et al., 2016; Kalsotra et al., 2018; Khullar and Reddy, 2018, 2019a, b).

Metallothioneins (MTs) are a superfamily of low molecular weight proteins, ubiquitous and polyphyletic, that coordinates heavy metal ions by establishing the metal-thiolate bond through their highly abundant cysteine residues (Calvo et al., 2017). These metalloproteins are then accumulated into the vacuoles and later released as metallic complex (Bellion et al., 2006). They are usually characterized by highly conserved C-X-C, C-X-X-C, C-X-Y-C motifs (Ramesh et al., 2009; Reddy et al., 2014; Hložková et al., 2016; Zahid et al., 2016; Nguyen et al., 2017; Khullar and Reddy, 2018). It has numerous physiological and biological functions such as detoxification of toxic heavy metals, metal homeostasis, free radical scavenging and protection against oxidative stress. MTs are pervasive and present in both prokaryotes and eukaryotes. Different MTs exhibit different metal preferences under different conditions. Palacios et al. (2011) classified MTs into two categories as Cu-thioneins and Zn/Cd-thioneins as per their metal binding preferences (Palacios et al., 2011). Cu-thioneins form homometallic Cu-MT complexes when exposed to Cu and Zn/Cd thioneins form homometallic complexes when exposed to Zn or Cd, respectively with high degree of folding. However, when exposed to other metals they form heteronuclear complexes with a lower degree of folding and high thiol oxidation resulting in disulfide formation (Palacios et al., 2011). Inspite of all the information, MTs biological structure and their role in various physiological processes in diverse living systems are still a matter of debate. Although many studies have reported diverse MT genes isolated from prokaryotic bacteria (Solioz, 2018), mammals (Atiáñ-Blasco et al., 2017; Drozd et al., 2018), plants (Huang et al., 2018; Imam and Blindauer, 2018), animals (Li et al., 2016) and fungal species (Iturbe-Espinoza et al., 2016) etc, but not much has been reported in ectomycorrhizal systems. The metal binding properties of metallothioneins isolated from same organism are different for different metals and host species. Three MT isoforms isolated from *Amanita strobiliformis* (AsMT1, AsMT2, AsMT3) responded differently to different heavy metals. When subjected to different metals, AsMT1 was induced by copper and silver, whereas AsMT2 was induced by cadmium and AsMT3 was induced by zinc (Hložková et al., 2016). Similar observations were made in *L. bicolor*, where the two isoforms LbMT1 and LbMT2 were differentially induced by cadmium and copper, respectively (Reddy et al., 2014). Similar results have also been reported in *Hebeloma mesophaeum*, *Hebeloma cylindrosporum*, *Pisolithus albus*, *Paëllus involutus*, *Suillus luteus* and *Suillus himalayensis* (Bellion et al., 2007; Ramesh et al., 2009; Sacký et al., 2014; Reddy et al., 2016; Nguyen et al., 2017; Kalsotra et al., 2018).

Recently, a new species *Suillus indicus* was collected from the conifer forests of the north western Himalayas, India, forming ectomycorrhizal association with *Pinus wallichiana* and *Cedrus deodara* (Verma and Reddy, 2015). The fungus was shown to promote the growth of *Pinus wallichiana* seedlings in the nursery (data not provided). Observing the impact of *Suillus indicus*, we considered identifying its potential in metal detoxification. For this, two MTs (SuiMT1 and SuiMT2) were identified from the *S. indicus* cDNA, by designing the primers from the EST transcripts of *Suillus luteus* (Ruytinx et al., 2011). Further, the role of both MTs in copper detoxification was studied using real time PCR and yeast functional complementation.

**MATERIALS AND METHODS**

**Organisms, culture media and culture conditions:** The ectomycorrhizal fungus *Suillus indicus*, isolated from the north western region of Himalayas was maintained on malt
extract medium (2%) with pH 5.5 at 25°C in the dark (Verma and Reddy, 2015). The tolerance of *S. indicus* to copper was studied by growing the mycelium in MMN broth supplemented with increasing concentrations of copper (0, 100, 200, 300 and 400 μM as CuSO₄·5H₂O) at 25°C in dark for 21 days. After 21 days, the mycelium was harvested under each stress and washed with EDTA water followed by two washings with distilled water. The washed mycelium was then dried at 48°C and the dry weight was recorded as the effect of Cu on mycelial growth. Further, the intracellular metal accumulation was determined using atomic absorption spectroscopy.

The *E. coli* DH10β cells were used to maintain and propagate various plasmids according to standard protocols (Sambrook and Russell, 2001). The copper sensitive *Saccharomyces cerevisiae* strain used for yeast complementation assays- DTY4 (*cup1*), deleted the Cu MT CUP1 gene (*MATa*, *trp1-1*, leu2-3, leu2-112, gal1, his-, *ura3-50*, *cup1*::URA3) (Hamer et al., 1985) derived from DTY3 wild-type strain (*MATa*, *trp1-1*,leu2-3, leu2-112, gal1, his-, *ura3-50*, URA3). The DH cells were used to maintain and propagate the mycelium was performed in mastercycler® ep realplex real-time PCR system (Eppendorf AG, Hamburg, Germany) using SYBR® Green JumpStart™ TaqReady Mix™ (Sigma Aldrich) under the conditions recommended by the manufacturer. The reaction was performed in total volume of 25μL, consisting of 12.5 μL master mix, 1μL each of forward and reverse primer (Table 1), 0.75 μL cDNA template and 9.75 μL H₂O. The cycling program used for qPCR was as follow: 95°C for 2 min (1 cycle), 95°C for 15 s, 55°C for 15 s and 68°C for 20 s (40 cycles). The relative quantification of gene expression between samples was calculated using the comparative threshold (Ct) method (Heid et al., 1996). α-actin (SbAct) (Accession: AF155930) and β-tubulin (Accession: AY112730) of *Suillus bovinus* were used as reference genes. Since the NormFinder observed minimum stability value for α-actin, it was used for the comparative analysis. The amplification efficiency of gene was calculated by the equation E=[10⁻¹/ΔΔC⁻¹]. The E value so obtained (1.25) was used to calculate Cm value, where Cm=Cm×[log(1+E)/log2]. The Cm value was calculated for each sample and then the comparative expression level of the genes was given by the formula 2⁻ΔΔC (Livak and Schmittgen, 2001) where ΔΔCt was calculated by subtracting the baseline's ΔCt to the sample's ΔCt and where the baseline represents the expression level of the control. All measurements were performed on independent biological samples from three replicate experiments in three technical replicates.

### Bioinformatic analysis

The open reading frame (ORF) of both *SuiliMT1* and *SuiliMT2* sequences was identified using ORF finder. Both the sequences were then subjected to BLASTp analysis, to identify the homologous sequences. The homologous sequences so obtained were aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/) so as to identify the conserved C-x-C motifs. Further, the molecular weight and pI of the predicted proteins were calculated using Expasy tool (https://www.expasy.org/).

#### Expression analysis of *SuiliMT1* and *SuiliMT2* (qRT-PCR):

The two weeks culture of *S. indicus* grown on MMN agar plates overlaid with cellophane sheets was stressed with increasing concentrations of Cu (0, 100, 200, 300 and 400 μM) for 48 hours at 25°C. The stressed mycelium was then scrapped from the cellophane sheets and ground in liquid nitrogen. From each stressed sample total RNA was isolated and cDNA was synthesized as per the protocols mentioned previously. Gene expression analysis of *SuiliMT1* and *SuiliMT2* in the mycelium was performed in mastercycler® ep realplex real-time PCR system (Eppendorf AG, Hamburg, Germany) using SYBR® Green JumpStart™ TaqReady Mix™ (Sigma Aldrich) under the conditions recommended by the manufacturer. The reaction was performed in total volume of 25μL, consisting of 12.5 μL master mix, 1μL each of forward and reverse primer (Table 1), 0.75 μL cDNA template and 9.75 μL H₂O. The cycling program used for qPCR was as follow: 95°C for 2 min (1 cycle), 95°C for 15 s, 55°C for 15 s and 68°C for 20 s (40 cycles). The relative quantification of gene expression between samples was calculated using the comparative threshold (Ct) method (Heid et al., 1996). α-actin (SbAct) (Accession: AF155930) and β-tubulin (Accession: AY112730) of *Suillus bovinus* were used as reference genes. Since the NormFinder observed minimum stability value for α-actin, it was used for the comparative analysis. The amplification efficiency of gene was calculated by the equation E=[10⁻¹/ΔΔC⁻¹]. The E value so obtained (1.25) was used to calculate Cm value, where Cm=Cm×[log(1+E)/log2]. The Cm value was calculated for each sample and then the comparative expression level of the genes was given by the formula 2⁻ΔΔC (Livak and Schmittgen, 2001) where ΔΔCt was calculated by subtracting the baseline's ΔCt to the sample's ΔCt and where the baseline represents the expression level of the control. All measurements were performed on independent biological samples from three replicate experiments in three technical replicates.

### PCR primers designed for the amplification of *SuiliMT1* and *SuiliMT2* gene of *Suillus indicus* and for qPCR analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Forward Sequence</th>
<th>Primer Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>SuiliMT1</em></td>
<td>5’-CGGGATCCATATGATCACGCGTACAGGATGC-3’</td>
<td>5’-GGATCCATATGATCACGCGTACAGGATGC-3’</td>
</tr>
<tr>
<td><em>SuiliMT2</em></td>
<td>5’-GGATCCATATGATCACGCGTACAGGATGC-3’</td>
<td>5’-GGATCCATATGATCACGCGTACAGGATGC-3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Forward Sequence</th>
<th>Primer Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actin</td>
<td>5’-GTATGGCCACGGCATTGACGAG3’</td>
<td>5’-GGAGCAGCGAATCTGACTA3’</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>5’-GGAGTACGTACGGCGACTAC3’</td>
<td>5’-GGAGTACGTACGGCGACTAC3’</td>
</tr>
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Underlined sequences are BamH1 and EcoR1 sites.
Cloning of MT genes: Metallothionein genes SuiMT1 and SuiMT2 were amplified from the cDNA of Suillus indicus (as described previously). The genes so obtained, along with the yeast expression vector pFL61 were subjected to double digestion with restriction endonucleases EcoRI and BamHI for 4 hours at 37°C. The digested genes and plasmid were run on 1% agarose gel and the required bands were excised using Thermo Scientific GeneJet Gel Extraction kit as per the prescribed protocol. The digested genes were further ligated into pFL61 using T4 DNA ligase. Further, the ligated product (pFL61 + SuiMT1 and pFL61 + SuiMT2) was transformed into E. coli DH5α cells and the positive clones were screened on LA ampicillin plates by colony PCR followed by plasmid isolation. The plasmid so obtained (pFL61, pFL61 + SuiMT1 and pFL61 + SuiMT2) were further transformed into copper sensitive yeast mutant (cup1) using lithium acetate method (Stearns et al., 1990) and the positive clones were selected by their tendency to grow on complete synthetic medium without uracil (SD-Ura). The positive clones so obtained were further used for yeast functional complementation studies against copper stress.

Yeast functional complementation assays: For studying the functional complementation of SuiMT1 and SuiMT2 genes in yeast mutant cup1, two parallel experiments: drop test and growth kinetics were performed. For Drop test, cultures of cup1, yeast cells carrying pFL61, pFL61 + SuiMT1 and pFL61 + SuiMT2 were grown in SD-Ura media for 24 hours at 30°C and 220 rpm. After 24 hours, optical density of all the three cultures was adjusted to OD =1.0. The cultures were serially diluted (10⁴, 10⁻² and 10⁻⁴) and 5µL of each dilution was spotted on SD-Ura plates supplemented with and without 150 mM CuSO₄. Plates were incubated for 3 days at 30°C and photographed. In a parallel experiment, to analyze the growth kinetics of the SuiMT1 and SuiMT2 transformants, 20 mL of fresh SD-Ura media were inoculated with mid-exponential pre-cultures of cup1 containing pFL61, pFL61 + SuiMT1 and pFL61 + SuiMT2 to attain a starting optical density of 0.02 at 600 nm. All the cultures at O.D 0.02 were grown at 30°C in a rotary shaker (220 rpm) for 5 hours followed by addition of 150 µM CuSO₄ to transformants. The cells were allowed to grow for next 48 hours, where the growth of each culture was monitored by taking the O.D₆₀₀ at every 3 hours interval. The data obtained was plotted as a graph and analysed by ANOVA. The means were compared with Tukey’s test at P<0.05. All the analysis was performed by using Graph Pad Prism 5.1 software.

RESULTS
Effect of copper on the growth and metal uptake of S. indicus: When subjected to different concentrations of copper (0, 100, 200, 300, 400 µM), the growth of S. indicus was adversely affected. Approximately 16 mg of fungal biomass was procured after 21 days when grown in 50 mL unstressed MMN broth. However, the fungal biomass declined when the same broth was supplemented with different concentrations of copper. The half minimum inhibitory concentration of Cu (IC₅₀) was observed at 170 µM, where the fungal biomass was reduced to 50% (Fig. 1a). However, the Cu uptake by the mycelium was found to increase as a function of external Cu concentration. At 100 µM of external Cu, approximately 0.75 µg of Cu was uptaken per mg of fungal dry weight. However, at 400 µM of external Cu, the metal uptake was found to elevate up to 1.22 µg/mg of fungus (Fig. 1b).

Sequence analysis of SuiMT1 and SuiMT2: Five transcripts were identified from the putative MTs from the EST library of Suillus luteus (Ruytinx et al., 2011). Amongst the five transcripts, two distinct putative MTs were opted and their specific primers were synthesized (Table 1). Both MTs were amplified from the Suillus indicus cDNA and sequenced. The sequence analysis of both SuiMT1 and SuiMT2, revealed that both the sequences showed high homology with metallothionein genes reported from various basidiomycetes. Further, the multiple sequence analysis of the homologous

Fig. 1 Effect of different concentrations of copper on a) mycelium growth and b) metal uptake in ectomycorrhizal fungus Suillus indicus, when grown on MMN medium supplemented with CuSO₄.
sequences highlighted three conserved C-x-C motifs, which are the main characteristic to metallothioneins (Fig. 2). SuimT1 consists of 105 bp ORF coding for 34 amino acids with a predicted molecular weight of 3.4 kDa and pl 5.9. SuimT2 consists of 108 bp ORF coding for 35 amino acids with a predicted molecular weight of 3.5 kDa and pl 4.14. Both the sequences were rich in cysteine (20.5% cysteine) and had no aromatic amino acids.

Expression analysis of both SiMT genes using qPCR: The impact of Cu on the induction of both SuimT1 and SuimT2 genes was studied using quantitative real time PCR analysis. The mRNA accumulation of both the genes increased rapidly when exposed to increasing concentrations of Cu. At an initial stress of 100 µM, the expression of SuimT1 was induced 1.5 times whereas SuimT2 was induced 3 times of the control. Further, on increasing the Cu concentration to 400 µM there was increased SuimT1 mRNA accumulation by ~7 folds and that of SuimT2 by ~13 folds (Fig. 3). However, the expression of reference gene actin remained unaltered. This shows that the expression of SuimT2 is more induced under Cu stress than SuimT1.

Functional complementation in yeast mutants: The role of both SuimT1 and SuimT2 in Cu tolerance was validated by their functional complementation into the Saccharomyces cerevisiae mutant cup1Δ (sensitive to copper) and wild type DTY3. Both genes were ligated into pFL61 vector and transformed into cup1Δ and DTY3. The growth of transformants was then monitored on SD-ura medium supplemented with and without Cu (150 µM). Drop test analysis revealed that the transformants carrying SuimT1 and SuimT2 effectively restored the Cu tolerance in yeast mutant cup1Δ, whereas the same mutant when transformed with only pFL61 could not survive (Fig. 4a). Similarly, in liquid assay, yeast mutant cup1Δ when transformed with pFL61 could not grow at Cu -150 µM, whereas the transformants carrying pFL61+SuimT1 and pFL61+SuimT2 successfully restored the Cu tolerance in cup1Δ (Fig. 4b). The wild type DTY3 was used as a positive control.

DISCUSSION

ECM fungi are very well known for their potential role in protecting the host plant from heavy metal stress (Colpaert et al., 2011; Khullar and Reddy, 2018, 2019a, b). A new ECM species Suillus indicus has been recently isolated from the conifer forest of northwestern Himalayas, India (Verma and Reddy, 2015). The present study focuses on identifying the response of S. indicus to Cu stress and the detoxification mechanisms involved in it. When exposed to increasing concentrations of Cu, the growth of S. indicus abated. The half minimum inhibitory concentration was recorded at ~170 µM.
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which is almost half of the IC₅₀ value reported for *S. himalayensis* (322µM) (Kalsotra et al., 2018). The Cu uptake tendencies of *S. indicus* were also comparable with that of *S. himalayensis*.

Two MT genes SuIMT1 and SuIMT2 were identified from *S. indicus* cDNA using the primers designed from EST transcripts of *S. luteus*. Both the genes showed homology with the already reported MTs from various basidiomycetes such as *Amanita strobiliformis* (AsMT2; AG004615), *Serendipita indica* (SiMT1; ACT83730), *Laccaria bicolor* (LbMT1; AH143933), *Russula atropurpurea* (RaMT1; AHA31882), *Fomitopsis rosea* (FrMT1; TFY60696) and *Coprinopsis cinerea* (CcMT1; XP001833429) (Leonhardt et al., 2014; Reddy et al., 2014; Hložková et al., 2016). Binz and Kägi classified MTs into 15 families based on their length and primary structure of sequences (Binz and Kägi, 1999). The length of the MTs varied from 24 amino acids to 257 amino acids. Both SuIMT1 and SuIMT2 genes belong to the family 8 in the above-mentioned classification.

Copper and zinc are the two most potential inducers of metallothioneins in ectomycorrhizal systems (Palacios et al., 2011; Khullar and Reddy, 2018). The intracellular accumulation of metal ions triggers the rapid transcriptional induction of MTs, which in turn sequester metal ions, thus minimizing their toxicity. Cu-induced metallothioneins have been reported in various ECM fungi like *Pisolithus albus* (PaMT1) (Reddy et al., 2016), *Laccaria bicolor* (LbMT1 and LbMT2) (Reddy et al., 2014), *Amanita strobiliformis* (AsMT1c) (Osobova et al., 2011), *Hebeloma cylindrosporum* (HcMT1) (Ramesh et al., 2009), *Paxillus involutus* (PiMT) (Bellion et al., 2007), *Suillus luteus* (SIMT1 and SIMT2) (Nguyen et al., 2017), and *Suillus himalayensis* (ShMT1 and ShMT2) (Kalsotra et al., 2018). MTs are induced by the same metal ions that bind to the MT proteins, leading to the direct activation of the defense mechanisms (Waalkes and Goering, 1990; Khullar and Reddy, 2018). Both SuIMT1 and SuIMT2 are actively induced under Cu stress, thus they can be defined as potential Cu-thioneins. Since different MTs respond differently in the same organism (Khullar and Reddy, 2018), in *S. indicus*, SuIMT2 has been more rapidly induced than SuIMT1 under Cu stress. Many studies have reported the presence of C-X-C, C-X-X-C or C-X-Y-C motifs in proteins primary sequence as the main characteristic feature of metallothionein. The primary protein sequence of *L. bicolor- MT1, A. strobiliformis MT1 and MT2, P. albus MT1, P. albus MT1, S. himalayensis MT1 and MT2* had three conserved C-X-C motifs where as *L. bicolor MT2, H. cylindrosporum MT2, H. mesophaeum MT2 and MT3* had six conserved C-X-C motifs and *S. luteus MTa and Mtb, A. strobiliformis MT3* had five C-X-C motifs with one C-X-Y-C motif conserved (Bellion et al., 2007; Ramesh et al., 2009; Reddy et al., 2014; Sácky et al., 2014; Hložková et al., 2016; Reddy et al., 2016; Nguyen et al., 2017; Kalsotra et al., 2018). In both SuIMT1 and SuIMT2, three conserved C-X-C motifs were found.

The function of both SuIMT1 and SuIMT2 in Cu-detoxification was validated using a functional complementation assay in Cu-sensitive yeast mutant cup1Δ. Transformation of the yeast mutant with SuIMT1 and SuIMT2 under the control of phosphoglycerokinase (PGK) promoter successfully restored the Cu tolerance capability of *S. cerevisiae* mutant cup1Δ. Similar observations have been reported in *L. bicolor, H. cylindrosporum, S. himalayensis, S. luteus, P. albus and P. involutus*, where the yeast mutant when transformed with the fungal MT gene successfully restored its metal tolerance ability (Bellion et al., 2007; Ramesh et al., 2009; Reddy et al., 2014; Reddy et al., 2016; Nguyen et al., 2017). Thus, it can be concluded that the MTs- SuIMT1 and SuIMT2, contribute to Cu homeostasis and detoxification in ectomycorrhizal fungus *S. indicus* and may be responsible for protecting the host plant under the Cu stress. The present study provided insight into the mechanisms involved in Cu detoxification in ectomycorrhizal fungus *S. indicus*. This understanding can further help in using fungus as the model organism for studying Cu detoxification mechanism or in developing various bioremediation technologies.

**CONCLUSION**

The present study provides insight into the molecular mechanism involved in copper detoxification in ECM fungus *S. indicus*. The characterization of two genes SuIMT1 and SuIMT2 highlighting their potential role in complementing the Cu sensitivity in cup1Δ of *S. cerevisiae* proves that they are Cu-thioneins and could play an important role in Cu homeostasis in *S. indicus*.

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