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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 *cup1*$^{\Delta}$, 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 (Atrián-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*, *Paxillus involutus*, *Suillus luteus* and *Suillus himalayensis* (Bellion *et al.,* 2007; Ramesh *et al.,* 2009; Sácký *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 $CuSO_4.5H_2O$) 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 washing 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*$^{\Delta}$), deleted the Cu MT CUP1 gene (*MATα*, *trp1-1*, *leu2-3*, *leu2-112*, *gal1*, *his−*, *ura3-50*, *cup1*$^{\Delta}$*::URA3+*) (Hamer *et al.,* 1985) derived from DTY3 wild-type strain (*MATα*, *trp1-1*, *leu2-3*, *leu2-112*, *gal1*, *his*$^{-}$, *ura3-50*, *URA3*$^{-}$).

RNA isolation, cDNA synthesis and gene amplification: Mycelial discs (7mm diam.) of *Suillus indicus* were cultured on MMN-agar plates overlaid with cellophane sheets for 15 days at 25˚C in the dark. After 15 days, the mycelium was transferred along with cellophane sheet to MMN medium supplemented with different concentrations of copper (0, 100, 200, 300 and 400 µM as $CuSO_4.5H_2O$) and incubated for 48 hours at 25˚C in the dark. The mycelium was then scrapped and crushed in liquid nitrogen. The total RNA was extracted from the crushed mycelia using the QiAzol lysis reagent (QIAGEN, Germany). The integrity of the isolated RNA was checked by formaldehyde agarose gel electrophoresis and RNA concentration was determined by measuring the absorbance at 260 nm on nanodrop. cDNAs were synthesized from approximately 5 µg of total RNA using "The revert aid first strand cDNA synthetic kit" (Thermo Fisher Scientific, U.S) as per manufacturer's instructions. Amplification of *SuiMT1* and *SuiMT2* genes were performed with the primers (**Table 1**) designed from EST sequences (GR975901, GR975896, GR975714, GR975715, GR975716) of putative MTs of *S. luteus*.

PCR reactions were carried out in 25 µL reaction containing 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP mix, 1µL of forward and reverse primer (10µM) (**Table 1**),1.5 U Taq polymerase (Sigma-Aldrich), 1µL template and nuclease free water. Starting with an initial denaturation at 94°C for 3 min, *SuiMT1 and SuiMT2* fragments were amplified for 35 cycles comprising 1 min at 94°C, 1 min at 55°C and 1 min at 72°C followed by final extension at 72°C for 8 min. PCR products were run on 1.5% (w/v) agarose gel and visualized by ethidium bromide staining. The amplified product was purified by using GeneJet PCR purification kit (Thermo scientific) and ligated in pMD20 vector (TA cloning kit, Takara). The ligated products were transformed into *E.coli* DH10β $CaCl_2$ competent cells by heat shock method (Singh *et al.,* 2010) and plated on Luria Agar plates supplemented with ampicillin, IPTG and X-gal for blue-white screening. The white colonies were randomly selected and verified by colony PCR. The positive colonies so obtained were sequenced and analyzed using various bioinformatic tools.

Table 1: PCR primers designed for the amplification of *SuiMT1* and *SuiMT2* gene of *Suillus indicus* and for qPCR analysis

Gene Name	Primer	Sequence
SuiMT1	*SuiMT1*F	5'-CGGGATCCATGTCCACCGCTACTGAAGTC -3'
	*SuiMT1*R	5'-CCGGAATTCTCAACATTTGCACTCTCCAGG -3'
SuiMT2	*SuiMT2*F	5'-CGGGATCCATGTCCACCGCTACTGAAGTC -3'
	*SuiMT2*R	5'- CCGGAATTCTCAATCAACATTGCACTCTCCAG -3'
α-actin	SactF	5'-GTATTGCCGACCGTATGCAG -3'
	SactR	5'-GGAGCGACGATCTTGACCTA -3'
β-tubulin	StubF	5'-GTGGACTCTAGCGGACCTAC -3'
	StubR	5'-CCCTGAGTCGCTAGTGAAGT -3'

Underlined sequences are *Bam*H1 and *Eco*R1 sites

Bioinformatic analysis: The open reading frame (ORF) of both *SuiMT1* and *SuiMT2* 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 *SuiMT1* and *SuiMT2* (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 grounded 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 *SuiMT1* and *SuiMT2* 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 H_2O. The cycling program used for qPCR was as follow: 95^0C for 2 min (1 cycle), 95^0C for 15 s, 55^0C 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 $E=[10^{(-1/slope)}]$. The E value so obtained (1.25) was used to calculate C_{t1} value, where $C_{t1}=C_{te}x$ [log(1+E)/log2]. The C_{t1} value was calculated for each sample and then the comparative expression level of the genes was given by the formula $2^{-\Delta\Delta CT}$ (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.

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 *Eco*R1 and *Bam*H1 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*$^{\Delta}$) 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*$^{\Delta}$, two parallel experiments: drop test and growth kinetics were performed. For Drop test, cultures of *cup1*$^{\Delta}$, 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$_{600}$=1.0. The cultures were serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and 5μL of each dilution was spotted on SD-Ura plates supplemented with and without 150 mM CuSO$_4$. 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*$^{\Delta}$ 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 CuSO$_4$ to transformants. The cells were allowed to grow for next 48 hours, where the growth of each culture was monitored by taking the O.D$_{600}$ 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 (IC$_{50}$) 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

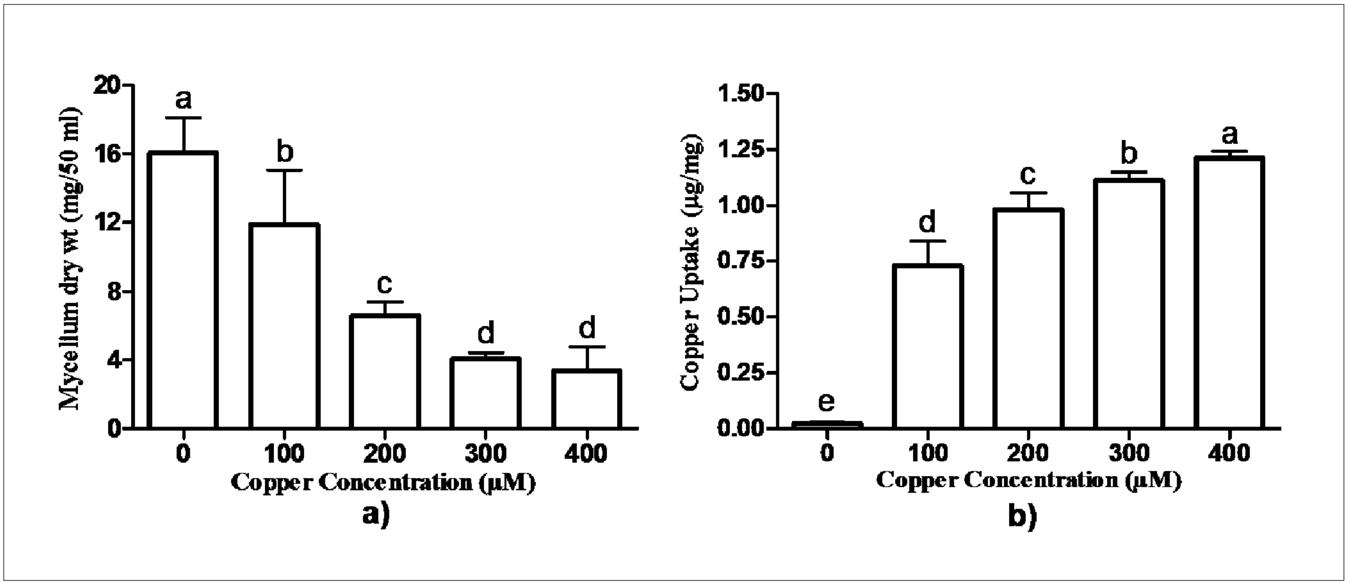
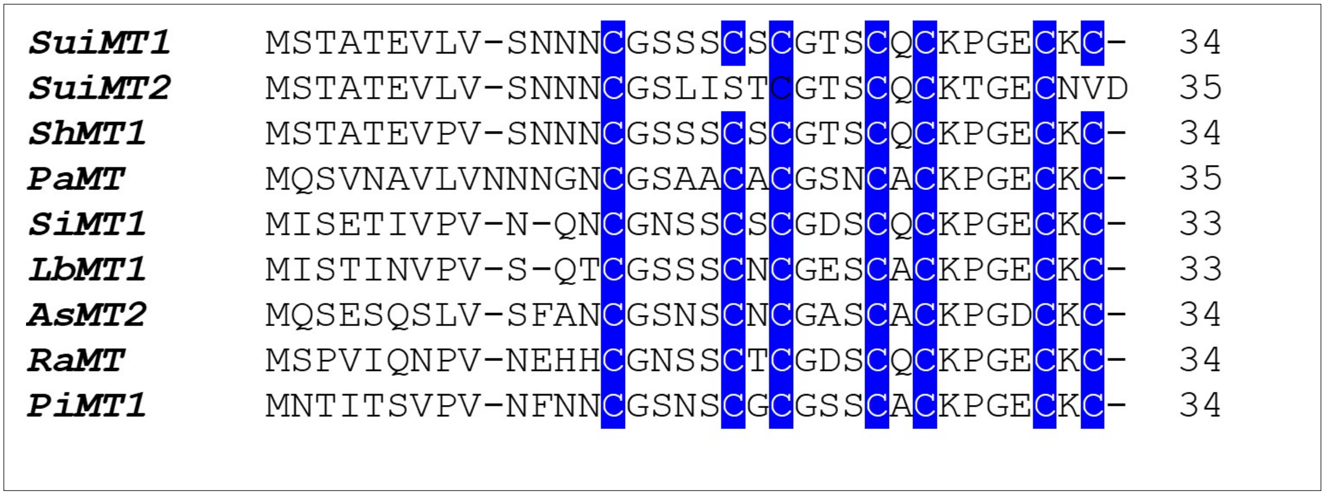


Fig. 1 Effect of different concentrations of copper on **a**) mycelium growth and **b**) metal uptake in ectomycorrhizal fungus *Suillus indicus*, when grown on MMN medium supplemented with CuSO$_4$.



SuiMT1	MSTATEVLV-SNNNCGSSSCSCGTSCQCKPGECKC-	34
SuiMT2	MSTATEVLV-SNNNCGSLISTCGTSCQCKTGECNVD	35
ShMT1	MSTATEVPV-SNNNCGSSSCSCGTSCQCKPGECKC-	34
PaMT	MQSVNAVLVNNNGNCGSAACACGSNCACKPGECKC-	35
SiMT1	MISETIVPV-N-QNCGNSSCSCGDSCQCKPGECKC-	33
LbMT1	MISTINVPV-S-QTCGSSSCNCGESCACKPGECKC-	33
AsMT2	MQSESQSLV-SFANCGSNSCNCGASCACKPGDCKC-	34
RaMT	MSPVIQNPV-NEHHCGNSSCTCGDSCQCKPGECKC-	34
PiMT1	MNTITSVPV-NFNNCGSNSCGCGSSCACKPGECKC-	34

Fig. 2. Multiple sequence alignment of *SuiMT1* and *SuiMT2* genes with their homologous sequences retrieved through BLASTp analysis. The conserved C-x-C motifs amongst metallothioneins from different basidiomycetes i.e *Suillus himalayensis* (*ShMT1*; AUS94321), *Pisolithus albus* (*PaMT*; AJO67962), *Serendipita indica* (*SiMT1*; ACT83730), *Laccaria bicolor* (*LbMT1*; AHI43933), *Amanita strobiliformis* (*AsMT2*; AG004615), *Russula atropurpurea* (*RaMT*; AHA31882), *Paxillus involutus* (*PiMT1;* AAS19463) were highlighted in blue.

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 pI 5.9. *SuiMT2* consists of 108 bp ORF coding for 35 amino acids with a predicted molecular weight of 3.5 kDa and pI 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 *cup1*$^{\Delta}$ (sensitive to copper) and wild type DTY3. Both genes were ligated into pFL61 vector and transformed into *cup1*$^{\Delta}$ 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 *cup1*$^{\Delta}$, whereas the same mutant when transformed with only pFL61 could not survive (**Fig. 4a**). Similarly, in liquid assay, yeast mutant *cup1*$^{\Delta}$ 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 *cup1*$^{\Delta}$ (**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

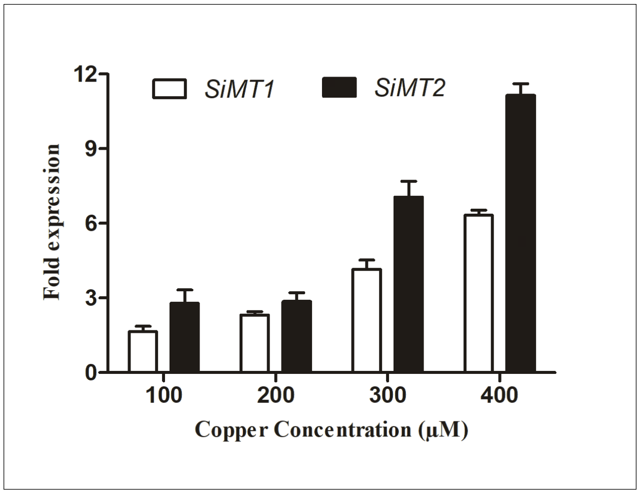


Fig. 3. Fold increase in the expression of *SuiMT1* and *SuiMT2* genes in *Suillus indicus*, when stressed with copper (0, 100, 200, 300, 400 µM) for 48 hours. The values are in reference with the control conditions (expression in free living fungus without metal stress) and are average of three biological replicates. Error bars are ±SD.

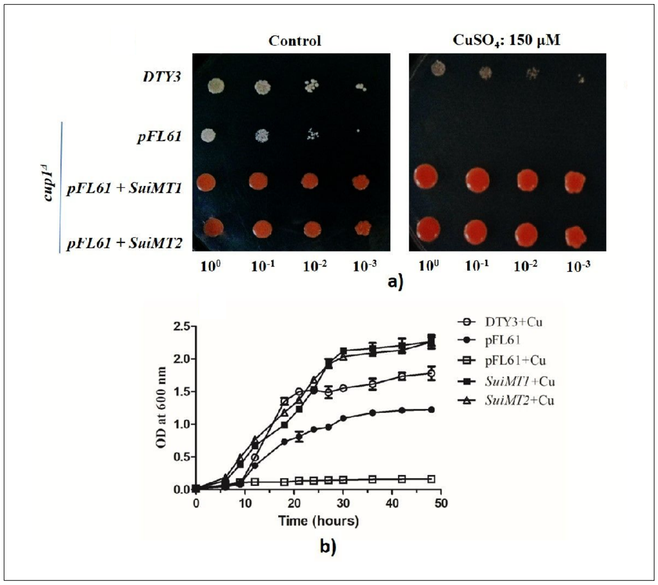


Fig. 4. Functional complementation of *SuiMT1* and *SuiMT2* genes in *S. cerevisiae* mutant *cup1*$^{\Delta}$ by a) drop test and b) liquid broth assay on SD-ura medium supplemented with and without 150 µM $CuSO_4$. The wild type DTY3 was used as the positive control.

which is almost half of the IC_{50} 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*; AHI43933); *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* (*SlMT1* and *SlMT2*) (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. involutus 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. stobiliformis 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; Sácký *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 *cup1*$^{\Delta}$. 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 *cup1*$^{\Delta}$. 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 *cup1*$^{\Delta}$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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