Cloning and characterization of metallothionein gene LbMT3 isolated from ectomycorrhizal fungus *Laccaria bicolor*

A Dissertation

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Submitted by

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(July 2016)

DECLARATION

I hereby declare that the work being presented in the M.Tech dissertation entitled "**Cloning and characterization of metallothionein gene LbMT3 isolated from ectomycorrhizal fungus *Laccaria bicolor***" has been carried out by me during the period of July 2015 to July 2016, under the guidance of Dr. M.S Reddy, Professor, Department of Biotechnology, Thapar University, Patiala. Further, I declare that I have not submitted the matter embodied in this dissertation for the award of any other degree or any other qualification of any university or examining body in India/elsewhere.

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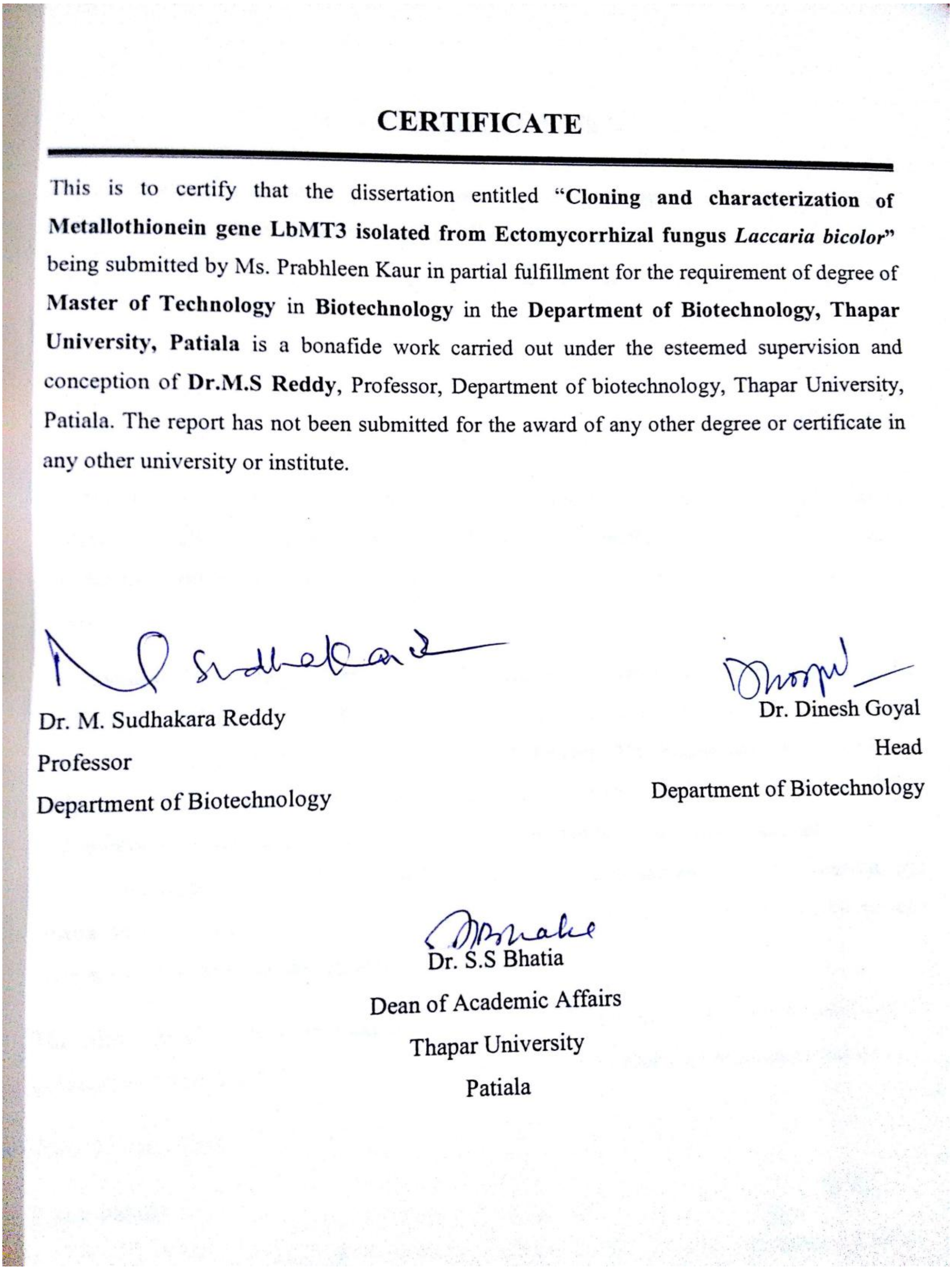

CERTIFICATE

This is to certify that the dissertation entitled "**Cloning and characterization of Metallothionein gene LbMT3 isolated from Ectomycorrhizal fungus *Laccaria bicolor***" being submitted by Ms. Prabhleen Kaur in partial fulfillment for the requirement of degree of **Master of Technology** in **Biotechnology** in the **Department of Biotechnology, Thapar University, Patiala** is a bonafide work carried out under the esteemed supervision and conception of **Dr.M.S Reddy**, Professor, Department of biotechnology, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in any other university or institute.

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Place: Patiala

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ABSTRACT

Heavy metals are the main source of soil contamination which effects the growth and development of living organisms. To tackle this problem a large number of remediating processes are available out of them the use of ectomycorrhizal fungi for detoxifying the heavy metals, is the most reliable method. They enhance the plant growth and wellness in severely contaminated soils against metal toxicity. The ectomycorrhizal fungi follow many molecular mechanisms to avoid the uptake or to tolerate the heavy metal stress. In the current study, *Laccaria bicolor* (ectomycorrhizal fungus) was studied for metal tolerance at different metal concentrations. In many eukaryotes, the cysteine rich peptides i.e. metallothioneins involved in metal tolerance and metal homeostasis are present. Total six putative metallothionein genes of *Laccaria bicolor* have been reported till date and two genes (LbMT1 and LbMT2) out of them have already been studied. In the present study LbMT3 gene (35 aa long) from *Laccaria bicolor* was cloned and characterized under metal stress conditions. In the metal tolerance studies, a significant decrease in the growth of mycelium was observed with the increase in metal concentration and from the experimental data LD_{50} value was also calculated for copper and cadmium for *Laccaria bicolor*. The gene expression of LbMT3 was studied with the help of qRT-PCR after isolating the RNA and synthesis of cDNA from it. LbMT3 gene showed different expression patterns to different metal stresses. The maximum expression was observed at the highest concentration of metals (40 µM for Cd and 400 µM for Cu). Yeast complementation studies in metal sensitive yeast strains indicated that LbMT3 gene is resistant to both the metals (cadmium and copper) but LbMT3 gene showed significantly higher expression levels in cadmium as compared with copper. This study proves to be highly useful in understanding the different mechanisms involved metal detoxification in fungus which can be further used as potential tool against cadmium and copper stress can be used in bioremediation.

ABBREVATIONS

RNA	Ribonucleic acid
DNA	Deoxy Ribonucleic acid
cDNA	Complementary Deoxy Ribonucleic acid
ECM	Ectomycorrizal
MT	Metallothionein
PC	Phytochelatin
ROS	Reactive oxygen species
TBE	Tris Boric acid EDTA
dNTP	Deoxy Nucleotide tri phosphates
LB	Luria Broth
LA	Luria agar
YPD	Yeast peptone dextrose
Cu	Copper
Cd	Cadmium
µg	Microgram
M	Molar
Cys	Cystein
Bp	Base pair
ORF	Open reading frame
PCR	Polymerase chain reaction
%	Percentage

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1. INTRODUCTION

1.1 Heavy Metals

The heavy metals are the naturally occurring components of earth's crust, which cannot be degraded or destroyed. Heavy metals are metals and the specific density of heavy metals is higher than 5 g cm^{-3}. Fifty three out of the 90 naturally occurring components are heavy metals, however not every one of them is of organic significance. Heavy metals can be both essential as well as non-essential. Heavy metals, for example, Zinc and Copper are crucial for ordinary plant development and improvement, since they are components of numerous compounds as well as various proteins. There are two primary ways of the metal penetrate to the soil are anthropogenic sources and mineral rock weathering. Ross (1994) studied that the anthropogenic sources of metal tainting can be separated to five fundamental groups: (1) industry (cadmium, copper, arsenic, chromium, nickel, cobalt, mercury, zinc; (2) metallic-ferrous mining and purifying (arsenic, cadmium, mercury and lead); (2); (3) farming (arsenic, cadmium, lead, copper, selenium zinc and uranium,);(4) atmospheric disposition (arsenic, uranium, cadmium, copper, lead, mercury and chromium,); (4) and (5) waste disposal (cadmium, chromium, arsenic, copper, lead, zinc and mercury). Fe, Mo and Mn are essential as micronutrients. Some metals, for example, copper, cobalt, chromium, magnesium, calcium, nickel, potassium, zinc and sodium, are key, serve as micronutrients and are utilized for redox-processes; to settle particles through electrostatic collaborations; as a part of different proteins and for direction of osmotic pressure. Numerous different metals have no organic part, for example, silver, aluminum, cadmium, gold, lead and mercury, are unnecessary and possibly lethal to microorganisms. The poisonous quality side effects found due to the overabundance of heavy metals might be because of a scope of interactions at the cell/sub-atomic level. Poisonous metals can bring about destructive impacts from numerous points of view, mainly because of their strong co-ordinating abilities (Hall, 2002). Harmful impacts incorporate the alterations in the functioning of catalysts and transport framework for crucial supplements and particles, the removal and\or substitution of key metal particles from biomolecules, conformational changes, displacement of crucial metal particles from biomolecules, blocking of vital practical gatherings in biomolecules, denaturation and inactivation of compounds and disturbances in cell and organellar membrane integrity(Jentschke and Godbold, 2000; Muller et at., 2007). Moreover, the overabundance of heavy metal may fortify the development of free radicals and responsive oxygen species via auto-oxidation and Fenton response, bringing about oxidative stress (Dietz *et al*., 1999).

1.2 Methods to remediate heavy metals

Heavy metals are the main source of soil pollution. A number of chemical, physical and biological techniques are available to remove heavy metals from soil that are divided into two categories:

1) *Ex-situ* techniques under which the soil is treated and retured to the place after treatment. It includes methods like physical separation, excavation, vitrification, detoxification and/or the physically or chemically destruction of contaiminant, and hence the contaminant undergoes immobilisation, destruction, stabilisation, incineration or solidification.

2*) Insitu* techniques under which the treatment is given on the site of contamination itself which mainly includes transformation or destruction of the contaminant, immobilization to decrease the bioavailability and separating the contaminant out of the bulk soil (Vandergrift *et al.,* 1992). Bioremediation is one of the most effective *insitu* techniques in which consumption and breakdown of environmental pollutants by the use of either naturally occurring or deliberately introduced microorganisms. Plants plays an crucial role in removing heavy metals (Phytoremediation) from the soil through various processes like rhizofiltration, phytostabilisation, phytoextraction, phytovolatilization and phytotransformation (Table 1) and Microorganisms are also used in this area (Ghosh M and Singh SP. 2005).

Table1. Different mechanisms involved in phytoremediation.

S.No.	**Process**	**Mechanism**	**Contaminant**
5.	Phytotransformation	Degradation in plant	Organics
4.	Phytovolatilization	Volatilisation by leaves	Inorganics /Organics
1.	Rhizofiltration	Rhizosphere accumulation	Inorganics/Organics
3.	Phytoextraction	Hyper-accumulation	Inorganics
2.	Phytostabilisation	Complexation	Inorganics

Phytoremediation is more luring method due to its high cost and low reliability for large areas but it may take decades for plants to grow and to remediate polluted soil. Whereas microbial metal bioremediation, many microorganisms play important role in reducing the heavy metal content from soil very quickly (within days). Many rhizophere bacteria enhance phytoremediation by different mechanism like by releasing various organic acids like oxalic acid, tartaric acid, formic acid etc which act like chelators,by oxidation/reduction reactions or by changing the pH of soil. *P.aeruginosa* produces surfactants like rhamnolipids which are specific to some metals like Cd and Pb. Bioemulsifiers of higher molecular weight like emulsan can be helpful in metal removal. According to sand *et al.*, 1992 *Thiobacillus ferroxidans* and *Leptospirillum ferroxidans* have the capability to oxidize iron and sulfur. Mycorrhizal fungi play an immense role to detoxify the heavy metals. (Rajendran *et al.*, 2003)

1.3 Mycorrhiza

Mycorrhiza alludes to an affiliation or beneficial interaction amongst fungi and plants that colonize the cortical tissue of roots amid times of dynamic plant development. The expression "mycorrhiza", which truly signifies "fungus root", portrays a cozy mutualistic relationship amongst fungi and plant roots. And around 95% of plant species characteristically form mycorrizae (Smith and Read, 2010). Mycorrhiza is partitioned into endomycorrhiza and ectomycorrhiza (Figure 1). Endomycorrhizal fungi penetrate the cortical cells that are present in the vascular plant roots and form a shrubby structure called arbuscule. On the other side ectomycorrhizal fungi do not penetrate inside the root cells and they form hartig’s net, an intracellular hyphae. Mycorrhizal organisms assume a vital part in giving access to mineral supplements at all phases of plant advancement. This is accomplished to a great extent through their capacity to mobilize key supplements, for example, phosphorus and nitrogen (Harley and Smith, 1983). Thus mycorrhizal growths impact plant wellness and survival and the improvement of plant community structure (Read, 1991). While upgrade of supplement uptake, both key and insignificant components might be lethal at high concentrations and in a few circumstances improved uptake could be harmful for both hosts and symbionts. In addition, collection of heavy metals in soils could antagonistically influence arrangement and advancement of ectomycorrhizas of tree species developing in contaminated soils (McCreight and Schroeder, 1982). There is significant enthusiasm for the collaboration amongst mycorrhizas and contamination.

The mycorrhizas have been grouped into various sorts. The arrangement depends on parasitic partners and basic qualities of mycorrhizas at development. The most old, across the board, and concentrated on mycorrhizal class is arbuscular mycorrhiza. Ectomycorrhiza is a typical type of beneficial interaction in backwoods trees. Different types of mycorrhizas are arbutoid, monotropoid, ericoid, and orchid mycorrhizas. Both the organism and the plant may influence the kind of mycorrhiza framed. Along these lines the mycorrhizal order must be considered mostly unmistakable. Furthermore, numerous parasites can shape distinctive sorts of mycorrhizas relying upon the host species, and the types of the genera Salix, Prunus and Acacia structure both ectomycorrhizas and arbuscular mycorrhizas. The hosts contain most types of angiosperms, all gymnosperms, pteridophytes and a few bryophytes. All the major taxonomic gatherings of parasites (Ascomycotina, Basidiomycotina and Zygomycotina) structure mycorrhizas. Some of these are commit symbionts that can't make food without the host plant. Mycorrhizal growths have wide host range and for the most part don't indicate strict advantageous connections. The species structure of mycorrhizal growths is subject to host plant age and natural conditions.

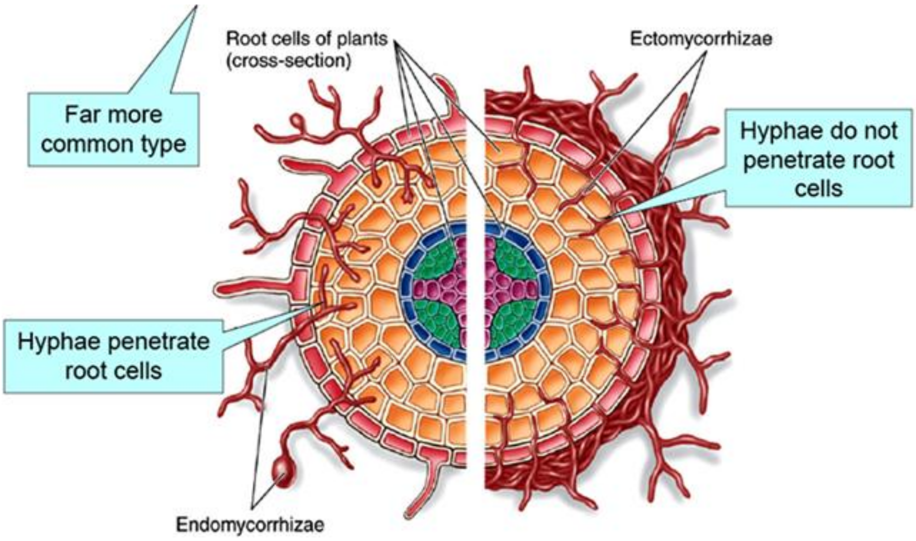


Figure1. Comparative diagram of ectomycorrhiza and endomycorrhiza.

1.4 Ectomycorrhiza

It is a type of advantageous relationship that happens between a contagious symbiont and the roots of different plant species. Ectomycorrizal fungus is responsible for the nutrients and metal transfer into their hosts (Khosla and Reddy, 2008). Ectomycorrhizas (ECMs), the ruling mycorrhizal advantageous interaction in boreal, mild and some tropical woodlands, are formed by 5000-6000 types of ascomycetes and basidiomycetes (Buscot *et al.*, 2000) and has that can be either gymnosperms or woody angiosperms, particularly individuals from the families Betulaceae, Pinaceae, Fagaceae, Salicaceae and Dipterocarpaceae (Smith and Read, 1997). Fossil records and sub-atomic clock dating propose that ECMs developed around 200 million years back (Cairney, 2000). ECM does not penetrate the cortical cells and forms an interface called hartig net. The hyphae are completely intercellular and structure a thick sheath (mantle) around parallel roots. From this mantle, individual hyphae or organized hyphal aggregates called rhizomorphs grow out and get exposed to soil. The mantle can have changed thickness, texture and shade depending upon the specific interaction of any particular plant and fungus. The mantle expands its surface and regularly influences fine-root morphology, thus is helpful in exchange of essential supplements. Ectomycorrhizal growths can impact the host plant from various perspectives. Decades back, it was shown that ectomycorrhizal organisms enhance plant sustenance by improving nitrogen, Phosphate and potassium uptake, amongst others (Harley and Smith, 1983). Supplements, especially nitrogen and phosphate, are taken up by ectomycorrhizal mycelium through dynamic retention and particular cell layer transporters taking after debasement by exoenzymes (e.g. phosphatases) (Smith and Read, 1997). Supplement trade amongst organism and plant happen utilizing cell-to-cell contact as a part of ectomycorrhizas, particularly in the Hartig net. Hartig net is an interface where nutrients,water etc get exchanged between plant roots and fungi (Bonfante and Genre, 2010). Ectomycorrhizal fungi, expand the plants access to soil water altogether. Ectomycorrhizas additionally protects host plant roots against root pathogens (Smith and Read, 2010).

Mechanisms of metal tolerance

Metals are very crucial and required in all parts of cellular development, metabolism and differentiation (Gadd, 1986). This prompts expression of a detoxification mechanism for survival of life form. All the organisms become resistance to heavy metals by “avoidance” when the organism becomes able to restrict metal uptake, or by “tolerance” when the organism survives in the presence of high internal metal concentration (Hall, 2002). The avoidance includes lessening the grouping of metal entering the cell by: extracellular precipitation, biosorption to cell dividers, decreased uptake, or expanded efflux. In the second circumstance, metals get chelated intracellular through the combination of ligands, for example, metallothioenins, phytochelatins, polyphosphates and\or compartmentation inside vacuoles (Figure 2) (Jentschke and Godbold, 2000; Muller *et at*.,2007).

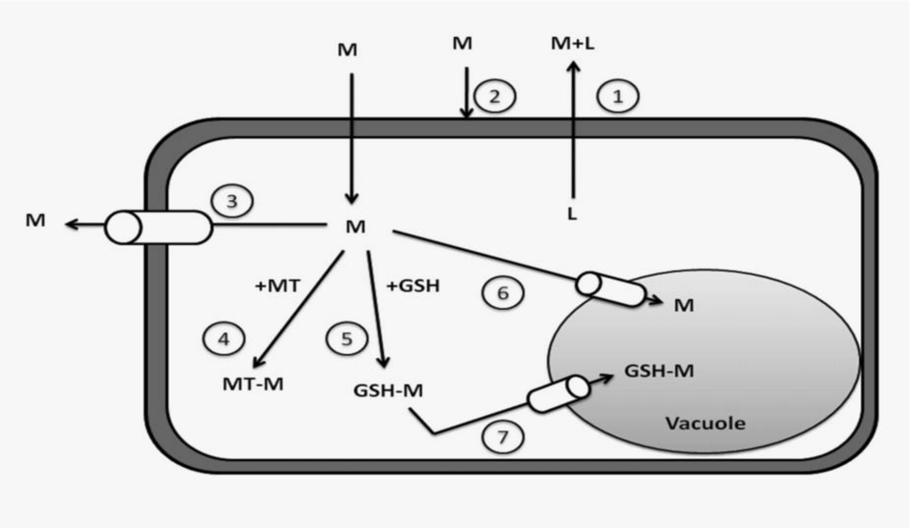


Figure 2: Schematic diagram of potential cellular mechanisms responsible for metal detoxification and tolerance in higher plants. 1) enhanced efflux 2) extracellular chelation by secreted ligands 3) cell wall binding 4) intracellular chelation by metallothioneins (MT) 5) intracellular chelation by glutathione (GSH) 6) subcellular compartmentation 7) compartmentation of GSH-M in vacuoles. (Khullar and Reddy, 2016)

1.5 *Laccaria bicolor*

Laccaria bicolor is a mycorrhizal individual from the Basidiomycota (Table 2). It has been accounted for to be palatable. *Laccaria bicolor* was the primary mutualistic organism to have its whole genome sequenced. This 65-megabase genome contains approximate 20,000 anticipated protein-encoding genes and an expansive number of transposons and repeated sequences. This species makes ectomycorrhizal relationship with a wide number of tree species, for example, red pine, jack pine and dark spruce.

Table2. Taxanomical classification of *Laccaria bicolor*.

Kingdom	**Fungi**
Class	Agaricomycetes
Division	Basidiomycota
Family	Hydnangiaceae
Order	Agaricales
Genus	*Laccaria*
Species	*L. bicolor*

Laccaria bicolor gets sugars from photosynthesis while supplying the plant with vital minerals and expanded water uptake. *Laccaria bicolor* upgrade the development and wellness of their host plant generally by enhancing their mineral sustenance. Mostly ectomycorrhizal fungus cannot be grown in culture but *Laccaria bicolor* can be grown (from basidiospores or tissue samples) and also with the mycorrhizal tree's roots. *Laccaria bicolor* have violaceous gills and at the base of the stem the color of mycelium is violet. Its cap is of 2-4.5cm wide, it can either convex or flat (figure 3). They likewise expand their host plant's resistance to heavy metals and enhance the survival and development of trees in soil contaminated by heavy metals. This ability of *Laccaria bicolor* is of extraordinary significance in light of the fact that there is a considerable chance of dispersal of heavy metals in the environment by human exercises. Metal resilience is accomplished by oxidative stress reaction catalysts, increment in the level of cell glutathione (GSH), metal efflux framework (Colpaert *et al.*, 2011; Ruytinx *et al.*, 2013) and sequestration of overabundance metals by compartmentalization. There are three primary intracellular peptides ie.GSH, phyochelatins and metallothioneins which chelate the metal particles present in abundance. Our emphasis is on metallothioneins which are cystiene rich particles, having little sub-atomic weight and chelate the intracellular concentrations of free thiophilic metal ions (Cu,Cd etc.) (Clemens,2001).

Figure 3: *Laccaria bicolor* (www.mushroomexpert.com)

1.6 Metallothioneins

Metallothioneins (MTs) come under super family of intracellular metal-binding proteins, present in almost every single living being. Metallothionein was initially isolated from the kidney of a horse. Normally, MTs have low atomic weight polypeptides (< 10 KDa), high metal substance containing dominatingly Zn, Cu or Cd, very monitored cysteine buildups and no aromatic amino acids or histidine. They have normal Cys-X-Cys and Cys-Cys bunches, where X is an amino acid other than cysteine. The prevalent component of MTs is that 33% of its amino acids are cysteine, which are sulfydryl buildups ready to tie substantial metals, for example, Cu, Cd and Zn. Cysteine is a sulfur-containing amino acids, thus the name "-thionein". In any case, the support of inorganic sulfide and chloride particles has been proposed for some MT frames. MTs in contrast to different metalloproteins show high proclivity (affinity) towards both the key (e.g. Cu and Zn) and unimportant metal particles (e.g. Hg and Ag) and have vast metal-restricting limits. Subsequently, they plays an essential part in metal homeostasis and detoxification i.e. MTs give security against metal lethality, be included in control of physiological metals (Zn and Cu) and give assurance against oxidative stress (Gadd, 1993; Cobbett & Goldsbrough, 2002). Metallothioneins were appeared to tie high concentrations of heavy metals (HMs) in metal thiolates and metal thiolate groups. All Cysteines present in the reduced form and are attracted to the metal particles through mercaptide bonds.

Objectives

- Identification of metallothionein genes in *Laccaria bicolor* genome
- Cloning and characterization of metallothionein gene LbMT3
- Role of LbMT3 in metal tolerance

2. REVIEW OF LITERATURE

Heavy metal toxicity is becoming one of the main reasons of contamination of environment by human activities. According to Gadd (1993) Zn, Cu, Mn, Ni and Co are essential metals and required in micro concentrations while Cd, Pb, Hg etc are non essential metals and all these metals are toxic above the threshold concentration.

Clemens *et al.*, 2013 explained that heavy metals are present in soil naturally with great variations in their concentrations. Due to their non degradability and human activities heavy metals get accumulated in the environment. He concentrated his studies on cadmium exposure and its adverse effects to human body. Humans are exposed to Cd so there is urgent need to lower the Cd intake. By the advancement in molecular biology the adverse effects of Cd exposure can be tackled.

2.1 Ectomycorrhiza

Smith and Read (2010) described more about the word ectomycorrhiza which is the symbiotic association between fungi and the roots of different plants belonging to families like *Fagaceae, Dipterocarpaceae, Pinaceae* and *Caesalpinoidaceae.* The physical, chemical and biological properties of soil get influence by the plant roots.

Bingham and Simard (2011, 2012a, 2012b) reported importance of the ectomycorrhizal network which is formed by the colonization of roots of distinct tree species. The ecomycorrhizal network plays very important role in the exchange of water and nutrients between different plants and which results in enhancing the stability and fitness of ecosystem of forests against the adverse effects of the environment.

Bonfante and Genre (2010) described that Mycorrhizal fungus is a heterogeneous group of diverse fungal taxa which are associated with the roots of about 90% of all plant species. The different Signalling pathways that occur between plants and fungi have now been explained and several novel nutrient transporters have been identified that revealed some cellular processes that underlie symbiosis. Hence, the contributions of each transporter in this type of mycorrhizal associations are starting to be unravelled. This new knowledge available is highly useful in agricultural practices.

Fomina *et al*., (2004) studied the ability of ectomycorrhizal fungi and ericoid mycorrhizal fungi to solubilize different metals like Cd, Cu, Pb etc. Metal tolerance and ability to solubilize vary widely for different metals and fungus species. Measurements of dry weight and radial growth were helpful in understanding the nature of ectomycorrhizal and ericoid fungi against different metals. Pb phosphate, carbonate, sulphide and tetraoxide exhibited the high toxicity while zinc phosphate showed the least toxicity in the test ectomycorrhizal fungal isolates. But in Pb-mineral-amended media ericoid mycorrhizal isolates were able to grow. Interestingly neither ectomycorrhizal nor ericoid mycorrhizal isolates produced clean zone on Pb-mineral-amended media. In a nutshell the metal tolerant fungus strains grew and solubilized the heavy metals unlikely non tolerant fungus strains.

Colpaert and Vanassche (1992) carried out study on 6 strains of ectomycorrhizal fungi to study the ability of zinc tolerance in *Pinus sylvestris* L. seedlings. Every mycobioant showed different results in their protection of plants against zinc toxicity. But *Thelephora terrestris* (Ehrh.) Fr. Strain showed no protection at all, it increased the zinc concentration in *P.sylvestris* seedlings and also enhanced the zinc toxicity. The experimental was carried out to find out the zinc tolerant strains which can be helpful in treating the zinc polluted soils effectivilty. These mycorrhizal fungi act as a filter against the zinc that can be immobilized thus it prevents the transportation of zinc to the hosts.

2.2 Extracellular and cellular mechanisms

Bellion *et al*., (2006) identified potential extracellular and cellular system that might be included in the resistance of ectomycorrhizal growths to abundance metals in their surroundings. These incorporate mechanisms that diminish uptake of metals into the cytosol by extracellular chelation through expelled ligands and restricting onto cell-divider segments. Intracellular chelation of metals in the cytosol by a number of ligands (glutathione, metallothioneins), or expanded efflux from the cytosol out of the cell or into sequestering compartments are likewise key components giving resilience. Bellion (2007) characterized Pimt1 gene, coding for a metallothionein from the ectomycorrhizal fungus *Paxillus involutus*.

Joerger *et al.,* (2001) revealed that *Pseudomonas stutzeri* AG259 have the capability to produce silver based crystals which are helpful in reducing the metal toxicity.

Hall (2002) discovered the function of various cellular systems in heavy metal detoxification. These roles are as like this: for mycorrhiza and for binding to cell divider and extracellular exudates; for lessened uptake or efflux pumping of metals at the plasma layer; for chelation of metals in the cytosol by peptides, for example, phytochelatins; for the repair of stress harmed proteins; and for the compartmentation of metals in vacuole by tonoplast found transporters. This review gives an expansive overview of the proof for a contribution of every mechanism in heavy metal detoxification and resilience (Table 3).

2.3 Molecular and physiological mechanisms

Ma *et al.,* (2013) reported the physiological changes and molecular changes that occur during the establishment and functioning of ectomycorrhizal fungi, plant roots and also at the whole plant level. He demonstrated that *P.involutus* inoculated with *P. canescens* showed larger root surface area. On increasing the concentrations of P, Ca, Fe and Zn enhances the photosynthetic rates, alters the concentrations of free radicals and H_2O_2 and elevates levels of ascorbate and glutathione which improves the carbohydrate and nutrient status, increases the rate of detoxification and defence mechanisms in the host plants which directly or indirectly helps the host to fight against the higher concentrations of Cd. He also elaborated certain processes at molecular level under which the ectomycorrhizal plants can alter the activity of different metal uptake transporters and can even modify the transportation processes at transcript and protein levels. Moreover the ectomycorrhiza can regulate the different chelating molecules for binding heavy metals in cells (figure 2).

Jacob *et al.,* (2004) found that complexation of cadmium by various phenolic compounds, or by complexing peptides, for example, MTs, glutathiones etc is the most common cellular response to cadmiumin in *Paxillus volutus*. But at molecular level in this fungus the different mechanisms are hard to understand. 2040 cDNAs of the ectomycorrhizal fungus *Paxillus involutus* were screened to recognize cadmium-responsive genes by utilizing differential hybridization. Forty nine (2.4%) out of the 2040 cDNAs were differentially expressed, among which transcripts coding a laccase, an aconitase, and a metallothionein were upregulated by 3.9-, 3.7-and 2.8-fold, individually, while genes coding hydrophobins and threonine dehydratase were firmly downregulated. Likewise, the present study proposes that the production of hydrophobins might be proficiently decreased, so that’s why there is more synthesis of Cys-enriched compounds.

Hernendez *et al.,* (2015) told the importance of glutathione in the mechanism of resistance that rely on upon biothiols in plant cells. The aggregation of lethal metals and metalloids, for example, cadmium (Cd), mercury (Hg), or arsenic (As), as an outcome of different anthropogenic exercises, represents a genuine risk to the earth and human wellbeing. The capacity of plants to take up mineral supplements from the soil can be expoilated to create phytoremediation innovations ready to mitigate the negative effect of dangerous components in terrestrial ecosystems. In any case, we should choose plant species able to tolerate the high concentration of heavy metals. The resilience of plant cells to heavy metals is exceedingly reliant on glutathione (GSH) metabolism. GSH is a biothiol tripeptide that assumes a central double part: to start with, as an antioxidant agent to alleviate the redox imbalance created by dangerous metal(loid) aggregation, and second as a precursor of phytochelatins (PCs), ligand peptides that control the free ion cellular circulation of these contaminants.

Ray and Adholeya (2009) investigated effects of coal ash on exudation of different organic acids and uptake of heavy metals by ectomycorrhizal fungus. He conducted experiments on *Pisolithus tinctorius, Scleroderma verucosum* and *Scleroderma cepa* which were grown on modified melin-norkans medium (MMN). HPLC was used to detect the exudation of different organic acids like formic acid, malic acid and succinic acid. Atomic absorption spectrophotometer was used to assay the mycelium accumulation of Al, As, Cd, Cr, Ni and Pb. By use classical multivariate linear regression model the relationship between metal uptake and exudation of organic acids was established.

2.4 Metallothioneins

Carpene *et al.,* (2007) characterized the metallothioneins functions and structural characteristics, metallothioneins described as low molecular weight proteins rich in cysteine content (Figure4). He also characterized the four subfamilies of metallothioneins designated MT-1 through MT-4. MT-3 and MT-4 are expressed in brain and differentiating stratified squamous epithelial cells. The relations between MTs and diseases can be studied as metallothioneins are induced by various stimuli so can be considered as valid biomarkers in medicine and environmental studies.

Osobova *et al*., (2011) reported 3 isoforms of MTs in *Amanita strobiliformis* that sequester intracellular Ag in mycelium. He proposed that MTs having a stretch of 10–13 amino acids lacking Cys deposits could be considered as a particular characterstic of MTs from the ectomycorrhizal basidiomycestes. Despite the fact that LbMT1 qualifies to this characteristic alongside MTs of *A. strobiliformis* and *P. involutus,* this is not valid for different MTs of ectomycorrhizal basidiomycetes including LbMT2.

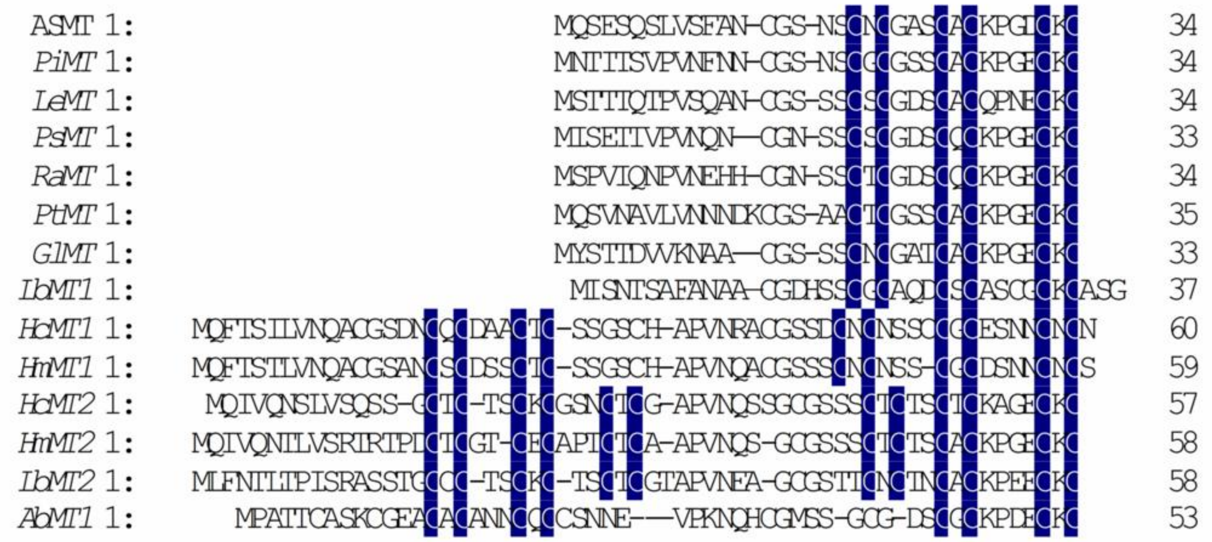
Loebus *et al.,* (2013) found the first exclusively cadmium-inducible MT and demonstrate mechanism on the protein level to segregate between harmful Cd and vital Zn particles inaquatic fungus. He used high concentrations of metals i.e. 40 mM ZnII, 208 μM CuII, 61 μM AsV, and 25 μM CdII, This makes it a testing environment for living life forms as they need to adapt to high metal concentrations in spring water. One of the surviving animal varieties found is the aquatic fungus *Heliscus lugdunensis*.

Ramesh *et al*., (2009) portrayed two MT genes from the ectomycorrhizal fungus *Hebeloma cylindrosporum*. The full length cDNAs were utilized to perform complementation assays in yeast mutant strains. As uncovered by heterologous complementation assays in yeast, HcMT1 and HcMT2 each encode a useful polypeptide fit for providing more resistance against Cd and Cu individually. The expression levels of HcMT1 was seen highest at 24 hours and increased as the Cu concentration was increased. HcMT2 was also actuated by Cu however the expression levels were less as contrasted with HcMT1.

Jaeckel (2005) reported a potential function of MTs and phytochelatins for *in vivo* heavy metal detoxification in aquatic fungus. In liquid medium, 0.1 mM cadmium concentration increased the glutathione (GSH) content and resulted in the production of extra thiol peptides. HPLC, electrospray ionization mass spectrometry, and Edman degradation affirmed that a novel metallothionein and phytochelatin (PC2) were produced. The metallothionein has a high homology to family 8 metallothioneins.

Ngu and Stillman (2009) studied the tertiary structures are absent when the metallothioneins are in its native forms without metals and in the presence of metal ions it forms metal-thiolate clusters because of its binding to metal ions as per the metal coordinates. Optical spectroscopy was used to understand the metalation of metallothioneins and for better understanding of mechanisms metalation of metallothioneins electrospray ionization mass spectrometry was used. From this paper he concluded that metallothioneins undergo metal induced protein folding.

Tan *et al*., (2016) cloned MT2 gene of size 667 bp from the grass carp (*Ctenopharyngodon idellus*). Similarity of this gene was found with the reported vertebrae MT gene having 3 exons which are interrupted with 2 introns. With the help of quantitative real-time PCR (qRT-PCR) the basal levels and the transcriptional responses of MT2 gene to cadmium (Cd) in different organs were measured. The inducibility of MT2 gene to Cd showed that it has a huge part to be played in the metal detoxification in *Ctenopharyngodon idellus* and in the liver the mt2 gene was the most sensitive to Cd.



```
ASMT 1:                           MQSESQSLVSFAN-CGS-NSCNCGASCACKPGICKC       34
PiMT 1:                           MNTTTSVPVNFNN-CGS-NSCGCGSSCACKPGECKC       34
LeMT 1:                           MSTTTQTPVSQAN-CGS-SSCSCGDSCACQPNECKC       34
PsMT 1:                           MISETTVPVNQN--CGN-SSCSCGDSCQCKPGECKC       33
RaMT 1:                           MSPVIQNPVNEHH-CGN-SSCTCGDSCQCKPGECKC       34
PtMT 1:                           MQSVNAVLVNNNDKCGS-AACTCGSSCACKPGECKC       35
GlMT 1:                           MYSTTDVVKNAA--CGS-SSCNCGATCACKPGECKC       33
LbMT1 1:                           MISNTSAFANAA-CGDHSSCGCAQDCSCASCGCKCASG    37
HcMT1 1: MQFTSILVNQACGSDNCQCDAACTC-SSGSCH-APVNRACGSSDCNCNSSCCGCESNNCNCN        60
HmMT1 1: MQFTSTLVNQACGSANCSCDSSCTC-SSGSCH-APVNQACGSSSCNCNSS-CGCDSNNCNCS        59
HcMT2 1:  MQIVQNSLVSQSS-GCTC-TSCKCGSNCTCG-APVNQSSGCGSSSCTCTSCTCKAGECKC       57
HmMT2 1: MQIVQNILVSRIRTPICTCGT-CECAPTCTCA-APVNQS-GCGSSSCTCTSCACKPGECKC       58
LbMT2 1: MLFNTLTPISRASSTGCCC-TSCKC-TSCTCGTAPVNEA-GCGSTTCNCTNCACKPEECKC       58
AbMT1 1:    MPATTCASKCGEACACANNCQCCSNNE---VPKNQHCGMSS-GCG-DSCGCKPDECKC       53
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Figure 4: An example showing multiple sequence alignment of MT proteins of basidiomycetous fungi sequences. Gaps introduced to provide the best alignment are indicated by dashes. Fully conserved Cys residues are highlighted in blue; name of fungi are: *Amanita strobiliformis* (AsMT), *Paxillus involutus* (PiMT), *Lentinula edodes* (LeMT), *Piriformospora indica* (PsMT), *Pisolithus tinctorius* (PtMT), *Ganoderma lucidum* (GlMT), *Laccaria bicolor* (LbMT1 & 2), *Hebeloma cylindrosporum* (HcMT1 & 2), *H. mesophaeum* (HmMT1 & 2) ,*Agaricus bisporus* (AbMT1).(Reddy *et al*., 2014).

2.5 Laccaria bicolor

Martin *et al.,* (2008) studied the whole genome sequence of ectomicorrhizal basidiomycete *laccaria bicolor* which is 65 mega base assembly and have 20,000 protein encoding genes. He also detected many repeated sequences and transposons. One of the important features was the presence of effector type small secreted proteins which are of unknown function but mostly get expressed in symbiotic tissues. The unexpected finding was the lack of carbohydrate active enzymes which cannot degrade the cell wall but can degrade non plant cell wall polysaccharides, so due to which this fungus can easily grow within both soil and living plant roots and that explain the dual biotropic and saprotropic life style of mycorrhizal fungus. Hence, this study enhances the knowledge of previously known and unknown mechanisms of symbiosis. The availability of genome sequence provides intense opportunities to get a deeper knowledge of different processes of symbiosis.

Reddy *et al*., (2014) reported the role of metallothionein genes in shielding plants from heavy metal stress. The cloning and characterization of two MT genes LbMT1 and LbMT2 from the ectomycorrhizal fungus *Laccaria bicolor* under different metal stress conditions. The expression levels of both LbMT1 and LbMT2 increases as a function of Cu concentration, the expression levels for LbMT2 were dependably higher as contrasted to those of LbMT1. Just LbMT1, but not LbMT2, reacted to Cd supply in the range of 25–100 mM while Zn did not influence the translation of either LbMT1 or LbMT2. Both genes reacted to oxidative stress, yet to a lesser degree contrasted with their reactions to either Cu or Cd stress (figure 4).

Colpeart *et al.,* (2011) discovered that heavy metal pollution causes evolutionary adaptations in many organisms. This review paper enhances our knowledge regarding the toxic effects of heavy metals when present in high concentrations. Under harsh environment condition the adaptation mechanisms are discussed and out of them metal exclusion strategy was focused. This strategy is found in *Suillus* species that can grow on metalliferous soils. The metal efflux system in *Suillus* may be responsible for reducing the transportation of heavy metals at plant fungus interface, without effecting the normal transportation of nutrients to the host plant.

Courbot *et al.*,(2004) told about the ectomycorrhizal fungus *Paxillus Involutus* when exposed to cadmium, the contents of glutathione and γ – glutamylcysteine increased. With the use of high performance liquid chromatography these thiol containing compounds (glutathione, γ – glutamylcysteine and phytochelatins) were measured. The thiol compounds are involved in metal detoxification by chelating the metal ions in cytosol. Mycelia on exposure to cadmium produced large amount of metallithioneins while phytochelatins were lacking, which suggest that ectomycorrhizal fungi can use different ways to tolerate heavy metal stress.

Pocsi *et al*., (2004) told about glutathione, a non protein thiol, present in yeast and filamentous fungi in high concentrations. Glutathione plays important role in cellular functions and in maintaining membrane integrity, structure of mitochondria and in differentiation and development of cells. Glutathione is an antioxidant which is helpful against heat shock and osmotic stress. Glutathione is involved in many detoxification processes under which the toxic endogeneous metabolites such as formaldehyde produced by methylotrophic yeasts (a by-product of glycolysis), get eliminated by glyoxalase pathway. Glutathione has anti-apoptotic activity and controls aging and autolysis in fungal species.

Table 3: Different mechanisms involved in metal tolerance (Hall, 2002)

Mechanism	Metal	Key reference
Mycorrhizas	Zn, Cu, Cd	Jentschke and Godbold (2000)
Cell wall, exudates	Various, including Ni, Al	Salt *et al*. (2000); Ma *et al*. (1997)
Plasma membrane	Arsenate	Meharg and Macnair (1992)
Reduced uptake	Ni	Arazi *et al*. (1999)
Active efflux	Various, including Zn	Silver (1996)
	(evidence not for plants)	Palmiter and Findley (1995)
Phytochelatins	Cd	Cobbett (2000)
Metallothioneins	Cu	Murphy and Taiz (1995)
Organic acids, amino acids	Various	Rauser (1999)
Heat shock proteins	Various, including Cd	Neumann *et al*. (1994)
Vacuolar compartmentation	Zn	Van der Zaal *et al*. (1999)

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Fungal strain

Basidiomycete fungus *Laccaria bicolor* (haploid strain S238) was used for this study. The culture was maintained at 25°C on Modified Melin Norkran's (MMN) medium with heller's micronutrients solution (Appendix).

3.1.2 Bacterial strain and culture conditions

In the present study the *E.coli* DH5α was used. This strain was maintained on luria agar plates at 37°C.

3.1.3 Yeast and culture conditions

Two copper-sensitive strains DTY3 (*MAT_, leu2-3, gal1CUP1s, 112his3_1, trp1-1, ura3-50)* and DTY4 (*MAT_, leu2-3, cup1::URA3, 112his3_1, trp1-1, ura3-50, gall)* referred as *cup1s* and *cup1_* and one cadmium sensitive *yap1* mutant (*MAT_, ura3D0, his3D1, leu2D0, YML:: kanMX4, met15D0)* derived from wild type BY4741 of were used in this study. These strains were maintained on YPD medium (agar supplemented plates or broth) at 30°C. For complementation studies the cells transformed of these strains were grown and tested on complete synthetic medium SD medium without uracil (SD –Ura) and are maintained in dark conditions.

3.1.4 Heavy metals

Copper and cadmium metals were used to study the tolerance and expression levels of LbMT3 gene.

3.2 Methods

3.2.1 Bioinformatics analysis

The search for the putative metallothionein genes of *Laccaria bicolor* were searched from the JGI genome portal (http://genome.jgi.doe.gov/). After doing the search 6 putative genes were retrieved with protein IDs 399685, 388087, 388066, 399683, 399677 and 399685. In the present study one gene with protein ID 399677 was selected for cloning and characterization. The open reading frame of the gene was obtained from the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) was used to get the homologous sequences to the LbMT3 gene and multiple sequence alignment of these sequences was done with the help of MULTALIN tool (http://multalin.toulouse.inra.fr/multalin/). To find the evolutionary relationships among these sequences phylogenic tree was constructed by using MEGA 7.

3.2.2 *Laccaria bicolor* tolerance to different heavy metals

To check the response of *Laccaria bicolor* to different concentrations of metals (Cd, Cu) the fungus was grown in MMN medium with Heller's micronutrients. MMN medium was prepared (50 ml in 250 ml flasks). 2-3 mycelium discs were inoculated from the actively growing mycelium plates and put in the flasks having MMN medium (pH 5.6) and incubated for 3 days to allow the mycelium to initiate the growth and on 4th day the respective metal in varying concentration was added in the broth. The concentration of metals used were: Cd^{2+} 0, 10, 20, 30, 40 µM/ml as $3CdSO_4$ Cu^{2+}; 0, 3, 6, 9, 12, 15 µM/ml as $CuSO_4.5H_2O$. After the addition of metal the flasks were incubated in dark at 25°C for next 17 days. On 21st day the mycelium was harvested and dried and total weight of mycelium at every particular concentration was noted. In order to find the metal uptake capacity of fungus, the dry mycelium was digested with nitric acid & perchloric acid and the total metal content per mg of mycelium was calculated by atomic absorption spectroscopy.

3.2.2.1 Nitric acid and Perchloric acid digestion

- 1 gm sample of air dried fungus was weighted in digestion tube and 10 ml of concentrated nitric acid was added and the flask was placed on electric heater for 1 hour at 145°C in acid proof digestion chamber having fume exhaust system.
- Then it was allowed to cool and 10 ml of concentrated nitric acid and 5 ml of perchloric acid were added and heated at about 100°C for first 1 hour and then the temperature was raised up to 200°C.
- The digestion was continued until the contents become colorless and only white fumes appeared.
- Then acid content was reduced till the white matter was left in the digestion tube.
- After it was removed from the heating mantle and was cooled. 50% diluted HCl was added and filtered through whatman filter paper number 42.
- The final volume was made 50 ml with MQ water.

3.2.2.2 Processing of cellophane sheets

In 1 litre boiling water, 0.4 gm EDTA Disodium salt was added and mixed well. 5 to 10 cellophane sheets of diameter 80mm were dipped in the water and boiled for 2 to 3 minutes (repeat the same with the remaining sheets).The cellophane sheets were washed in a running tap water for 5 minutes followed by the final washing with distilled water and then the sheets were autoclaved for 15 minutes.

3.2.3 Isolation of nucleic acids

3.2.3.1 Preparation of mycelium for nucleic acid isolation

7mm two or three discs were taken from actively growing mycelium and were inoculated on the MMN agar plates overlaid with cellophane sheets. The fungus was allowed to grow for 15 days and then the cellophane sheets were transferred on new plates supplemented with required metal and was allowed to grow for next 2 days. After incubation period the mycelium were scrapped and the mycelium was collected in glass vials. The mycelium was crushed with liquid nitrogen to make powder of it. This mycelium powder was used further for RNA isolation and DNA isolation.

3.2.3.2 RNA isolation

The crushed samples of *Laccaria bicolor* stored in -80°C were used to isolate RNA with Qiazol (QIAGEN) method.

- About 100 mg of liquid nitrogen crushed mycelium sample were taken and 1 ml of Qiazol reagent was added in it and mixed well.
- The samples were vortexed, incubated at 15°C for 10 minutes and were centrifuged at 12000g for 10 minutes.
- After the centrifugation the supernatantwas collected in fresh 2 ml eppendrof and then 200µl of chloroform was added and mixed well.
- The mixture was incubated at 15°C for 2-3 minutes and Centrifuged at 12000 g for 15 minutes.
- The aqueous phase was collected in a fresh 1.5 ml eppendrof and then 500 µl of ice cold iso-propanol was added in it.
- The mixture was kept at -20°C for about 15 minutes.
- Then it was centrifuged at 12000 g for 10 minutes and the supernatant was discarded.
- The pellet was washed with 1 ml of 75% ethanol and centrifuged at 7500 g for 10 minutes and the supernatant was discarded.
- The pellet was dissolved in 20-50 µl DEPC treated water and then it was stored at -80°C.

3.2.3 cDNA synthesis using RT-PCR

cDNA was synthesized from total RNA by reverse transcription using oligo (dT) primer with the help of Revert AID^{TM} first strand cDNA synthesis kit (Thermo scientific). RNA of 5µg concentration was taken and 1µl of oligo dT was added and MQ water added to make up the volume up to 12 µl and then placed at 65°C for 5 minutes. In this mixture 4 µl of 5X buffer, 1 µl of Ribolock RNase, 2 µl 10 mM dNTPs and 1 µl of Reverse Transcriptase were added and mixed well. The mixture was incubated at 42°C for 1 hour followed by 5 minutes incubation at 70°C. The prepared cDNA was used for amplification of genes (figure 5).

3.2.4 Gene expression analysis using qRT-PCR

After the synthesis of cDNA from RNA, the qRT-PCR was performed. This technique is used to detect the RNA expression. The expression of gene was quantified using a non specific double stranded DNA binding dye SYBR Green.

The reaction mixture for qPCR for each reaction of total volume of 25 µl in which 12.5 µl of SYBR mix, 1 µl each of forward and reverse primer (LbMT3 or housekeeping gene specific, Table 4), 0.75 µl of cDNA template and 9.75 of water was added followed by the gene specific primers and cDNA. PCR tubes were placed in the thermal cycler to begin cycling. The first step of the cycle was initial denaturation. The next 40 to 50 cycles were the amplification program, which consists of three steps: (1) denaturation, (2) annealing (3) elongation. After the completion of PCR program the results were analysed by realplex instrument.

Table 4: Primers for Real Time PCR - actin (housekeeping genes) and gene specific LbMT3.

LbactF	5’- TGGAAGCGTAATGGGGAAGT -3
LbactR	5’- CAGTGTTGGTGATGTGCGAA -3’
LbMT3 F	5’-GGAATTCCATGCCATGATTTCTACCATCAACGTACC -3’
LbMT3 R	5’-CGGGATCCCGTTAGACAGCTTAGTTCTTTCCAGCATCA-3’

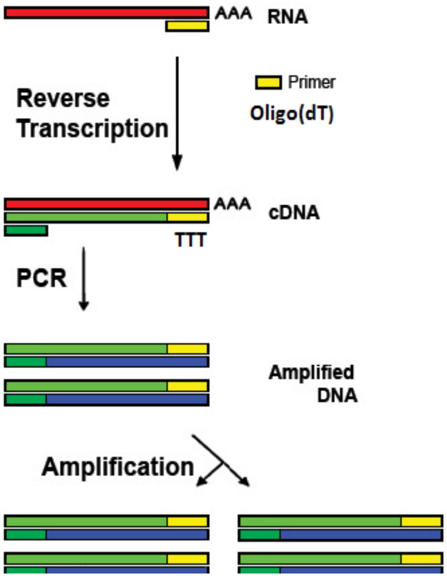


Figure 5: Schematic diagram of cDNA synthesis using reverse transcriptase and gene amplification

3.2.5 Genomic DNA isolation

- One-third part of liquid nitrogen crushed mycelium was taken in 1.5 ml centrifuge tube.
- 0.5 ml extraction buffer was added, mixed well and kept at 65°C for 15-20 minutes.(Appendix)
- 0.5 ml equilibrated phenol was added, mixed well and incubated for 15 minutes at room temperature.
- 0.5 ml chloroform : isoamyl (24:1) was added, mixed by inversion and incubated at room temperature for 15 minutes.
- The mixture was centrifuged at 12000 g for 20 minutes and the upper aqueous layer was collected in new tube.
- To the aqueous layer 400 µl of chloroform : isoamyl (24:1) was added and mixed by inversion.
- Then the mixture was centrifuged at 12000 g for 10 minutes and supernatant was collected in new tube.
- 0.54 volume of isopropanol was added, incubated at room temperature for 15 minutes and centrifuged for 10 minutes at 12000 g.
- The pellet was washed with 100 µl of 70% ethanol.
- The pellet was re-suspended in 300 µl of 0.2 M ammonium acetate and incubated overnight at 4°C.
- The DNA was precipitated by adding 600 µl of ethanol and centrifuged at 10000 g for 15 minutes at 4°C followed by dissolving the pellet in 50 µl of autoclaved water/ TE buffer (pH-8).

3.2.6 Plasmid (pFL61) isolation from bacterial cells

- 10 ml of luria broth was prepared and inoculated with 1% of culture and kept at 37°C for overnight.
- About 1.5 ml of overnight grown bacterial culture was taken in the ependroff and pelletized by centrifugation at 12000 rpm for 1 minute and the supernatant was discarded.
- About 200 µl of solution I was added and the pellet was dissolved into it completely by vigorous shaking and was kept at room temperature for 3 minutes.

- The 400 µl of solution II were added and were shaken for 10-15 minutes gently.
- The whole mixture was kept on ice box for 3 minutes.
- 300 µl of solution III were added in the mixture, shaken vigorously.
- Then the mixture was kept on ice box for 15 minutes and centrifuged at 12000 rpm for 10 minutes and supernatant was transferred in fresh ependroff.
- The 400 µl of Phenol: Chloroform: Isoamyl (25:24:1) was added in it and mixed well.
- The mixture was centrifuged at 12000 rpm for 10 minutes resulting in 3 layers, the upper aqueous layer having the plasmid was recovered in fresh eppendroff.
- Equal volume of ice chilled isopropanol was added and kept at -20°C for 10 minutes to 1 hour.
- Then it was centrifuge at 10000 rpm for 10 minutes and the supernatant was discarded.
- For washing the pellet 100 µl of ethanol were added and then it was centrifuged at 8000 rpm for 5 minutes, the supernatant was discarded.
- The pellet was air dried and dissolved in TE buffer or MQ water (Appendix).

3.2.7 Methods to analyze nucleic acids

3.2.7.1 Electrophoresis of nucleic acids

Procedure to make agarose gel (0.8% to 2%) for nucleic acids

For 0.8% agarose gel, 0.32 gm agarose was dissolved in 40 ml of 0.5x TBE buffer. It was microwaved for 1-1.5 minutes and then allowed to cool.1 µl of Ethidium bromide (EtBr) (0.5 µg/ml) was added and casted in gel cast tray. The nucleic acid samples (4 µl) were mixedwith 6X loading dye (4 µl) and loaded in the well. The gel was run at 70V voltage and 40mA current and the migrated nucleic acids were seen in U.V. transilluminator (312 nm).

3.2.7.2 Quantification of nucleic acids

A 1 µl drop of nucleic acid sample was placed on nanodrop padestel and O.D. was measured at 260 nm . One absorption unit simply means that the quantity of RNA or ssDNA is 40 µg/ml and 50 µg/ml of double stranded DNA (Sambrook *et al.*, 1989). For checking the purity of nucleic acids and contamination of polysaccharides and proteins the ratio of O.D.

260/230 nm and O.D. 260/280 nm were measured. The value closer to or greater than 1.8 indicates the purity of DNA and 2.0 indicates the purity of RNA.

3.2.8 Gene amplification by polymerase chain reaction (PCR)

For amplifying the LbMT3 sequences the gene specific primers were designed, LbMT3F (5'-GGAATTCCATGCCATGATTTCTACCATCAACGTACC -3') and LbMT3R (5'-CGGGATCCCGTTAGACAGCTTAGTTCTTTCCAGCATCA-3') primers introducing BamHI and EcoRI sites (sites underlined). PCR reactions were carried out containing 1x reaction buffer, 2 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 µl of each primer, 1 µl of cDNA and 0.3 µl of Advantage Taq DNA polymerase (Invitrogen) and MQ water to make up the volume upto 25 µl. The amplification of genes of interest from genomic DNA was carried out in PCR. The amplified DNA was visualized on 1.5% agarose gel.

3.2.9 Gene purification

3.2.9.1 Restriction digestion and ligation of isolated gene and plasmid

In the restriction digestion the DNA samples (pFL61 or LbMT3 gene) were digested with two enzymes EcoR1 and BamH1 (Thermo Scientific Restriction and Modifying Enzymes).The different reaction components were added in each reaction in the following sequence-

- Firstly, DNA sample was taken and in it 2x tango buffer, BamH1 and distilled water was added (Table 5).
- The mixture was incubated for 1hour at 37°C.
- After incubation, EcoR1 was added and the mixture was incubated at 37°C for 3 hours (EcoR1 shows the star activity so it was added after 1 hour).
- After the completion of the digestion protocol, the enzymes were heat inactivated at 80°C for 10 minutes. (figure 6)

Table 5: The components of digestion mixture and their amount.

DNA Sample	10 µl
2x tango buffer	4 µl
BamH1	1 µl
EcoR1	1 µl
MQ water	To 20 µl

Ligation: Ligation of plasmid and insert (gene) was done in a ratio 1:3 by the following formula

$$ng\ insert = \frac{ng\ vector\ \times Kbp\ onsert}{Kbp\ vector}$$

The reaction mixture for ligation is given in table 6.

Table 6: Different components of ligation reaction mixture

Components	Concentration
Plasmid DNA (PFL61)	20-100 ng
Insert DNA (LbMT3)	3:1 ratio of plasmid
10x T4 DNA Ligase buffer	2 µl
Thermo Scientific T4 DNA Ligase	1 µl
Water (nuclease free)	To 20 µl
Total volume	20 µl

The reaction mixture was incubated at 16°C for 20 minutes and then at 4°C for overnight. After incubation, the enzyme was inactivated by placing it at 65°C for 10 minutes.

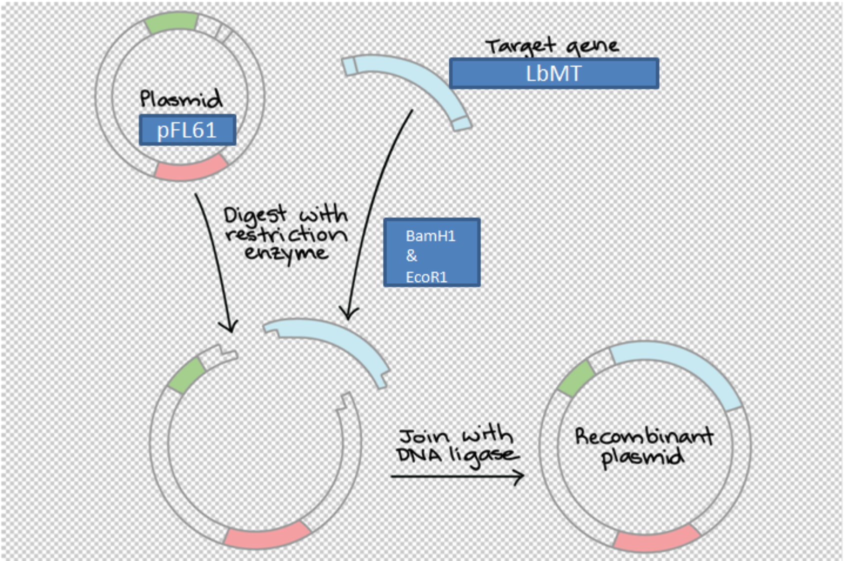


Figure 6: Schematic diagram showing the restriction digestion of pFL61 and LbMT3 with EcoR1 and BamH1 followed by ligation with T4 DNA ligase.

3.2.9.2 Purification of digested DNA

After restriction digestion of the DNA, it was purified with the use of Thermo Scientific GeneJET Gel Extraction Kit (Thermo scientific) because by using it the samples get purified and no traces of undigested vector were left that can interfere during transformation.

- The DNA samples were run on 1% agarose gel at 70V. The gel part containing the DNA fragment was excised and weighted.
- Then binding buffer was added in to the eppendrof having gel slice in 1:1 volume and incubated at 50°-60°C for 10 minutes until the gel slice was melted.
- 800 µl of gel solution was transferred in to the purification column and the column was centrifuged for 1 minute. Flow-through was discarded and column was packed in the tube again.
- 700 µl of wash buffer was added in the column and flow through was discarded.
- For one more time the column was centrifuged to remove the residual wash buffer.
- Then the column was transferred into a clean 1.5 ml microcentrifuge tube and elution was done with 20-30 µl of lukewarm MQ water and then it was stored at -20°C.

After gel elution procedure the concentration of eluted samples was measured on nanodrop.

3.2.10 Bacterial cell transformation

Competent cells preparations

- The fresh luria broth was prepared and cultured with single colony of *E.Coli* DH5α cells. It was incubated at 37°C for 16 to 20 hours with vigorous shaking.
- The 20 ml of LB was inoculated with 200 µl of overnight grown culture of *E.coli* DH5α and incubated at 37°C for 2-3 hours till the $O.D._{590}$ reaches 0.5.
- After incubation, the culture was poured into ice cold 50 ml aukrages.
- The tubes were kept on ice for 10 minutes and were centrifuged at 5000 rpm for 10 minutes at 4°C and the supernatant was discarded.
- The tubes were kept in inverted positions for 1 minute and the pellet was resuspended in 10 ml of ice cold 0.1 M $CaCl_2$.
- The tubes were kept on ice for 10-15 minutes and centrifuged at 5000 rpm for 10 minutes at 4°C.
- The supernatant was discarded and the tubes were kept in inverted position for 1 minute.

- The pellet was resuspended in 1 ml of ice cold 0.1 M $CaCl_2$ and stored on ice for 12-24 hours.

Transformation

- About 100 µl competent cells were taken in pre-chilled microfuge tubes.
- Then 100 ng (5µl) of plasmid in first tube having competent cells was added and mixed and then it was labeled as test vial. In another vial of competent cells no insert was added and vial was labeled as control.
- The mixture was stored on ice for 30 minutes.
- After incubation the vials were kept in water bath at 42°C for exact 2 minutes.
- Rapidly the vials were transferred to ice box and the cells were chilled for 1-2 minutes.
- Then 1 ml of LB was added to each vial and incubated for 45-60 minutes at 37°C.
- The vials were centrifuged at 10000 rpm for 1 minute and then the pellet was dissolved in 100 µl of LB.
- About 100 µl of transformed cells were spreaded on LA + Amp plates (Appendix) and incubated at 37°C for 12-16 hour.

3.2.10.1 Bacterial colony PCR

- Pin heads of colonies was dissolved in 5µl of distilled water in PCR vials. The PCR vials were incubated at 98°C for 10 minutes and then quickly transferred on ice.
- About 19 µl of reaction mixture into the same PCR vial was added and then the PCR was run according to the gene program.
- After running the PCR the samples were loaded on 1.5% of agarose gel and the amplified DNA bands were visualized in U.V. light.

3.2.11 Yeast transformation

The plasmid (pFL61+LbMT3) was isolated from positive colonies after bacterial transformation. Cadmium sensitive strain *yap1* and copper sensitive strain DTY4 and a wild type BY4741 cells were used for transformation. The transformed cells were selected on SD-Ura only and SD-Ura with metal plates.

- YPD medium (20 ml) was prepared and inoculated with yeast colony and incubated overnight at 30°C with 200-250 rpm shaking.
- After one day incubation the O.D. at 600 nm of culture was measured and the required amount of culture in 40 ml of YPD flask was added so that its final O.D. becomes 1 and final volume becomes 50 ml.
- Then it was incubated at 30°C for 2 hours at 200 rpm so that the final O.D. reaches up to 2.
- The culture was transferred into 50 ml falcon tube and centrifuged for 5 minutes at 3000 rpm at room temperature.
- The pellet was re-suspended in 20 ml of distilled water and centrifuged for 5 minutes at 3000 rpm.
- The salmon sperm DNA (10 µl) was placed at 100°C for 5 minutes and then quickly transferred on ice for 5 seconds for further use.
- The supernatant was discarded and the pellet was re-suspended in 1 ml of water and the yeast suspension was transferred in 1.5 ml ependroff.
- It was centrifuged for 30 seconds with the bench centrifuge. The supernatant was discarded and the pellet was re-suspended in 1 ml of water.
- About 100 µl of aliquots in ependroff were made of yeast competent cells and were centrifuged for 30 seconds with bench centrifuge and the supernatant was discarded.

The following mixture was added in each tube, 240 µl of PEG 3500- 50% W/V, 360 µl of LiAC (1 M), 10 µl of salmon sperm DNA (10 mg/ml) 4-1 µl of plasmid DNAl of autoclaved distilled water

- This mixture was incubated in water bath for 1 hour at 42°C followed by centrifugation for 30seconds.
- Then 1 ml of YPD media was added, the pellet was re-suspended in it and was incubated for 1 hour and then centrifuged for 30 seconds and the supernatant was discarded.
- Then 1 ml of water was added in each tube and again centrifuged for 30 seconds, the supernatant was discarded.
- 1 ml of water was added and the pellet was re-suspended in it. Spreading was done with the transformed yeast cells on metal amended plates which were placed at 30°C for 2 days.

3.2.11.1 Yeast colony PCR

- Pin heads of colonies was dissolved in 10µl of 20mM NaOH in PCR vials. The PCR vials were incubated at 98°C for 30 minutes and then quickly transferred on ice.
- The vials were spinned for 30 seconds. 1 µl of supernatant from the vials was taken as template transferred into new PCR vials containing 19 µl of reaction mixture explained in section 3.2.8 and then the PCR according to the gene program was run.
- 1.5% agarose gel was used to visualize the amplified DNA bands in U.V. light.

3.2.12 Yeast functional complementation assays

To study the functional complementation properties of LbMT3 gene DTY4 and *yap1* yeast cells culture having DNA (pFL61, pFL61+LbMT3) and BY4741 which were obtained after successful transformation, were grown in SD-Ura (SD without uracil) medium at 30°C with 200 rpm for 2 days. After incubation the O.D. was adjusted same for all yeast cultures and 5 µl of 10^0-10^{-4} dilutions were dotted on SD without Uracil and SD without Uracil supplemented with 150 µM $CuSO_4$ and 40 µM $CdSO_4$ plates. Plates were incubated at 30°C for 3 days and growth pattern was studied.

For growth kinetics, flasks containing 50 ml of SD-Ura medium were inoculated with mid-log precultures of *yap1* and DTY4 cells having (pFL61, pFL61+LbMT3) and BY4741 with (PFL61 only) was used as control. After incubation the initial O.D. of yeast cultures was set to 0.02 at 600 nm and the cells were allowed to grow for 6 hours at 30°C and then 150 µM $CuSO_4$ and 40 µM $CdSO_4$ were added. The O.D. at 600 nm of all the cultures was measured after 3 hours interval for next 36 hours.

3. RESULTS

4.1 Bioinformatic analysis

The LbMT3 gene (protein ID 399677) was characterized using various bioinformatics tools. Firstly, the transcript of the gene was obtained from ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The ORF of the gene consists of 102 bps which encode 35 amino acids (figure 8). The homologous sequences of the gene were obtained through BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (figure 9).

399677

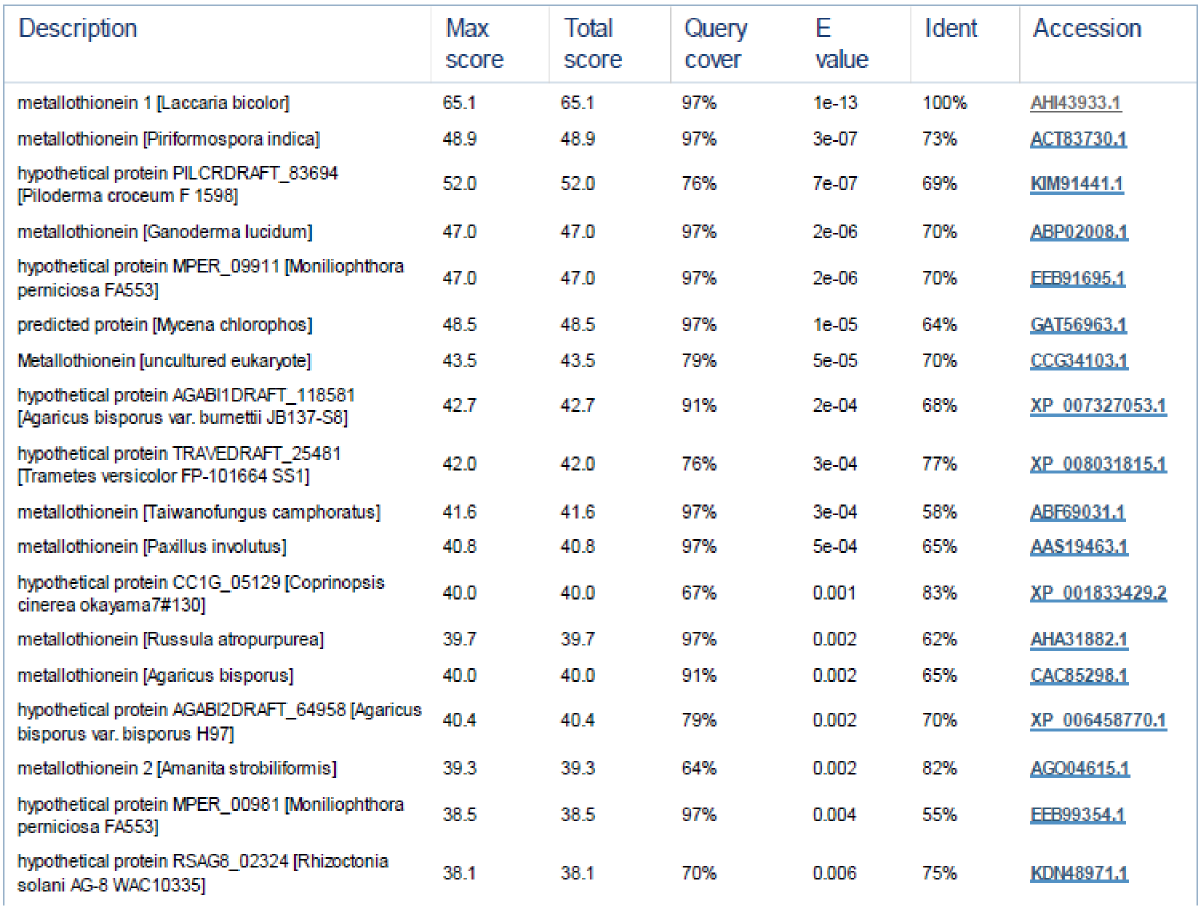
GGAAGAGATGGGCGGGTGATGGTGGAAAGAAACAGAGCCCGCCGTCGTCCGAATTTTCAGTCCTT
ACAGCAACGAGCAGCGAGGTCAAACACGCACAGAACACGAGGATTATCGGCAACTCCCGAAAAC
AAATACCACCAAATACTGATTATCTATTTATGTGGTCCAATCCGAGATCAAGGTGAACATAATCTC
TTGTGTAAACCATGATTTCTGTGTGTACCTCTTTCATTGGTATCGTTACAAACTCACCCTTCTTTAG
ACCATCAACGTACCCGTCTCCCAGACTTGCGGCAGCAGCTCTTGCAACTGCGGCGAAAGGTGACCT
TACTGTTTCCCTCCTGTCGTTCTCATCTCATCCGTACTTTGCTCAGTTGTGCCTGCAAGCCTGGTGA
ATGCAAGTGCTGATCGGAATTTTACTCCTAATTGTACATTGTATCTTAAGAAATTGATGCTGGAAA
GAACTAAGCTGTCACCTCTCTTCCTGCAAGAATATGAATGATCGGCCGCAAGTCATTTCAACAAAG
TACTTACAAGGTCTTCATATCGCCTACGTCTAAGCGGATCCAATTCTTGAAGTTGCTGCAGCATGTT
GTTGCATTCTCTTGTCAACATCGCCAGGTCAACGTCAGTGGCGTGGTCCTTTATTAGCATTCGCTTG
TAGCAGACGATCG

Figure 7: Genomic sequence of LbMT3 gene

1 atgatttctaccatcaacgtacccgtctcccagacttgcggcagc
M I S T I N V P V S Q T C G S
46 agctcttgcaactgcggcgaaagttgtgcctgcaagcctggtgaa
S S C N C G E S C A C K P G E
91 tgcaagtgctga 102
C K C *

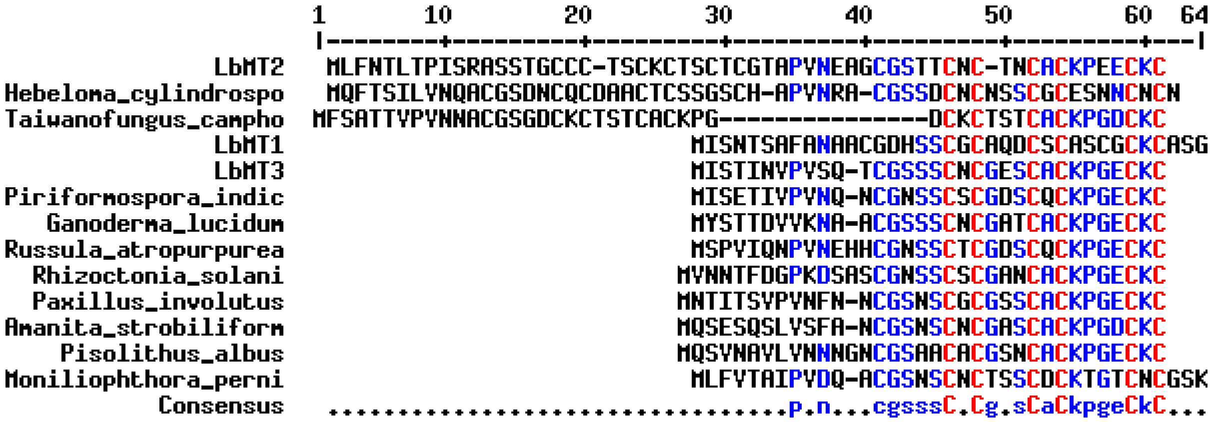
Figure 8: The transcript sequence of LbMT3 gene.

The homologous sequences of different species were retrieved in the FASTA format. The so obtained sequences were then analysed by doing multiple sequence alignment using MULTALIN software (http://multalin.toulouse.inra.fr/multalin/). The multiple sequence alignment of homologous sequences showed seven conserved cysteine residues, out of which 6 forms C-x-C (figure 10).



Description	Max score	Total score	Query cover	E value	Ident	Accession
metallothionein 1 [Laccaria bicolor]	65.1	65.1	97%	1e-13	100%	AHI43933.1
metallothionein [Piriformospora indica]	48.9	48.9	97%	3e-07	73%	ACT83730.1
hypothetical protein PILCRDRAFT_83694 [Piloderma croceum F 1598]	52.0	52.0	76%	7e-07	69%	KIM91441.1
metallothionein [Ganoderma lucidum]	47.0	47.0	97%	2e-06	70%	ABP02008.1
hypothetical protein MPER_09911 [Moniliophthora perniciosa FA553]	47.0	47.0	97%	2e-06	70%	EEB91695.1
predicted protein [Mycena chlorophos]	48.5	48.5	97%	1e-05	64%	GAT56963.1
Metallothionein [uncultured eukaryote]	43.5	43.5	79%	5e-05	70%	CCG34103.1
hypothetical protein AGABI1DRAFT_118581 [Agaricus bisporus var. burnettii JB137-S8]	42.7	42.7	91%	2e-04	68%	XP_007327053.1
hypothetical protein TRAVEDRAFT_25481 [Trametes versicolor FP-101664 SS1]	42.0	42.0	76%	3e-04	77%	XP_008031815.1
metallothionein [Taiwanofungus camphoratus]	41.6	41.6	97%	3e-04	58%	ABF69031.1
metallothionein [Paxillus involutus]	40.8	40.8	97%	5e-04	65%	AAS19463.1
hypothetical protein CC1G_05129 [Coprinopsis cinerea okayama7#130]	40.0	40.0	67%	0.001	83%	XP_001833429.2
metallothionein [Russula atropurpurea]	39.7	39.7	97%	0.002	62%	AHA31882.1
metallothionein [Agaricus bisporus]	40.0	40.0	91%	0.002	65%	CAC85298.1
hypothetical protein AGABI2DRAFT_64958 [Agaricus bisporus var. bisporus H97]	40.4	40.4	79%	0.002	70%	XP_006458770.1
metallothionein 2 [Amanita strobiliformis]	39.3	39.3	64%	0.002	82%	AGO04615.1
hypothetical protein MPER_00981 [Moniliophthora perniciosa FA553]	38.5	38.5	97%	0.004	55%	EEB99354.1
hypothetical protein RSAG8_02324 [Rhizoctonia solani AG-8 WAC10335]	38.1	38.1	70%	0.006	75%	KDN48971.1

Figure 9: Homologous sequences of LbMT3 gene obtained by BLASTp analysis.



```
                     1       10        20        30        40        50        60  64
                     |--------+---------+---------+---------+---------+---------+---|
               LbMT2  MLFNTLTPISRASSTGCCC-TSCKCTSCTCGTAPVNEAGCGSTTCNC-TNCACKPEECKC
Hebeloma_cylindrospo  MQFTSILVNQACGSDNCQCDAACTCSSGSCH-APVNRA-CGSSDCNCNSSCGCESNNCNCN
Taiwanofungus_campho MFSATTVPVNNACGSGDCKCTSTCACKPG---------------DCKCTSTCACKPGDCKC
               LbMT1                            MISNTSAFANAACGDHSSCGCAQDCSCASCGCKCASG
               LbMT3                            MISTINVPVSQ-TCGSSSCNCGESCACKPGECKC
Piriformospora_indic                            MISETIVPVNQ-NCGNSSCSCGDSCQCKPGECKC
   Ganoderma_lucidum                            MYSTTDVVKNA-ACGSSSCNCGATCACKPGECKC
Russula_atropurpurea                            MSPVIQNPVNEHHCGNSSCTCGDSCQCKPGECKC
  Rhizoctonia_solani                           MVNNTFDGPKDSASCGNSSCSCGANCACKPGECKC
  Paxillus_involutus                           MNTITSVPVNFN-NCGSNSCGCGSSCACKPGECKC
Amanita_strobiliform                           MQSESQSLVSFA-NCGSNSCNCGASCACKPGDCKC
    Pisolithus_albus                           MQSVNAVLVNNNGNCGSAACACGSNCACKPGECKC
Moniliophthora_perni                            MLFVTAIPVDQ-ACGSNSCNCTSSCDCKTGTCNCGSK
           Consensus  .................................p.n...cgsssC.Cg.sCaCkpgeCkC...
```

Figure 10: Multiple sequence alignment of homologous sequences showing the presence of Cys residues and C-x-C motifs are represented by red color. Gaps are introduced to provide the best alignment which are indicated with dashes.

MEGA 7 software (http://www.megasoftware.net/) was used to construct the phylogenetic tree using maximum parsimony method. The tree obtained showed the evolutionary relationship of LbMT3 gene with metallothionein genes of other species (figure 11). LbMT3 gene showed maximum homology and evolutionary relationship with MT genes from basidiomycetes.

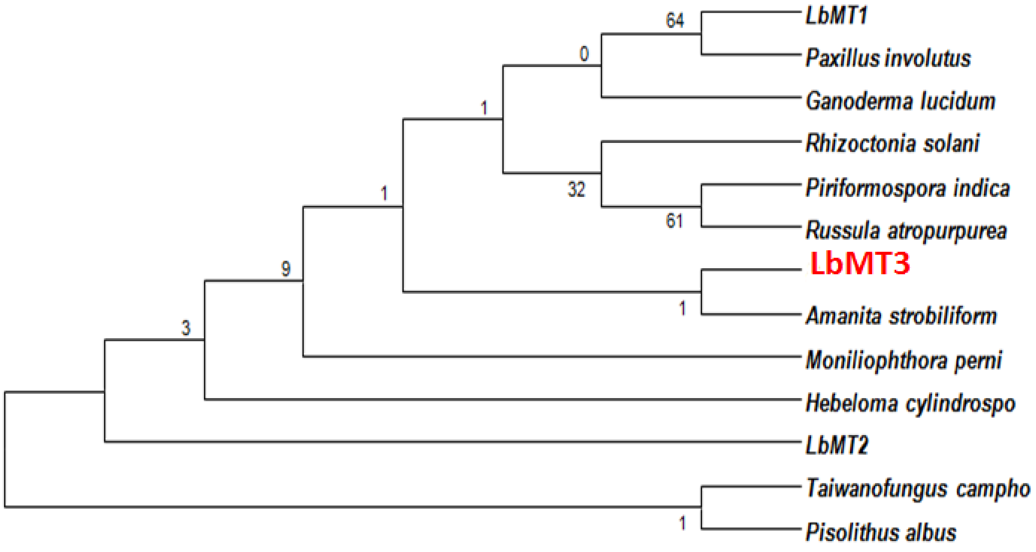


Figure 11: Phylogenetic tree showing various homologous sequences obtained by MEGA 7. The comparative study of homology can be done among the 3 putative genes of *Laccaria bicolor* (LbMT1, LbMT2, LbMT3(red)).

The homology among LbMT1, LbMT2 and LbMT3 was studied from phylogenetic tree using maximum parsimony. The LbMT3 is homologous to *amanita strobiliform.*

The genomic DNA of *L. bicolor* was isolated the LbMT3 gene was also amplified from genomic DNA . A significant difference was observed in the size of amplifications so obtained. The amplification obtained from cDNA was of 102bps whereas that obtained from genomic DNA was of ~200bps (Figure 12).

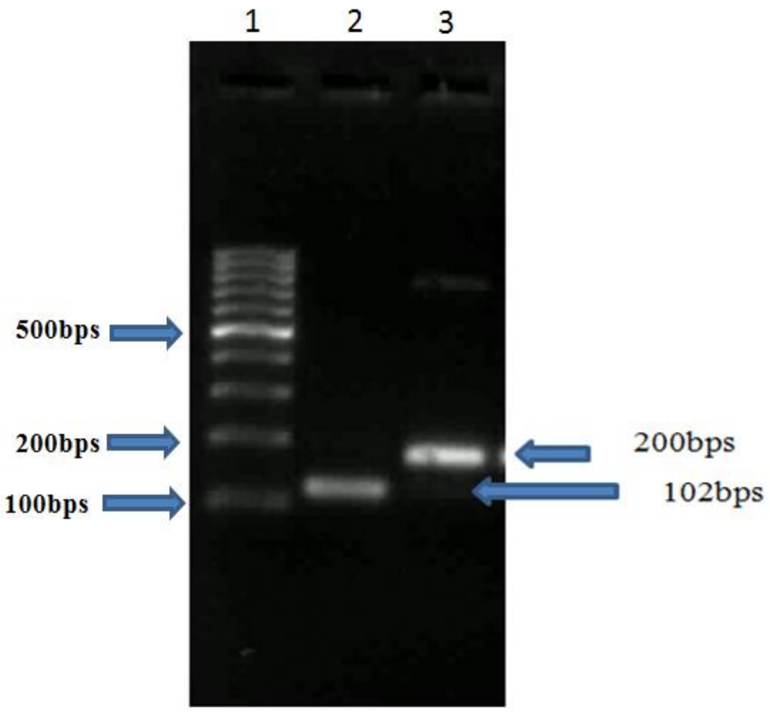


Figure 12: LbMT3 gene amplified by cDNA in 2nd lane and genomic DNA in 3rd lane.

The amplified products so obtained were purified and sequenced by Sangar DNA sequencing services. The sequences so obtained were aligned using multiple sequence alignment tool Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). On comparing both cds and genomic amplification of LbMT3 genes two introns as shown below in figure 13

```
  1 ATGATTTCTGTGTGTACCTCCTTTCATTGGTATCGTTACAAACTCACCCTTCTTTAGACC  60
     M   I   S  -   -   -   -   -    INTRON   -   -   -   -   -   -   -   -   T
 61 ATCAACGTACCCGTCTCCCAGACTTGCGGCAGCAGCTCTTGCAACTGCGGCGAAAGGTGA 120
     I   N   V   P   V   S   Q   T   C   G   S   S   S   C   N   C   G   E   S -
121 CCTTACTGTTTCCCTCCTGTCGTTCTCATCTCATCCGTACTTGCTCAGTGGCTGCATGCA 180
    -   -   -   -   -   INTRON   -   -   -   -   -   -   -   -   -   -   C   A
181 AGCCTGGTGAATGCAAGTGCTGA                                       203
     C   K   P   G   E   C   K C
```

Figure 13: The genomic amplified product showing the coding and non coding regions

4.2 Tolerance of *Laccaria bicolor* to different heavy metals

The tolerance of *Laccaria bicolor* was analysed in the presence of metals by growing it in metal embended media for 21 days. The mycelium was harvested and dried. The dry weight was recorded for Cu (table 7) and for Cd (table 8).

Table 7: Dry weight of mycelium at different concentration of copper

Cu conc(µm)	**Dry weight(mg/50 ml)**
0	29.98±2.52
100	14.18±1.92
200	11.22±0.769
300	3.06±0.378
400	1.29±0.195

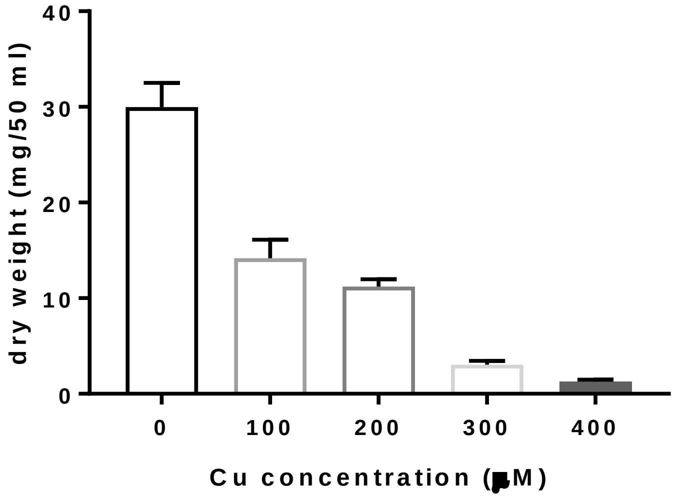


Figure 14 :Effect of different copper concentration on mycelium growth

With the increase in metal concentration the dry weight of mycelium decreases which means higher concentraions of metal effect the growth of *Laccaria bicolor (*figure14).

Table 8: Dry weight of mycelium at different concentration of cadmium

Cd concentration(μM)	Dry weight (mg/50 ml)
0	54.3±1.61
3	20.82±2.64
6	17.5±2.46
9	10.6±1.40
12	5.6±3.34
15	3.62±1.21

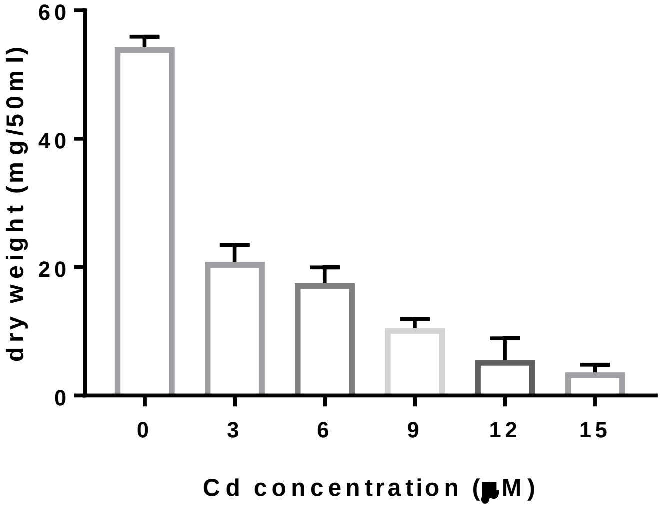


Figure 15: Effect of different Cd concentration on mycelium dry weight

With the increase in concentration of Cd the dry weight of mycelium was decreased. This shows that the biomass of mycelium get affected by metal presence (figure 15).

The dried mycelium was further used to calculate the uptake metal by the mycelium at different concentrations of metals Cu and Cd as shown in (table 9) and (table 10) respectively atomic absorption spectroscopy.

Table 9: Copper uptake in *Laccaria bicolor* at different copper concentrations

Copper Concentration(µM)	Weight (µg/mg)
0	0.0157±0.005
100	0.966±0.05
200	1.39±0.006
300	1.56±0.09
400	0.175±0.004

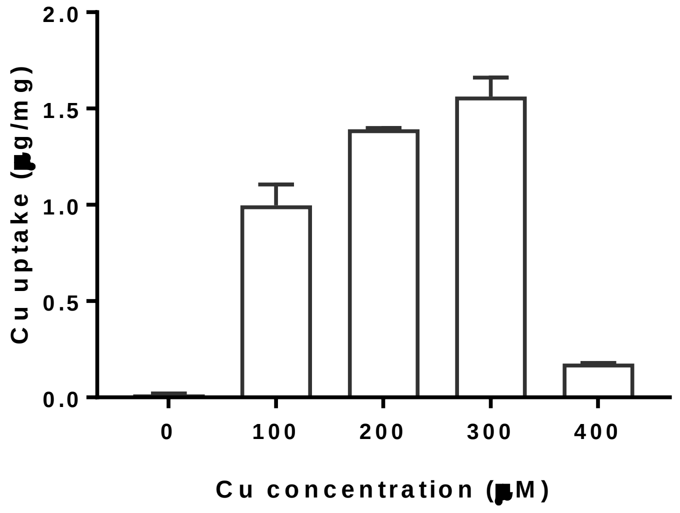


Figure 16: Accumulation of copper metal in *Laccaria bicolor* at different metal concentrations

The maximum copper was uptaken at 300 µM of Cu concentration which means that LbMT3 gene is getting expressed maximally at 300 µM of Cu concentration.

Table 10 : Accumulation of cadmium in *Laccaria bicolor*

Cadmium Concentration(µM)	Weight (µg/mg)
0	0.093±0.001
3	0.236±0.001
6	0.765±0.001
9	0.887±0.04
12	0.409±0.001

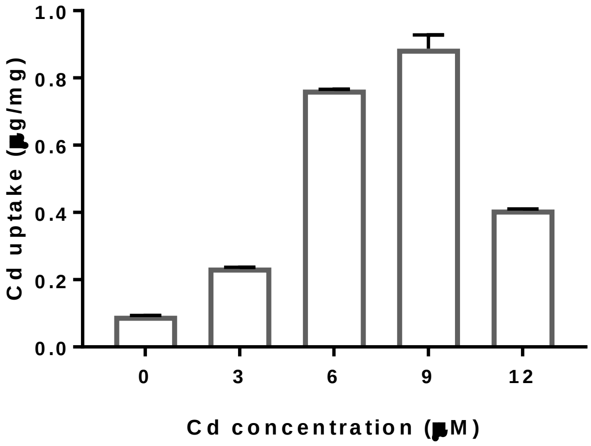


Figure 17 : Cadmium accumulation in *Laccaria bicolor* at different concentrations of Cd

The accumulation of cadmium in mycelium increases with the increase in cadmium concentration upto certain value (9 µM of cadmium and 300 µM of copper) but after that point the uptake of Cd and Cu decreases. It indicates the capacity of the mycelium to grow and uptake different metals.

4.3 RNA isolation

The RNA was isolated from the mycelium samples that was grown on the different concentrations of metals(table11 and table 12). For copper 0, 100, 200, 300, 400 µM (figure 18) and for cadmium 0, 10, 20, 30, 40 µM concentrations were used. The mycelium was crushed with liquid nitrogen and then cDNA was synthesized by Reverse transcriptase PCR. The cDNA was further used to analyse the gene expression at different metal concentrations.

Table 11 : Concentration of RNA at different concentrations of copper

RNA Sample	Metal Concentration (µM)	RNA concentration (µg/µL)
A	0	2.6
B	100	1.7
C	200	2.8
D	300	2.5

Table 12 : Concentration of RNA at different concentrations of cadmium

RNA Samples	Metal Concentration (µM)	RNA concentration (µg/µL)
A	0	3.6
B	10	4.1
C	20	3.9
D	30	3.7
E	40	3.9

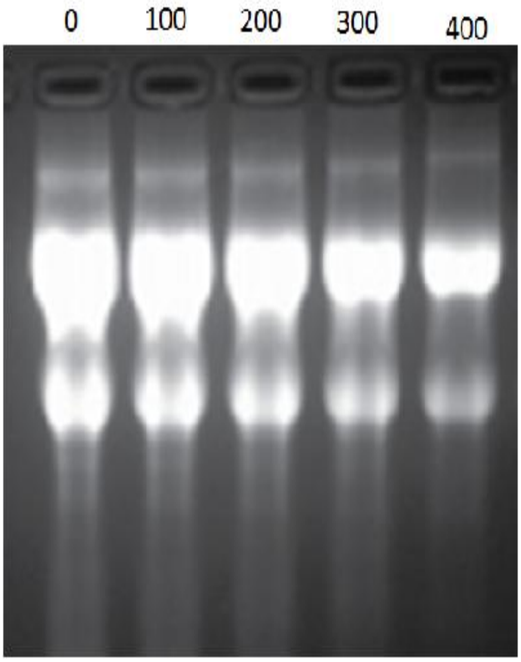


Figure 18 : RNA isolated from mycelium grown on different Cu concentrations(0, 100, 200, 300, 400 µM)

To check the quality of RNA it was run on 1% agarose gel because RNA is highly prone to degradation.

qRT-PCR analysis

The cDNA synthesized from RNA was used to run qRT-PCR where actin gene (housekeeping gene) was taken as control. The gene expresions were analyzed by the formula:

$$Expression\ fold = \frac{2^{(Target\ control - Target\ stress)}}{2^{(Housekeeping\ control - housekeeping\ stress)}}$$

Table 13 : Variation in gene expresion at different cadmium concentrations

Cd concentration(µM)	Fold expression
3	3.56±0.02
6	8.88±0.03
9	9.65±0.04
12	16.46±0.03

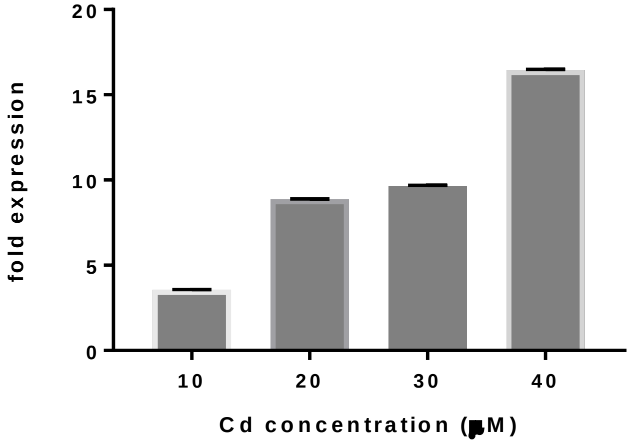


Figure 19: Change in fold expression with change of cadmium concentration

It was observed that with the increase in Cd concentration there was upregulation of LbMT3 gene in *Laccaria bicolor* (figure 19 and table 13). It indicates that LbMT3 gene is responsible for cadmium tolerance.

Table 14 : Change in fold expression at different concentrations of copper

Cu concentration (µM)	Fold expression
100	4.93±0.0152
200	5.76±0.060
300	8.73±0.097
400	8.88±0.105

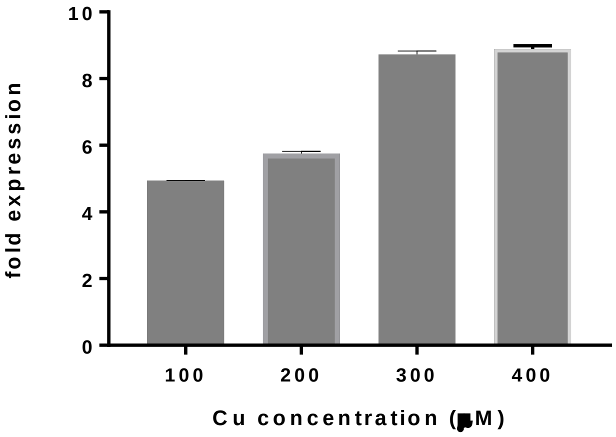


Figure 20: Change in fold expression with change of copper concentration

It was observed that the expression of LbMT3 gene was increased upto many folds and the highest level of gene expression was observed at 400 µM concentration of copper (figure 20 and table 14). It indicates that under metal stress conditions the metallothionein gene get expressed significantly.

4.4 Cloning of metallothionein genes

The amplified gene and plasmid pFL61 were double digested with EcoR1 and BamH1 and digested products were purified. Then the ligation of purified products (LbMT3 gene and pFL61) was done. The ligated product was used for transforming the *E.coli* DH5α cells. Colony PCR and plasmid isolation were used to check the positive clones (figure 21).

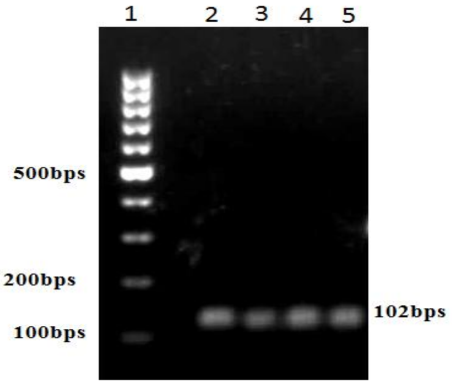


Figure 21: Positive clones of LbMT3 gene in 2,3,4 and 5 lanes showing exact size bands (102bps) against 100 bps ladder (1 lane)

From the confirmed positive clones the ligated plasmid was isolated and further transformed into metal sensitive yeast mutants.

4.5 Yeast complementation study

To validate the function of LbMT3 gene check at different concentrations of both copper and cadmium, yeast metal sensitive strains DTY4 and *yap* were used for transformation. Then positive colonies were selected by streaking them on SD-Ura embedded with respective metal plates.

Table 15 : Growth pattrens of different Cu sensitive yeast strains

Time (hours)	BY4741	DTY4	DTY4+Cu	LbMT3 Cu
6	0.046±0.006	0.069±0.010	0.072±0.004	0.0187±0.002
9	0.090±0.005	0.091±0.004	0.109±0.011	0.047±0.001
12	0.493±0.002	0.367±0.014	0.118±0.003	0.398±0.017
18	1.351±0.052	0.721±0.020	0.115±0.020	0.490±0.013
21	1.501±0.010	0.810±0.080	0.137±0.006	0.490±0.014
24	1.521±0.040	0.921±0.022	0.137±0.002	0.497±0.021
27	1.556±0.002	0.958±0.030	0.143±0.004	0.480±0.025
30	1.582±0.033	1.092±0.002	0.147±0.001	0.524±0.036
36	1.689±0.017	1.177±0.033	0.156±0.011	0.510±0.012
40	1.700±0.006	1.214±0.012	0.160±0.010	0.511 ±0.013

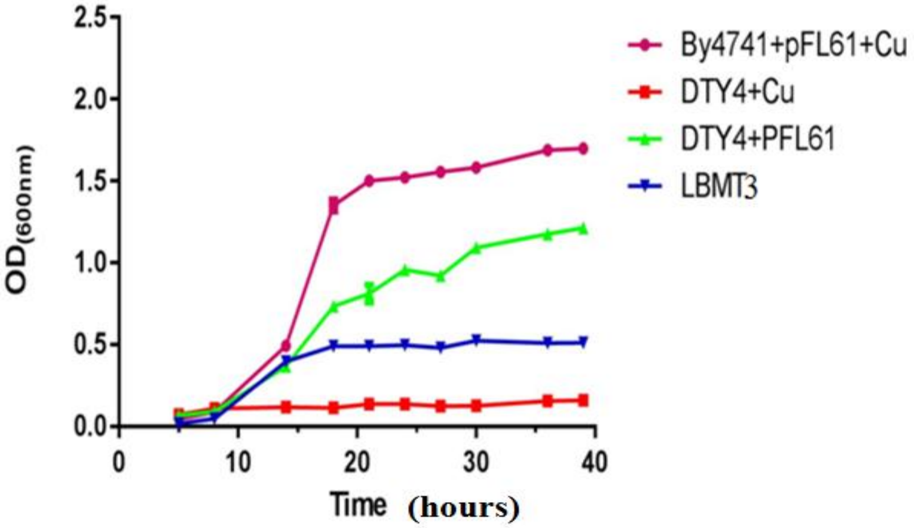


Figure 22 : Growth kinetics was studied of different yeast strains in presence of copper (150 µM).

Growth kinetics of LbMT3 gene in the presence of copper showed tolerance to this metal but its tolerance level was low whereas the wild type (BY4741) was tolerant and yeast mutant (DTY4) having no copy of metal resistance gene without pFL61 did not grow in Cu (figure 22). It means metallothionein gene is present in LbMT3 gene and responsible for metal tolerance(Table 15).

Table 16 : Growth pattrens of different Cd sensitive yeast strains

Time	**BY4741**	***Yap***	***Yap*+Cd**	**LbMT3 cad**
5	0.057±0.002	0.051±0.000	0.046±0.001	0.059±0.001
8	0.080±0.006	0.062±0.005	0.055±0.000	0.076±0.008
12	0.133±0.004	0.068±0.003	0.060±0.001	0.098±0.011
18	0.174±0.007	0.096±0.011	0.060±0.007	0.326±0.042
21	0.214±0.015	0.209±0.005	0.067±0.013	0.799±0.007
24	0.266±0.009	0.301±0.045	0.060±0.004	1.052±0.003
27	0.401±0.047	0.490±0.127	0.058±0.001	1.23±0.002
30	0.429±0.021	0.636±0.033	0.066±0.006	1.34±0.0262
33	0.438±0.018	0.772±0.002	0.073±0.009	1.50±0.133
36	0.420±0.006	0.866±0.045	0.072±0.006	1.61±0.021
48	0.427±0.001	0.942±0.013	0.061±0.004	1.45±0.112

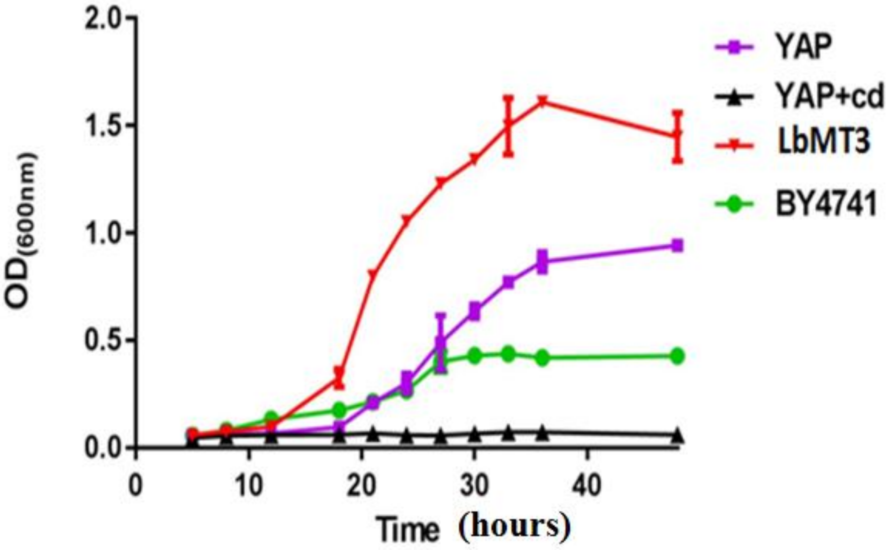


Figure 23: Growth pattrens of different yeast strains in presence of cadmium

The growth kinetics of LbMT3 gene in presence of cadmium showed sigmodal shaped curve which means it is highly tolerant to Cd. Cd resistant yeast mutant *yap* was used as a control which in presence of Cd did not grow while the growth of BY4741 was moderate. (figure 23 and table 16).

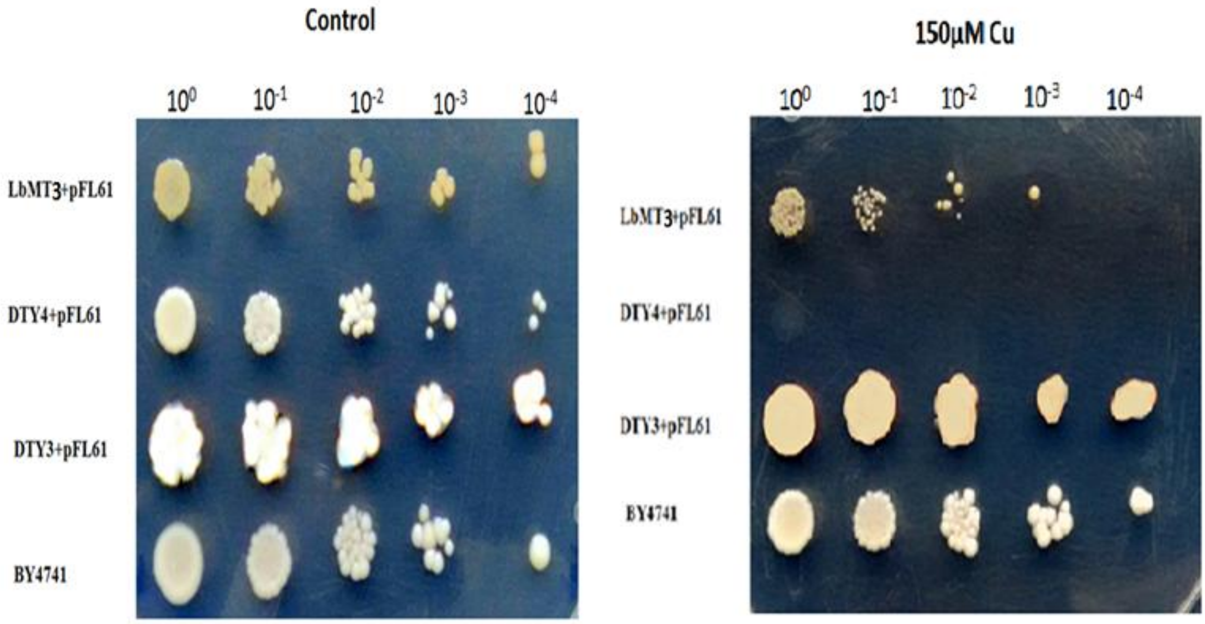


Figure 24: Functional complementation of copper sensitive yeast mutants on selective media. The diluted (10^0 to $10^{-4)}$ transformant cultures were spotted on SD-Ura medium with or without metal supplement as shown.

In the functional complementation studies of *Laccaria bicolor* the Mutant strains were transformed with empty vector pFL61 or with pFL61+LbMT1. In control (plate without metal) yeast mutant DTY4+pFL61 showed growth but on copper emended plate no growth was observed. DTY4 does not contain any gene copy which provide resistant to copper (figure 24). The positive transformants were tolerant to Cu but was moderately expressed.

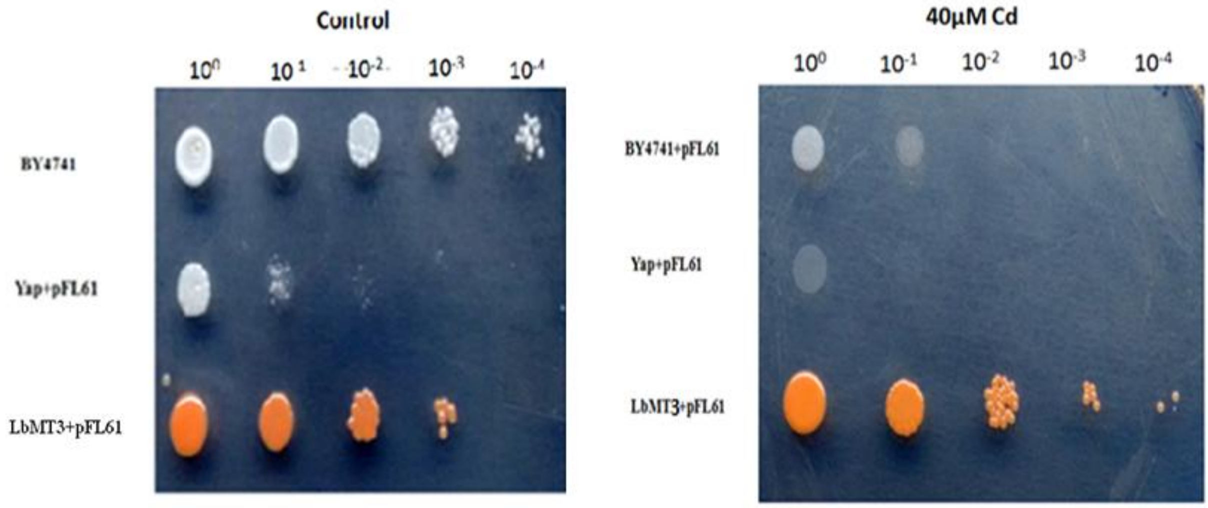


Figure 25 : Functional complementation of cadmium sensitive yeast mutants on selective media. The diluted (10^{0} to 10$^{-4)}$ transformant cultures were spotted on SD-Ura medium with or without metal supplement as shown

In case of cadmium, yeast mutant *yap* did not show resistant to cadmium whereas growth of the LbMT3 gene was observed. It indicates that LbMT3 gene is highly tolerant to cadmium (figure 25).

From the different yeast functional complementation studies it was observed that yeast strain containing empty vector (pFl61) did not grow on metal embedded plate while growth in the yeast strains containing ligated vector with LbMT3 gene was observed. So LbMT3 gene is responsible for metal tolerance.

5. Summary

Mycorrhizal fungi are an important part of the various activities that may improve the plant's wellness and growth. These activities are responsible for providing protection to the host plants against heavy metal stress. Ectomycorrhizal fungi are more tolerant to the higher concentrations of the metals as compared to their host plants. These fungi can be brought into use for studying the different metal tolerant mechanisms at the molecular level for better and deeper understanding. ECM fungi plays very crucial role in metal detoxification and in improving the growth conditions by transporting and mobilizing the different nutrients like phosphorus and nitrogen to roots. With time fungi have evolved and improved its various mechanisms involved in metal detoxification. In this study the effects of different heavy metals like Cd and Cu on *Laccaria bicolor* were assessed. The considerable variations in metal tolerance by this fungus were analyzed which shows that might be different mechanisms are employed by the fungus to tolerate the heavy metals.

Firstly the metal tolerance studies on mycelium grown in liquid broth under different metal stresses were performed. The dry weight of mycelium decreased with the increase in metal concentration. The metal accumulation in the mycelium at different metal concentrations showed the variations in the metal uptake. The metal uptake amount increased up to certain concentration (for cadmium 9 µM/ml and for Cu 300 µM/ml) and then it was decreased. The LD_{50} values were also calculated. The LD_{50} values of cadmium where 50% of the growth of mycelium was inhibited was at 3 µM Cd concentration and LD_{50} value of copper was found to be at 100 µM Cu concentration.

Further to study the expression of LbMT3 gene, qRT-PCR analysis was done on the cDNA isolated from mycelium grown on different metal concentrations. Fold expression was calculated at different concentrations of metal. The fold expression of metallothionein gene was increased with the increase in metal concentration. The maximum gene expression was observed at 400 µM of Cu concentration and at 40 µM of cadmium concentration. It indicates that LbMT3 gene get expressed at high metal concentrations with many fold increment.

There were variations in the induction of LbMT3 gene in response to different metals. LbMT3 gene is tolerant to both the metals (Cu and Cd) but its expression level varies. Bioinformatics analysis has proved to be very helpful in finding the homology of LbMT3 gene with other genes by which the structure or function of any unknown sequence can be predicted. The structural features of metallothionein genes by bioinformatics tools can be visualized easily. The metallothionein genes are cysteine rich molecules and this feature was analyzed in this study by multiple sequence alignment. Moreover evolutionary relationships can easily be studied after generating phylogenetic tree by maximum parsimony method using MEGA7. The homology was studied between LbMT3 gene and the already characterized genes of *Laccaria bicolor* (LbMT1 and LbMT2). Bioinformatics facilitates the study of LbMT3 gene at molecular level and reduces the complexity level.

For studying the genomic sequence of LbMT3 the genomic DNA analysis was done where the gene amplification of LbMT3 gene showed the presence of introns. Two introns in the LbMT3 gene were found due to which the size of the gene increased which was visualized on agarose gel. In checking the tolerance of *Laccaria bicolor*. For functional complementation studies the LbMT3 gene ligated with pFL61 vector was transformed into metal sensitive yeast strains. The comparative study of metallothionein gene with wild type and yeasts mutants on SD-ura with and without metal plates was done. The yeast mutants (DTY4 and *yap*) having no copy of metal resistant gene did not grow on metal embedded plates whereas the transformants having LbMT3 gene were able to grow. However, LbMT3 gene showed tolerance to both Cd and Cu metals but is more resistant to Cd as compared to Cu. From these studies it can be concluded that in the presence of cadmium the gene expression was higher than in Copper.

This study can be prove to be very beneficial in the future where there will be more need to control the soil pollution. *Laccaria bicolor* may be used as potential tool in bioremediation.

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APPENDIX I

Melin’s modified Norkans medium

$(NH_4)HPO_4$ 250 mg/l
KH_2PO_4 500 mg/l
$MgSO_4.7H_2O$ 150 mg/l
$CaCl_2.H_2O$ 50 mg/l
NaCl 25 mg/l
1 % (w\v) $FeCl_3$ 1.2 ml/l
Thiamine HCl 40 mg/l
Biotine 0.4 mg/l
Glucose 2500 mg/l
Heller’s micronutrient (100x) 10 ml/l
Agar 8 g/l

YPD medium
Peptone 20 g/l
Yeast extract 10 g/l
Agar (for plates only) 20 g/l
Add H_2O to 950 ml.
Allow medium to cool to55C and then add dextrose (glucose) to 2% (50 ml of a sterile 40% stock solution).

PEG/LiAc solution (polyethylene glycol/lithium acetate)
Prepare fresh just prior to use.
PEG 4000 40%
TE buffer 1X
LiAc 1X

LB broth
Bacto-tryptone 10 g/l
Bacto-yeast extract 5 g/l
NaCl 5 g/l
Adjust pH to 7.0 with 5 N NaOH.

Heller’s Micronutrient solution (Heller, 1953)
$FeCl_3.6H_20$ 100 mg/l
$ZnSO_4.7H_20$ 100 mg/l
H_3BO_3 100 mg/l
$MnSO_4.4H_20$ 10 mg/l
$CuSO_4 .5H_20$ 3 mg/l
$AlCl_3$ 3 mg/l
$NiCl_3.6H_20$ 3 mg/l
KI 1 mg/l

Genomic DNA Extraction buffer
Sodium acetate 100 mM
Na_2EDTA 50 mM
NaCl 500 mM
SDS 1%

LB/amp$_+$ agar plates
Prepare LB broth as above. Add agar (18 g/l), autoclave, and cool to 50°C. Add ampicillin to 50 µg/ml.

Plasmid extraction solution I (10X)
Tris-HCl 25 mM (pH 8.0)
Glucose 50 mM
Na_2EDTA 10mM

Plasmid extraction solution II
NaOH 5M
SDS 10%

Plasmid extraction solution III
5.0 M K-acetate (pH 4.5)

Extraction buffer
Sodium Acetate 100 mM
Na_2EDTA 50 mM
NaCl 500 mM
SDS 1%

Agarose gel loading dye (6X)
Bromophenol blue 0.25%
Xylene cyanol FF 0.25%
Glycerol in water 30.0%