Cloning and characterization of metallothionein genes of *Suillus sibiricus* an ectomycorrhizal fungus

A dissertation

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For the award of the degree of

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IN

BIOTECHNOLOGY

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I hereby declare that the work being presented in this thesis "**Cloning and characterization of metallothionein genes of *Suillus sibiricus* an ectomycorrhizal fungus**" submitted by the undersigned for the award of the degree of Master of Science in Biotechnology, at Department of Biotechnology is a true and independent work of my own carried out under the supervision of **Dr. M. Sudhakara Reddy**, Professor,

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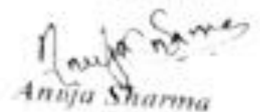

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ABBREVIATIONS

SNW06	*Suillus sibiricus*
dATP	2'-Deoxyadenosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
ssDNA	single stranded DNA
dTTP	2'-Deoxythymidine 5'-triphosphate
EDTA	Ethylenediamine Tetra acetic Acid
HCl	Hydrochloric Acid
Kb	Kilobase pairs
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
RNA	Ribonucleicacid
RNAase	Ribonuclease
TBE	Tris-Borate-EDTA
ME	Malt Extract
YPD	Yeast Potato Dextrose
YNB	Yeast Nitrogen Broth
SD Ura^-	Synthetic Dextrose media lacking uracil
MT	Metallothioneins

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ABSTRACT

Heavy metal pollution is one of the major environmental hazards concerning the world today. Heavy metals are continuously mobilized into our food chain through metal contaminated soil, air and water. Heavy metal contaminated soil hinders the plant growth by entering into the plant cells and altering the cellular metabolism. In such cases mycorrhizal fungi plays a vital role. The mycorrhizal fungi form a symbiotic relationship with the plant roots and protect them from various heavy metal stresses. The ectomycorrhizal fungi have the inbuilt defence mechanism to chelate the heavy metals by producing the metal chelators like, metallothioneins, glutathione, phytochelatins etc. It has been observed that plants associated with the mycorrhizal fungi can sustain high concentrations of heavy metals. Metallothioneins, cysteine-rich polypeptides, are quoted to be responsible for metal detoxification by chelation and homeostasis in eukaryotic organisms. This study focuses on the cloning and characterization of the metallothionein genes, SsMT1 and SsMT2 from *Suillus sibiricus* into the metal-sensitive yeast mutants to study the expression of the genes in eukaryotic system. The metallothionein genes were firstly identified in the EST library of *Suillus* species. These EST sequences were characterized using the multiple sequence alignment and phylogenetic analysis. Using these analysis two metallothionein genes were selected and the gene specific primers were designed as SsMTF1, SsMTR1, SsMTF2, SsMTR2. Restriction sites were added to the 5' end of these primers. The metallothionein genes were amplified using these primers and restriction digestion was performed along with pFL61 using the restriction enzymes EcoRI and BamHI. The ligated product was transformed into *E.coli* DH5α cells and then into the *Saccharomyces cerevisae* mutant DTY4 (Copper sensitive strain) which is a mutant of BY4741(Copper tolerant). Expression of SsMT genes was studied by applying yeast functional complementation assay. In this approach the expression of both the genes in response to Cu stress was studied as compared to that of the BY4741 (wild, heavy metal-tolerant) strain. We also studied the level of gene induction under Cu stress at different concentrations of the metal. The mycelium was exposed to different concentrations of $CuSO_4$ and then the biomass was acquired and studied for the effect of different concentrations on the growth of the mycelium.

1. Introduction

1.1 Heavy Metal Pollution

Deteriorating environmental condition is the biggest threat to the society today and a big topic of discussion too. Heavy metal pollution is one of the major causes of declining health of our environment. These include metals like Lead, Copper, Cadmium, Zinc, Cobalt and other heavy metals that mainly breach through anthropogenic activities like industrial exhaust and drainages (Survey by INSA in 2011). Scientists are continuously indulged in finding a solution to this ever growing issue that is claiming our lives slowly. Developing countries like India must know how to tackle the growing concentrations of this poison in the human system. Sources of the pollution may be any, anthropogenic or natural where natural sources include seepage of these metals from the rocks, volcanic activities, forest fires, etc.

Toxic Elements & Heavy Metals

What limits have been established so far?

	EPA	FDA	USP	WHO/FAO		EU	EU	CA PROP 65
	Drinking Water		Oral Limit	Food	Water	EU Directive 1881/2006	EFSA/ CONTAM Panel	
	mg/L (unless specified)		ppm			mg/kg wet weight	Total Weekly Intake (TWI)	
Aluminum	50-200 µg/L	—	5,000	—	100-200 µg/L	—	—	—
Arsenic (inorganic)	0.01	Apple Juice: 10 ppb	1.5	Provisional Tolerable Weekly Intake (PTWI) 15 µg/kg body weight	10 µg/L	—	No limit; panel says it needs more data.	10 mcg daily intake
Cadmium	0.005	Food Color Additives: 15 ppm	2.5	Provisional Tolerable Monthly Intake (PTMI) 25 µg/kg body weight	3 µg/L	.05 – 3.0	2.5 µg/kg per bw	4.1 mcg daily intake
Copper	1.3	—	50	—	2,000 µg/L	—	—	—
Lead	0.015	Total Daily Intake (TDI) 75 mcg/day Bottled Water: 5 µg/L Candy: 0.1 ppm Fruit Juices: 50 ppb	1	Previous limit withdrawn in 2011.	10 µg/L	.02 – 3.0	Previous limit withdrawn in 2013.	0.5 mcg daily intake
Mercury	0.002	Elemental: 1 ppm	1.5	PTWI 1.6 µg/kg per bw	Inorganic: 6 µg/L	0.1 – 1.0	Inorganic Mercury: 4 µg/kg bw Methylmercury: 1.3 µg/kg bw	0.3 mcg daily intake
Tin	—	—	3,000	PTWI 14 mg/kg per bw	—	50 – 200	—	—

Figure 1 Different heavy metals and their permissible limits in food as updated by some of the regulatory bodies as updated on January 10, 2014. source: labs.natralnews.com

Heavy metal toxicity can cause degenerative diseases with symptoms like mental disorder, GI disorders, muscle and joint pains, poor vision, decreased physical stamina and susceptibility to fungal infections (Bamji, 2011). The metals are more likely to affect small malnourished children and pregnant women much more strongly as compared to any healthy individual (Lal, 2011). Many physiochemical and bioremedial measures are being followed top check the entry of the metal pollutants right at their site of production or storage in the environment (big rocks).

Various methods have been proposed to cut short the release of these metals at toxic levels in the environment. We can go for remediation technologies which involves treating the already leached out metal in such a way that it does not harm our systems in any way and other method widely being used for the purpose is the bioremediation method which involves use of living organisms to check the outflow of metals from the earth's crust as well as industrial exhausts. Bioremediation involves use of plants and microorganisms in an association with each other to reduce the metal toxicity levels in the environment particularly soils (Lal, 2011).

Microflora such as mycorrhizae, possess a great deal of potentiality in degrading the metals *in-vivo* in an association with the host plants, the phenomenon that is known as "Rhizoremediation" (Bamji, 2011). Various plants and microorganisms have been modified genetically for detoxifying the polluted soils. To know the exact effects of heavy metals on the health needs further more research since metal toxicity levels depends upon the age, physiological and nutritional status and genetic factors which means we still need to study the related effects of these metals.

1.2 MYCORRHIZAE

These are some complex symbiotic relations found between the plant roots and the fungi but sometimes these may become parasitic (Pringle & Wolfe, 2011). For these mutual symbioses the word "Mycorrhiza" was first defined and used by a German researcher named A. B. Frank in 1885 (Muchovej, 2011). These are particularly characterized by their three main features which Firstly, includes the invasive nature of these symbioses i.e. they themselves can invade new habitats. Secondly, after forming an association with the plant roots they can also assist the plant to invade new locations or new habitats. Thirdly, they prevent the host plant from any foreign invasions by any of the soil pathogens (Pringle et al, 2011).

These symbiotic associations work in such a way that the fungal counterpart absorbs nutrients such as nitrogen and phosphorus from the soil for the plant and in return the plant caters for its carbon requirements (Pringle et al, 2011).

Mycorrhizae are classified mainly in two categories they are the ones that grow inside the roots of the plants and called as "Endomycorrhiza or Arbuscular mycorrhiza" and the second ones are called as "Ectomycorrhizae" that grow outside the plant roots. Endomycorrhizae generally grow inside the root cortex of the plant. These are characterized by the formation of arbuscles in the root region, giving them the name Arbuscular Mycorrhizae (AM). Generally these mycorrhizae are found in vegetable crops and fruits (Muchovej, 2011).

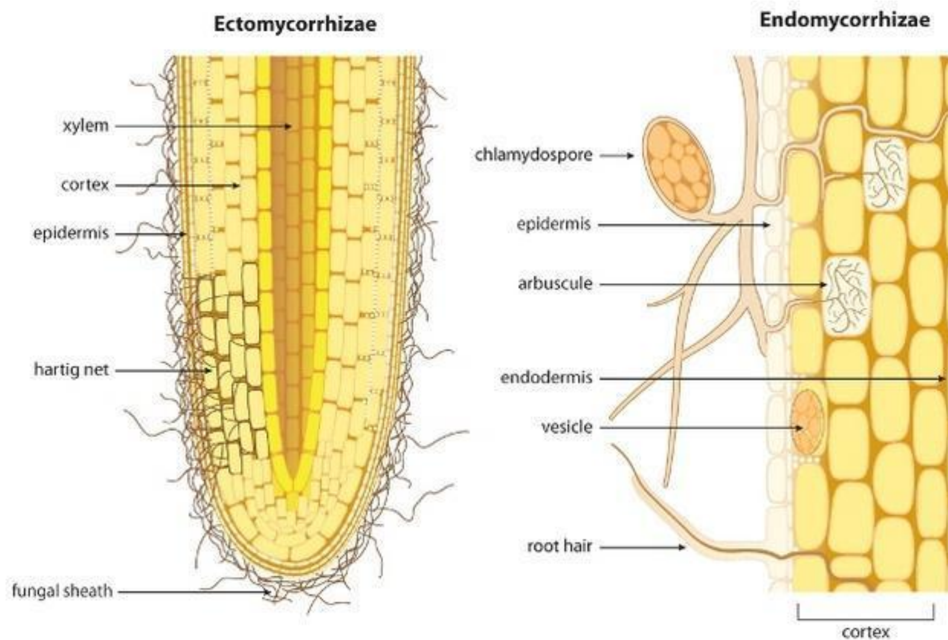

Figure 2 A schematic showing the difference between ectomycorrhizal and endomycorrhizal colonization of plant roots © 2013 Nature education Bonfante, P. and Geri. Mechanisms underlying Plant-fungus interactions in mycorrhizal symbioses

1.2.1 Ectomycorrhizae

These symbioses are characterized by the formation of a sheath around the root tip in the form of a "Hartig net" (Hock and Bertold, 2012) which means the hyphae penetrate the root structure but grows on the outside and covers the whole root. Most of the ectomycorrhizae are found in association with the Pines, Birches, Willows and Oaks (Hock and Bertold, 2012). Old forests include large individuals as a part of the ectomycorrhizal community.

Suillus is one of the most commonly found ectomycorrhizal fungi associated with the pine trees, especially in the mountainous regions of Europe, North America, India and Siberia. It has the potential to extend over an area of $100m^2$ (Harley and Harley, 1987). *Suillus sibricus* also called the "Siberian Slippery Jack", was isolated from the roots of the pine trees of Siberia (Singer,1938). It is characterized by fruit bodies that have slimy caps during the wet weathers with a dia. upto 10cm. On the underside of the caps it posses yellow bruises that are angular in shape and impart a pinkish to cinnamon color to the fruit bodies (Harley and Harley, 1987).

Figure 3 An image showing the fruit bodies of *Suillus sp.* source: (Inzenge) Kuntze amantafoto.it

Not much research work have been done over the metal resistive nature of this particular species whereas a lot of work have been carried out on *Suillus luteus* and researchers have already found certain proteins that can metabolise heavy metals and thus reduce the heavy metal pollution from the soil. Proteins such as Metallothionein and Phytochelatins are held responsible for the metabolism of the toxic metals (Hall, 2001) allowing the healthy survival of the plants even in the soils highly polluted with such metals.

1.3 Metallothioneins and Phytochelatins

The most common methods of metal detoxification include the process of immobilization exclusion, chelation and compartmentalization of the metal ions which includes the binding of

the metal ions with certain peptide ligands as well as the expression of other stress response mechanisms which includes the release of certain stress proteins and ethylene (Bellion et al, 2005).

Two types of metal binding ligands are produced by the plants that help in phytoremediation, they are: metallothineins and phytochelatins both of them are functional equivalents of each other. metallothineins are gene encoded cys- rich polypeptides and phytochelatins are enzymatically synthesized polypeptides (Cobbett, 2000).

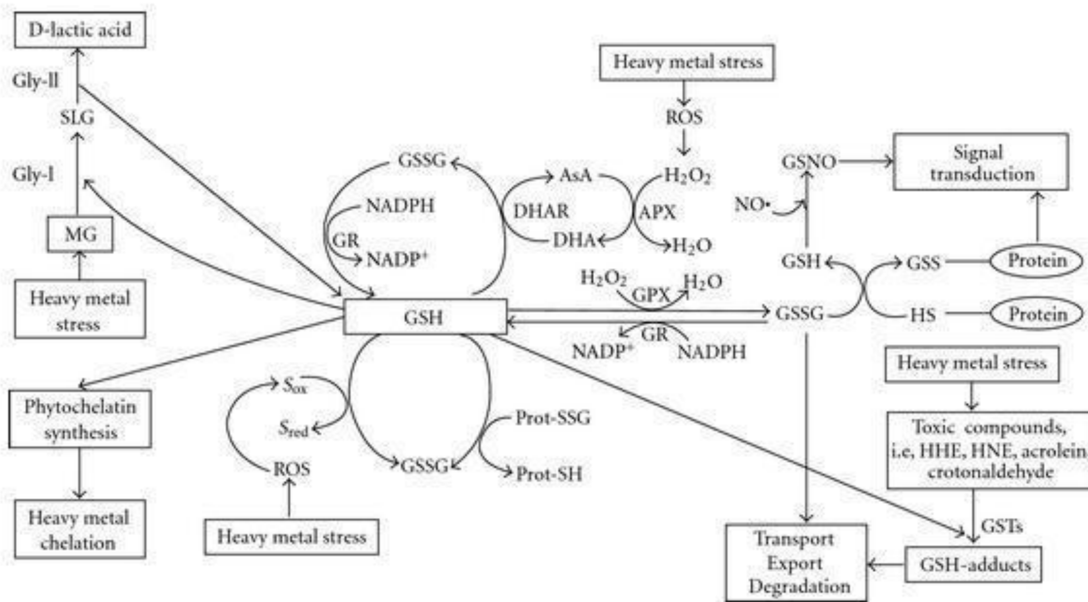

Figure 4 Regulation of GSH and heavy metal metabolizing enzymes adapted from Hossain and Fujita

1.3.1 Metallothioneins

The peptide consists of more than 30% cysteine content with a low molecular mass of 6000-7000Da. It is characterised by the formation of thiol residues with all the metals of groups 11 and 12. Metal thiolate clusters in the metal binding site are responsible for immobilizing and chelating the metal from the surroundings (Stillman, 1995).

The peptide sequence is characterized by the presence of 30% of the cysteinyl residues by number of amino acid residues present with absence of any of the aromatic amino acids.

Metallothineins are classified under three classes based on the source of extraction. Class I metallothioneins come from mammalian sources, Class II come from yeasts and fungi and Class III includes γ-glutamyl cysteinyl isopeptides which comes from a wide range of sources such as yeasts to plants. Studies have shown that a 3-D structure of metal free metallothionein is a random chain as instead of presence of thiol groups there are no di-sulphide bonds present in the molecular structure of the polypeptide. MTs have shown an *in-vitro* scavenging of the free oxygen radicals which hypothesizes that these may neutralize the hydroxyl radicals. Metallothineins derive their antioxidant properties from their characteristic sulfhydryl nucleophilicity and metal complexation (Stillman, 1995).

In the present study the metallothionein genes from the ectomycorrhizal fungus were cloned and characterised.

2. Review Of Literature

2.1. Heavy Metals

No particular, criterion to define heavy metals have been obtained yet but these were classified as metals with high densities (Srivastava and Goyal, 2010). Heavy metals generally posses a density, of more than 5g/ cm^3 (Jarup, 2003).

In periodic table the metals that posses incomplete d-orbitals have been classified as the heavy metals, which includes lanthanides and actinides. Fifty three of the naturally occurring ninty elements are the heavy metals (Weast, 1984).

According to 2015 WHO survey, many heavy metals are required for the normal metabolism of our body but they are toxic or poisonous once they reach a high concentration. There are several essential and non essential metals that have been listed as toxic to the environment such as cadmium, zinc, copper, manganese, nickel, cobalt, chromium, selenium, silver, antimony, thallium, mercury, lead and arsenic.

Howe et al. (1997) stated that although heavy metals are already found in nature in normal concentrations but their breaching from the resources such as metal rocks increases their concentration in the environment leading to the hazardous effects of these on the environment.

Bruins et al (2000) heavy metals such as calcium, cobalt, chromium, copper, potassium, magnesium, sodium, nickel and zinc are essential, either as micronutrients or may be used for several metabolic processes such as redox-processes, molecular stabilization, as components of various enzymes and for regulation of osmotic pressure.

Jarup (2003) since already a part of the environment the heavy metals become toxic when they leach out in the environment mainly due to anthropogenic activities such as mining, vehicle emissions, etc. Other activities continuing the list of potent contamination sources might be combustion, extraction of resources, runoff and release from storage and transport.

The entry routes of heavy metals into the plants and animals are mostly the uptake of these through air inhalation, water uptake, ingestion and direct contact. (Balasubramanian et al., 2009) The uptake of heavy metals by animals, plants and humans is mainly via inhalation, ingestion with food and manual handling.

2.1.1 Heavy Metal toxicity: As already stated above heavy metals are toxic when they cross certain permissible levels. For the proper growth and development of plants and Cu and Zn are required in trace amounts (Hall, 2002). Even in certain cellular processes such as N_2 fixation, breakdown of water molecules during oxygenic photosynthesis, oxygenic respiration, certain catalytic processes, C-C bonds re-arrangement, etc. (Ramesh et.al, 2009) the heavy metals play important role by making certain metal complexes.

Ochiai (1987) metal toxicity may be due to blocking of functional groups, displacement of essential metal ions modification in conformation of biomolecules, denaturation of enzymes and loss of integrity of cell or organ wall.

Ramesh (2008) stated that, the presence of characteristic incomplete d-orbital may result in the formation of complex metal compounds which may or may not show any redox activities.

Hall (2002); stated that the heavy metals form complexes by binding to the sulfhydryl groups of the protein motifs which disrupts the structure of the proteins leading to the inactivation of various enzymes involved in major metabolism processes. As well as these may lead to the elimination of certain essential elements that leads to the deficiency disorders.

Dietz et al. (1999 the heavy metal concentrations beyond permissible limits may lead to the formation of reactive oxygen species along with an emission of certain free radicals which then leads to the generation of an oxidative stress.

The toxicity levels of the pollutants depend upon the bioavailability of the metal which, further depends upon various factors including the metal concentration and the other biological factors such as biosorption, bioaccumulation and solubilisation (Berthelin, 1995). These factors control the level of exposure of the metals to the animals and their environment, whereas, the term exposure is defined as to be the combined effect of concentration of the meal and the time of contact "An event that occurs when there is a contact at the boundary between a human and the environment with a contamination of a specific concentration for an interval of time." (NRC Human exposure assessment for airborne pollutants).

2.2 Mycorrhizae

The term refers to the symbioses between fungi and plant roots. The name mycorrhizae itself is derived from this mutualistic relationship in greek "Myco" means fungi and "Rhyza" means roots.

Pringle and Wolfe (2011) stated that mycorrhizae are ubiquitous organisms found associated to the roots of forest trees such as Pines, Birches, orchids, oaks, etc. Mycorrhizae is a condition which is necessary for the survival of plants living in soils with poor nutrient content or which have nutrient content with a very low bioavailability (Malloch et al., 1980).

Mycorrhizae are quoted to be the main nutrient providing organ in the terrestrial plants (Smith and Read, 1997). These associations shows a characteristic bi-directional movement of the nutrients which is responsible for the symbiotic relationship shown by these associations (Smith and Read, 19917). *H. cylindrosporum* can form mycorrhiza with vide range of gymnosperms (Giltrap, 1982). *H. cylindrosporum* is a pioneer species which can inhabitate new forests and regions of low accumulation (Guidot et al, 2002).

Pringle and Wolfe (2011) although the associations are mutualistic in nature, they take up carbon from the plants in return to the soil nutrients absorbed by the fungal hyphae, but sometimes they may show parasitic relationships too. All the four known fungal cell types are known to be involved in the formation of mycorrhizae including the fungal species like ferns, liverworts, ferns and seed plants. Mycorrhizae can be classified into two distinct types they are: Arbuscular mycorrhizae and Ectomycorrhizae.

The classification of Mycorrhizae is based upon mature structural features and the fungal associate they form symbiose with (Isaac, 1992). Arbuscular mycorrhizae are generally found associated to the roots of shrubs and small plants, whereas Ectomycorrhizae are found associated to the woody trees. Other forms of ectomycorrhizae are arbutoid, monotropoid, ericoid and orchid ferns (Pringle and Wolfe, 2011).

Ectomycorrhizae are much more predominant as compared to arbuscular mycorrhizae. Over 5000-6000 ascoycetes and basidiomycetes form ectomycorrhiza (Buscot et al, 2000). According to the fossil records and carbon dating data, it can be inferred that the ectomycorrhiza evolved some 200 million years ago (Cairney, 2000).

Whitman (2009) in presence of mycorrhizae the plants can generate their own nutrition without the use of any fertilizers and regular watering. This is due to the property of the fungal cells to break down the organic compound complexes to make the nutrition present in the soil available to the plants.

Ectomycorrhiza have been reported to enhance the uptake of the nitrogen, phosphorus and potassium by plants along with some other essential nutrients (Smith and Read, 1997; Barker et al.,1998). This is further enhanced by their property of the mycorrhiza to expand over a vast area of land, they can extend upto an area of a $100cm^2$ as in some species of *Suillus* genera, thus getting the most suitable niche for their proper growth along with the photosynthetic carbon products from the plants (Whitman, 2009).

These mycorrhizae have played a very important role in the evolution of the plants by inferring them with a property of metal resistance. In more than 90% of the plants its seen that the mycorrhizae present are the Arbuscular type.

Khosla and Reddy (2014) Two third of the mycorrhizae forming plants are involved with the arbuscular fungal types, while Ectomycorrhizal fungal species although being less in numbers still rule the forest as they are involved with both the gymnosperms and angiosperms. To impart the metal tolerance property to the plants the fungal cells changes the morphology as well as the physiology of the plants. Primarily these plants enhance the metal tolerant property of their host plant by accumulating the metal in the walls of their hyphae growing outside the matrix as well as in the slime layer present outside the cell walls of the hyphae and thus the metal is kept away from its exposure to the plant tissue.

2.2.1 Mycorrhizae and Metal Toleranc

Although the exact mechanism of, imparting the metal tolerance to the plants by these fungal species have not been assessed properly yet, but it is believed to be a cumulative effect of various factors.

Khosla and Reddy (2014): The mycorrhizal fungi may show differential expression towards metal stress depending upon species i.e. different species may react differently towards a same concentration of the same metal. It may also depend upon the effect of different metals and metal

concentrations on same species. In a general approach the method or mechanism of detoxification of the metal by these fungi may include

(1) Metal mobility: the fungal hyphae adsorb the metals on their surfaces as well as they absorb the metal and store in their vacuoles in their cytosol. This way they act as a filtering barrier to the metal ions, which immobilize the metals and stop them from reaching to the plants. It have been observed that the metal binds to the cell wall of the hyphae due to a net negative charge on their surface an metal being positive in charge gets bound to the fungal hyphae and thus kept away from the plants. Higher concentrations of Zinc have been observed to be accumulated inside the cell vacuoles of the fungal cells.

(2) Hydrophobicity: by this we mean to say that the level of metal uptake by the plants, to an extent, also depends upon the hydrophilicity of the fungal apoplast.

(3) Chelation: another major method of metal detoxification is binding the metal ions before they reach the plant cells. For this purpose the hyphal cells release certain organic acids into the rhizosphere which react to the metal complexes present in the soil. These organic acids react with metal complexes to release free base cations that in turn mobilize the phosphate from iron and aluminium phosphates which otherwise is really difficult for plants to attain.

(4) Metal Sorption on External Mycelium: in this mechanism the fungal cells change the plant physiology such that the metals get bound to the cell walls of the hyphae or get trapped inside the cell vacuole, which directly affects the metal availability.

(5) Production of peptides: production of low molecular weight peptide molecules is the most commonly and widely used mechanism of the mycorrhizae to fight against the metal stress. It includes the release of metallothioneins and phytochelatins inside the cells to metabolise the metal ions present in the cytosol. Metallothioneins are characterised by low molecular weights and high cystein contents. The property of metal detoxification is due to the formation of thiol residues that bind to metal complexes.

When the effect of metals on ectomycorrhizae in relation to host is studied it was observed that most detoxification mechanisms involve the exclusion of the heavy metal such that it does not reach the plant cells (Jentschke and Godbold, 2000).

Jacob et al (2004) under Cadmium exposure polyphenolic compounds are released which may prove to be an important parameter of cellular response to excess concentrations of heavy metals in *P. involutus*.

Bellion et al. (2006): most of the organic acids that are released to chelate the metal ions are the di- and tri-Carboxylic acids. Metal solubilisation from the metal containing mi nerals is facilitated by the protons provided due to the exudation of the organic acids from the mycorrhizal cells. Melanisation of the cells increases the biosorption of the metals.

Green and Clausen (2003) it was observed that copper stress could induce the oxalic acid efflux from the Brown rot fungi. Sometimes soil acidification is caused by the exudates as they provide protons for metal solubilisation present in the metal containing minerals (Fomina et al., 2005).

Bellion et al. (2006) metallothioneins are present in the cellular vacuoles which results in the intracellular chelation of the biosorbed metals. When *Paxillus involutus* was given a cadmium stress, it was found that 20% - 30% of the metal was accumulated in the cytosol and vacuoles. The cadmium conjugated glutathione complexes transport the metal ions out of the cells.

2.3 Metallothioneins

Stillman (1995): These are small polypeptides of molecular weight of about 6000-7000 Da. Metallothioneins show a characteristic property of formation of metal-thiolate clusters. Metallothioneins show a wide occurrence, they have been isolated from yeasts, fungi and even higher eukaryotes such as plants, certain mammals, etc. Metallothioneins are known to isolate naturally present heavy metals. Three classes of metallothionein protein have been isolated.

<u>Class I Metallothioneins:</u> These have been isolated from the mammals. These are made up of 60-62 amino acid residues. Presence of CXC motifs characterizes the metallothionein proteins, where C refers to cystein and X refers to any amino acid. But no aromatic amino acid can be found in the sequence of these polypeptides. Class I amino acids are found to be most effective towards Zn and Cd metals. <u>Class II Metallothioneins:</u> Class II of metallothioneins have been chiefly found in yeasts and fungi. These have a characteristic low cystein content as compared to the class I metallothioneins. Involve a clustered metal thiolate structure in a single domain.

Class III Metallothionein: These are composed of γ-glutamyl cysteinyl isopeptides, isolated from a vast range of sources such as yeasts and plants. Class III metallothioneins show resistance towards Zn, Cd and Cu. Di-sulfide bonds are totally absent from the 3D structure of these proteins. A great deal of similarity can be observed between the metal thiolates formed by Metallothioneins and those which are formed naturally from inorganic thiolates.

Different isoforms of metallothioneins have different roles in detoxification of heavy metals, and the dependence on metallothioneins(MT) also varies environmentally. Metallothioneins can also be used as biomarkers (Amiard et al. 2005).

2.4 *Suillus* and Metal tolerance

Verma and Reddy (2014): *Suillus* have been found to promote the growth in pine trees plant height, biomass of roots and shoots as well as uptake of nutrients like N_2 and P from the soil. Different studies verified that the *Suillus* species shows the best growth on 2% ME media as compared to MMN media and no growth at all on PDA.

While isolating and characterising the *suillus* species total of the eight types of isolates have been isolated numbered from SNW01 to SNW08. Maximum plant and biomass growth was shown by SNW06 *Suillus sibiricus* followed by *Suillus indicus* SNW02, *Suillus granulatus* SNW04 and *Suillus sibricus* SNW07 (Verma and Reddy, 2014).

Van Tichelen et al (2001) Other species of *Suillus* have also been studied that shows metal tolerance, copper resistance was observed in Scots Pine trees infected with *Suillus bovinus* and *Tellephora terrestris*.

Verma and Reddy (2014) In all the ectomycorrhizal fungi *Suillus* is the most prominent and widely found genera in the order Boletales. The *Suillus* ECM is confined mainly to temperate, boreal and Mediterranean regions. Mostly spread along the Northern Hemisphere some may be present along the Southern Hemisphere too. Out of all the known species of *Suillus*, *sibiricus* is the one that is found in the environment most frequently and is the most widely spread species.

Colpaert et al. (2007): The species shows a significant level of adaptation towards heavy metal stress. Along with other factors heat shock proteins and metallothioneins may also be quoted to be responsible for repair of already damaged tissue as well as their antioxidative detoxification mechanisms may be held responsible for reduction in the accumulation of the reactive-oxygen

species. This have been observed that the tolerance of the isolates towards Zn increased when exposed to the soil contaminated with the heavy metals this may be due to enhanced Zn exclusion from the cells, which includes the functioning of metallothioneins and phytochelatins.

Colpaert et al., 2010 It was observed that the expression of two antioxidative proteins was lower in Cd-tolerant species as compared to the expression of a chaperon molecule. E.Krznaric et al. Environmental pollution, 2009: Presence of the heavy metals (in this study Cd) induces the tolerance in the ECMs. The studies showed that the cadmium present in the soils may result in the evolution of the species into cadmium tolerant species as they were observed to express high tolerance as compared to the isolates that were not exposed to Cd stress.

While studying adaptive zinc tolerance in *Suillus bovinus* (Ruytinx et al., 2013) observed that in Zinc sensitive isolates of the mycelium the efficient transfer of zinc into the vacuoles increases its zinc storage capacity as it increases the accumulation of zinc.

It has been observed that elevated levels of heavy metals results in the evolution of the metal tolerance. Same results were observed when isolates of *Suillus luteus* were isolated from high cadmium contaminated soils. They showed an increase in the Cadmium tolerance (Krznaric et al., 2009). Also, it have been observed that the mycelia isolates that show high tolerance *in vitro* pose high protection to the plant hosts (Krznaric et al., 2010). (Adriensen et al., 2006) showed in a recent study that when a less tolerant isolate UH-Sbo-Mg2 was inoculated with the pine roots, they also showed less tolerance, although there was a slightly better than non mycorrhizal plants but the protection was not as good.

Gaps in the study

Lot of work have been done on the metal resistance property of *Suillus luteus* but till date no work have been conducted on the most widely found species of the genus, *Suillus sibiricus* **.**

Metallothionein gene have been isolated from the *luteus* strain, this study is aimed at characterizing the MT gene in a related strain of *Suillus* and also identifiyng its expression pattern in higher eukaryotes.

Objectives

- Cloning and characterization of Metallothinein genes from *Suillus sibiricus.*
- Functional complementation by using metal sensitive yeast mutants.

3.1 Biological Material Used

(1) Culture of *Suillus sibiricus*: The mycorrhiza was isolated from regions of Siberia. We maintained the culture on 2% ME (Malt Extract) medium (pH 5.5), at 25°C for 24 days in dark and then was shifted to 4°C for its storage.

(2) E. coli DH5α: This is a mutatant of *E. coli* which carries certain selectable markers such as lacZ mutation, etc (dlacZ M15 (lacZYA-argF)U169 recA1 end A1 hsdR17 (rK-mK+) sup E44 thi-1 gyr A96 relA1) (Taylor et, al., 1993). The cells were maintained at 37°C for 12-16 hours on 2% LA (Luria Agar) medium and then stored at 4°C. These cells were further used for cloning purpose by making them competent.

(3) Yeast culture: DTY4 (MAT, leu2-3, 112his31, trp1-1, ura3-50, gal1, cup1::URA3) strain of *Saccharomyces cerevisae* was used for showing functional complementation in yeast cells. The wild strain of DTY4 showed copper tolerance therefore this mutant form is used.

3.2 Metal tolerance

Suillus sibiricus culture was first maintained on 2%ME solid medium for 15 days. A pre-processed cellophane sheet was laid on the solid medium plate and the culture disc was inoculated over it. After 15 days the sheet was shifted to a plate containing medium supplemented with five different concentrations of copper sulfate (0, 100, 200, 300, 400µM) and incubated at 25°C for another 3 days. After the incubation period the culture was scrapped off from the cellophane sheet and crushed using liquid nitrogen and preserved at -80°C.

3.3 Bioinformatic techniques

We acquired the EST library of *Suillus luteus* from NCBI website (www.ncbi.nlm.nih.gov). Five EST sequences with GenBank accession numbers as GR975901, GR975896, GR975716, GR975715, GR975714 were retrieved. A reverse compliment of these sequences was acquired and submitted to ORF finder (www.ncbi.nlm.nih.gov/gorf/) and analysed the ORFs present in all

the sequences. The transcripts so obtained were submitted for BLASTp to find out the homologous sequences. The EST sequences retrieved were:

GR975901

GTATGCATCTAGATTGATGAGTCCTGAGTAAAACGCCTCTGCTCGACGAACATCCATACA
TCTCCTACTATAATCATAGGCCGACAACATAATACACGACGAGGCCGAGTTCAAGATTCG
ATATTCAACGTTCAATCAACATTTGCACTCTCCAGGCTTGCATTGGCACGAAGTGCCGCA
TGAGCAGCTCGACGAGCCACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGACAT
ATTGTGATAGATCACTACGCAGT

GR975896

GCGATGCATCTAGATTGACTGCGTAGTGATCTATCACAATATGTCCACCGCTACTGAAGT
CCTTGTTTCTAACAACAACTGTGGCTCGTCGAGCTGCTCATGCGGCACTTCGTGCCAATG
CAAGCCTGGAGGGTGCAAATGTTGATTGAACGTTGAATATCGAATCTTGAACTCGGCCTC
GTCGTGTATTATGTTGTCGGCCTATGATTATAGTAGGGGATGTATGGATGTTCGTCGAGC
AGAGGCGTTTTACTCAGGACT

GR975716

CCTGGTACTCGCGATGCATCTAGATTGACTGCGTAGTGATCTATCACAATATGTCCACCG
CTACAGAAGTCCTTGTTTCTAACAACAACTGTGGCTCGTTGATTAGCACATGCGGCACTT
CGTGCCAATGCAAGACTGGAGAGTGCAATGTTGATTGAACGTTGAATATCGACTCTGGAA
CTCGGCCTCCTCGTGTATTATGTTGTCGGCCTTTG

GR975715

GATGAGTCCTGAGTAATACGCCTCTGCTCGACGAACATCCATACATCTCCTACTATAATC
ATAGGCCGACAACATAAAACACGACGAGGCCGAGTTCAAGATTCGATATTCAACGTTCAA
TCAACATTTGCACTCTCCAGGCTTGCATTGGCACGAAGTGCCGCATGAGCAGCTCGACGA
GCCACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGACATATTGTGATAGATCAC
TACGCAGT

GR975714

GCTCGGTACTCGCGAATGCTCTAGATTGACTGCGTAGTGATCTTCTGCGACTGCGTCTGG
TCTACCGCTTCTGGAGCTAAAACCTCTGCCACTACCACTGCATCTTCTGGTACAACTCAG
AAGACCGGCGCTGCCAGTAGCCTTTCTGTCTCTTCGGCAATGGGTGTTGCCGGTGTGATG
AGTCCTGAGTAATACGCCTCTGCTCGACGAACATCCATACATCTCCTACTATAATCATAG
GCCGACAACATAATACACGACGAGGCCGAGTTCAAGATTCGATATTCAACGTTCAATCAA
CATTTGCACTCTCCAGGCTTGCATTGGCACGAAGTGCCGCATGAGCAGCTCGACGAGCCA
CAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGACATATTGTGATAGATCACTACG
CAGT

The homologous sequences thus obtained were subjected to multiple sequence alignment using Multalin software and we found that there was maximum similarity between four of the five

sequences while only one of them was distinct. The sequences were further characterized by the phylogenetic analysis.

3.4 Molecular techniques used

3.4.1 Isolation of total RNA (TRIzol method)

The culture of *Suillus sibiricus* was grown on 2% ME agar medium with cellophane sheets at 25°C in dark for 15 days and then was transferred to 2% ME agar media supplemented with different concentrations of $CuSO_4$ viz; 0, 100, 200, 300 and 400µM and incubated under same conditions for 3 days. After 3 days the culture was scrapped off the cellophane sheet and crushed under liquid nitrogen to form a powder of the culture and stored at -80°C. Now, the powdered mycelium was used to isolate RNA using TRIzol reagent (Invitrogen). The protocol followed was as per the manufacturer's instructions.

1. Approximately 100mg of the powdered mycelium was taken.
2. Added 1mL of TRIzol reagent and incubated at 15°C for 5 min.
3. Centrifuged at 12000 g, 4°C for 10 min.
4. Added 200µL of chilled chloroform and shaken vigorously.
5. Kept at 15°C for 2-3 min.
6. Centrifuged at 12000g, 4°C for 15 min.
7. Separated the aquous layer in a fresh eppendoerf tube.
8. Added 500µL of chilled Iso-propanol and incubated at -20°C for 10-15 min.
9. Centrifuged at 12000g, 4°C for 10 min.
10. Discarded the supernatant and washed the pellet with 75% ethanol (chilled).
11. Centrifuged at 7500g, 4°C for 5 min.
12. Air dried the pellet and dissolved in RNase free water and stored at -80°C.

4.4.2 Molecular analysis of RNA

This is required to check the purity of the Nucleic acids isolated, before they can be used for any further processing. For analysis of the isolates we can employ two techniques:

Electrophoretic analysis (using simple simple agarose gel electrophoresis): In this we used agarose gel electrophoresis to analyse the RNA quality. We prepared a 1% agarose gel in 0.5X TBE buffer and 0.5μL EtBr for staining purpose. The RNA sample was mixed with the tracking dye and loaded into the wells. The gel was viewed under Gel Doc system after 1 hour run and images were retrieved. The intensity of the bands can be compared to check the quantity of the RNA as well as the purity of the product.

Spectrophotometric analysis: This analysis is required for exact quantification of the nucleic acids. For this we used a nanodrop instrument. 1μL of RNA sample was loaded on the pedestal and reading was recorded. In general 1 unit O.D. at 260 nm is equal to 50 ng/μL of the DNA and 40 ng/μL of the RNA.

4.4.3 DNA Amplification

Reverse Transcription from RNA to cDNA

The cDNA were synthesised from the RNA isolated using Reverse Transcription PCR (RT-PCR) with the help of Reverse AID^{TM} first strand cDNA synthesis kit (Fermentas Life Sciences, USA). The protocol followed was as per the manufacturer's instructions. Approximately 5μg of RNA was taken, added 1μL of oligo dT and MQ water as required to make up the volume upto 12μL. The mixture was incubated at 65°C for 5 minutes then added 4μL of 5X Rxn Buffer (provided in the kit), 1μL Ribolock RNase, 2μL of 10mM dNTP mix and 1μL of RevAID Reverse Transcriptase mixed well and applied PCR with following reaction conditions

Table 1 Reaction conditions for the synthesis of cDNA

Initial Delay	42°C	10 min
Denaturation	42°C	10 min x 2 cycles
Annealing	42°C	10 min x 2 cycles
Elongation	42°C	5 min x 2 cycles
Further Elongation	70°C	5 min

Gene amplification from cDNA

For a single reaction of gene amplification we followed the procedure as was provided by the manufacturer of the gene amplification kit (Fermentas, Life sciences, USA). Added 25μL of the 10X PCR Buffer, 2μL dNTPs, 1μL of the forward primer, 1μL of the reverse primer, 0.3μL of Taq. Polymerase, 1μL of template and MQ water to make up the volume upto 25μL. And a reaction was set as:

Table 2 Reaction conditions for gene amplification from cDNA using gene specific primers.

Initial Delay	94°C	3 min
Denaturation	94°C	1 min x 35 cycles
Annealing	55°C	30 sec x 35 cycles
Elongation	72°C	1 min x 35 cycles
Further Elongation	72°C	8 min
Store	4°C	∞

The primers used were:

SsMT1:5’-CGGGATCCATGTCCACCGCTACTGAAGTC-3’

SsMT1R:5’-CCGGAATTCTCAACATTTGCACTCTCCAGG-3’

SsMT2F:5’-CGGGATCCATGTCCACCGCTACTGAAGTC-3’

SsMT2R: 5’-CCGGAATTCTCAATCAACATTGCACTCTCCAG-3’

This resulted in amplification of SsMT1 gene and SsMT2 gene from the cDNA molecule. This amplification was verified by using Agarose Gel Electrophoresis. A 100bp ladder was loaded along with the sample which validated the presence of a 115bp fragment of the gene.

3.4.4 Plasmid Isolation (Alkaline Lysis method)

For isolation of plasmid we followed two methods 1. Alkaline Lysis method & 2. Using the Qiagen mini prep plasmid isolation kit. In Alkaline lysis method, E. Coli cells containing the plasmid (pFL61) were inoculated in 20mL of Luria Broth supplemented with ampicillin this allows a selective growth of only cells containing the vector.

A. Alkaline lysis method:

1. 1.5-2mL of the liquid culture was taken in a 2mL microfuge tube.
2. Centrifuged for 1 min at 12000 rpm in a benchtop centrifuge.
3. The supernatant was discarded.
4. Added 200µL of solution I (50mM Glucose + 25mM Tris-Cl + 10mM EDTA).
5. Mixed well and allowed to stand for 3-5 sec at room temperature.
6. Added freshly prepared 400 µL of solution II (0.2M NaOH + 1% SDS).
7. Added a 300µL of solution III (5M potassium acetate + glacial acetic acid + water).
8. Stored on ice for 15 minutes and centrifuged at 12000rpm for 5minutes.
9. Added 400µL of P:C:I solution (Phenol:Chloroform:Isoamyl::25:24:1).
10. Centrifuged at 10000 rpm for 10 minutes.
11. Discarded the pellet, and retained the supernatant in a fresh tube.
12. Added an equal volume of chilled isopropanol, and allowed to stand for 5-10 minutes at -20°C.
13. Centrifuged at 12000rpm for 5 minutes at 4°C.
14. Washed the pellet with 300 µL of 75% ethanol.
15. Centrifuged at 8000rpm for 5 minutes and dissolved the pellet in 50 µL of MQ water.

4.4.5 Restriction digestion of the isolated gene and plasmid (Kit method)

The Genomic DNA needs to undergo restriction digestion if it is to be cloned in to a new cell. For restriction digestion we took both the genes and the vector (pFL61) and set up the reaction as follows. Since it was a double digestion we had to first check out the buffer that is compatible with both the enzymes. We took help of online buffer calculator by life sciences technologies and found that for EcoR1 and BamH1 Tango buffer is to be used.

Before we set up the reaction both the nucliec acids were quantified. Instructions were followed as provided with the enzymes by “Thermo fisher” the reaction was set up as, 10µL of each nucleic acid, 4µL of buffer 3µL of MQ water and 1.5µL of enzyme EcoR1

and incubated the mixture at 37°C for one and a half hour. After the incubation 1.5µL of enzyme BamH1 was added and incubated at 37°C for another 1.5- 2 hours and then kept at 80°C for 10 minutes to inactivate the enzymes.

3.4.6 Purification of digested Nucleic acids

The digested nucleic acids were purified using the Thermo Scientific Gene JET Gel exclusion kit. Instructions of the manufacturer were followed.

After the purified product was obtained it was quantified using a nanodrop.

3.4.7 Ligation of the purified product

The purified gene was ligated with the purified plasmid using the ligase enzyme. The reaction was set up using a formula, a 3:1 ratio of gene to plasmid should be maintained in order to achieve a better ligation. The reaction was setup as, using the formula $\frac{conc.of\ vector \times size\ of\ insert}{size\ of\ vector} \times 3$ this equation gives the concentration of the insert to be added. Added 2µL of app. 3 ng/µL of plasmid, 1µL app. 2ng/µL of insert and rest of enzymes as prescribed in the protocol provided with the kit as, 1µL of ligase and 4µL of buffer and incubated at 4°C overnight.

3.4.8 Bacterial Transformation ($CaCl_2$ method)

Competent cells were prepared using E. coli DH5α strain by $CaCl_2$ method. These cells were stored at -80°C in glycerol. Transformation was done as:

1. 100µL of competent cells were taken in pre-chilled microfuge tubes.
2. Added 5µL of ligate/plasmid to the cells, mixed gently and stored on ice for about 30 minutes. Set up a control in which neither plasmid nor insert will be added
3. Kept on water bath at 42°C for 2 minutes.
4. Rapidly transferred the tubes to ice bath and chilled for 2-3 minutes.
5. Added 1mL of LB to each tube and incubated at 37°C for 45-60 minutes.
6. Centrifuged at 8000rpm for 1 minute.
7. Removed 800µof the media and mixed the pellet in the remaining 200µL of the media.
8. Spread 100µL of the mixture each on two plates of LA+Amp.
9. Incubate for 12-16 hours at 37°C.

3.4.9 Yeast Transformation

Primary culture of DTY4 was maintained by inoculating 20mL of YPD broth with the culture, overnight. The O.D. of the culture was maintained at 2. Transferred the culture to falcon tubes and centrifuged at 3000rpm for 10 minutes. The pellet was suspended in 1mL of distilled water. Pelleted out the cells in microfuge tube. Centrifuged and discarded the supernatant and made up the final volume 1mL again. Added 100µL of the culture to the tube, 240µL PEG, 36µL 1M LiAc, 10 mL ssDNA(single stranded DNA), 1µL template and water to make up volume 360µL. Maintained at 42°C for 1 hour. Pelleted out the cells and mixed in 1mL of media and spread on SD media lacking Uracil which was supplemented with $CuSO_4$ in a concentration of 150µM and incubated at 30°C for 2-4 days.

3.5 Assays applied for confirmation of transformants:

3.5.1 Bacterial Colony-PCR

This was applied to confirm the presence of the metallothionein gene. A colony of the transformed cells was picked up and mixed in 5µL of the MQ water. The mixture was incubated at 95°C for 10 minutes, shifted on ice for 2-3 minutes and used as template just like in normal PCR.

3.5.2 Yeast functional Assay

A colony of transformed cells was suspended in 100µL of medium, serially diluted the medium and on a SD URA^- plate, placed drops of 5µL of the above dilutions at equal intervals. This was employed to compare the expression and growth of both the genes in different dilutions of the culture.

3.5.3 Biomass Assay

This assay was applied to check the effect of different concentrations of $CuSO_4$ on the growth of the fungal mycelium. The mycelium was inoculated in a liquid broth and kept for incubation at 25°C in dark for 3 days or till a significant growth is observed then added $CuSO_4$ to it in different concentrations 0, 100, 200, 300, 400µM and kept for incubation at same conditions for 21 days and after that the dry weight was recorded.

3.5.4 Yeast Growth Kinetics

In this assay we compaired the growth of the transformants with that of the non- transformants. Culture was inoculated in SD URA^- broth and incubated at 30°C with a shaking of 230 rpm for 5 hours. After 5 hours $CuSO_4$ was added and O.D. was measured after every 3 hours for 15 hours.

4.1 Designing the primers

After procuring the sequences from EST library of *Suillus luteus* we searched for the ORFs by submitting the sequences to NCBI's ORF finder (www.ncbi.nlm.nih.gov/gorf/). The transcripts so obtained were analysed using multiple sequence alignment tool 'MultAlin' (multalin.toulouse.inra.fr/multalin)

ORF analysis was as:

ORFs found in the sequences were as:

GR975901

```
24 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc M
   S T A T E V L V S N N N C G
     69 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga S
        S S C S C G T S C Q C K P G
    114 gagtgcaaatgttga
        128 E C K C *
```

GR975896

```
41 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc M
   S T A T E V L V S N N N C G
     86 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga S
        S S C S C G T S C Q C K P G
    131 gggtgcaaatgttga
        145 G C K C *
```

GR975716

```
atgtccaccgctacagaagtccttgtttctaacaacaactgtggc
   M  S  T  A  T  E  V  L  V  S  N  N  N  C  G
     96 tcgttgattagcacatgcggcacttcgtgccaatgcaagactgga S
        L I S T C G T S C Q C K T G
    141 gagtgcaatgttgattga
        158 E C N V D *
```

GR975715

```
225 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc M
      S T A T E V L V S N N N C G
    180 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga S
        S S C S C G T S C Q C K P G
    135 gagtgcaaatgttga
        121 E C K C *
```

GR975714

```
225 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc M
      S  T  A  T  E  V  L  V  S  N  N  N  C  G
    180 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga S
        S  S  C  S  C  G  T  S  C  Q  C  K  P  G
    135 gagtgcaaatgttga
        121 E  C  K  C  *
```

When multiple sequence analysis was performed following graph was observed:

```
>GR975901    MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
>GR975896    MSTATEVLVSNNNCGSSSCSCGTSCQCKPGGCKC-
>GR975714    MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
>GR975715    MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
>GR975716 MSTATEVLVSNNNCGSLISTCGTSCQCKTGECNVD
```

These were the five putative sequences identified and the similarity can be seen with the help of the multiple sequence alignment result. 7 Cys residues and 3 C-x-C motifs were observed in four of the sequences while one sequence have only one C-x-C motif. Four out of five sequences were highly similar so we identified two types of MT genes to be present in *Suillus sp*. That were designated as SlMT1 and SsMT2 genes. The primers were designed manually by adding EcoR1 and BamH1 restriction sites to 5' end of all the four types of primers. The primers sequences are as:

SsMT1F: 5'-CGGGATCCATGTCCACCGCTACTGAAGTC-3'

SsMT1R: 5'-CCGGAATTCTCAACATTTGCACTCTCCAGG-3'

SsMT2F: 5'-CGGGATCCATGTCCACCGCTACTGAAGTC-3'

SsMT2R: 5'-CCGGAATTCTCAATCAACATTGCACTCTCCAG-3'

A BLASTp was performed on one of the transcripts to characterize the gene and a homology was observed between the known sequences of the *Suillus* EST and metallothionein transcripts of some other basidiomycetes.

Description	Max score	Total score	Query cover	E value	Ident	Accession
metallothionein [Paxillus involutus]	35.4	35.4	91%	0.20	59%	AAS19463.1
metallothionein [Pisolithus albus]	34.7	34.7	91%	0.34	55%	AJO67962.1
metallothionein [Russula atropurpurea]	33.5	33.5	91%	0.94	50%	AHA31882.1
metallothionein 2 [Amanita strobiliformis]	32.0	32.0	91%	3.6	50%	AGO04615.1
hypothetical protein MPER_09911 [Moniliophthora perniciosa FA553]	31.6	31.6	82%	3.9	55%	EEB91695.1
Metallothionein [uncultured eukaryote]	31.2	31.2	94%	5.1	48%	CCG34103.1
hypothetical protein RSAG8_02323 [Rhizoctonia solani AG-8 WAC10335]	31.2	31.2	71%	5.7	56%	KDN48970.1
metallothionein [Piriformospora indica]	31.2	31.2	74%	6.0	54%	ACT83730.1
hypothetical protein CC1G_05129 [Coprinopsis cinerea okayama7#130]	31.2	31.2	82%	6.9	55%	XP_001833429.2
hypothetical protein JAAARDRAFT_29377 [Jaapia argillacea MUCL 33604]	30.8	30.8	85%	7.5	50%	KDQ63354.1
hypothetical protein PILCRDRAFT_83694 [Piloderma croceum F 1598]	32.7	32.7	68%	9.9	46%	KIM91441.1

Figure 5 Homologous sequences procurred from the BLASTp analysis of SsMT2 gene.

After the BLASTp analysis we submitted the homologous sequences were retrieved in FASTA format and were subjected to multiple sequence alignment using 'multalin' software and it was observed that all the sequences had C-xC residues at same locations, moreover same number of Cys residues were observed.

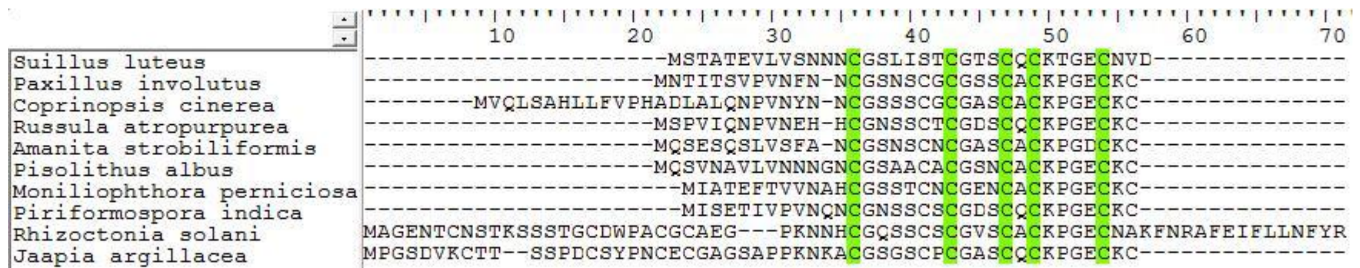

Figure 6 Multiple sequence alignment of MT2 gene of Suillus sp

1 C-x-C residue and total 5 Cys residues were observed in all the sequences. A characteristic feature of all the ectomycorrhizal metallothionein peptides is observed to be the presence of C-x-C motifs near the N-terminal of the peptide. With these aligned sequence a phylogenetic tree was constructed by NJ method. But sequences of *Coprinopsis cinerea*, Rhizoctonia solani and Jaapia argillacea are unrelated species so they were excludedfrom the phylogenetic tree.

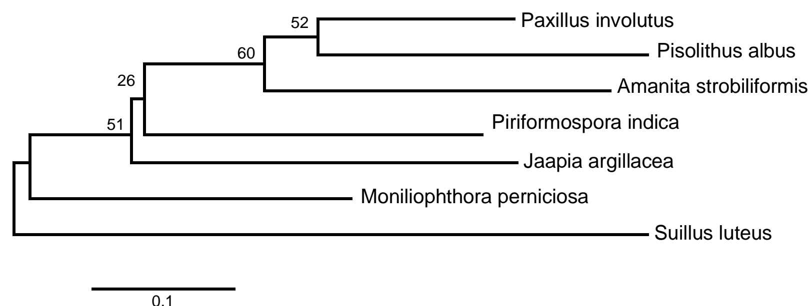

Figure 7 Phylogenetic analysis of metallothionein gene using neighbour joining method with the help of MEGA6 software

For molecular characterization of the putative metallothionein genes we entered the sequences in ExPASy software to analyse the molecular weights and pI values of the polypeptides. The results have been expressed as follows:

Sequenceaccession number	pI value	Mol. Weight (Da)
GR975901	5.88	3419.84
GR975896	7.52	3347.78
GR975716	5.88	3419.84
GR975715	5.88	3419.84
GR975714	4.14	3570.97

It was observed that all the putative metallothionein peptide sequences have the same molecular weight, GR975714 being the longest one with the least pI value. This result suggested that all the peptide sequences were close to metallothionein gene, having a molecular weight close enough to that of the metallothionein genes already sequenced.

4.2 RNA isolation

Total RNA isolated using TRIzol method was first quantified, the concentrations came out to be:

Table 3 Concentration of isolated RNA

Fungal Sample	Metal Concentration (µM)	RNA concentration (ηg/µL)
A	0	3065.7
B	100	2857.1
C	200	2458.4
D	300	2381.7
E	400	1864.3

It was also tested on an agarose gel to know whether the RNA was intact or not. Two bands were observed in the gel which shows that the mRNA is intact.

4.2 Bacterial Transformation: After 12-16 hour incubation small colonies were seen growing on the LA+Amp plate. Whereas no colonies were observed on the plates that were inoculated with cells containing no insert (plasmid/gene), and a significant growth was observed of cells containing only plasmid on ME media without metal. Patches of the colonies were made and colony PCR was performedwhich showed bands near the 100bp fragment. The plasmid of the positive colonies was isolated and transformed into yeast cells.

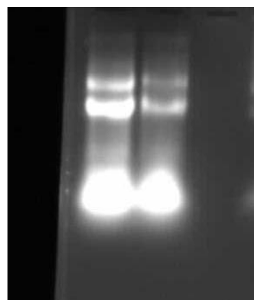

Figure 8 Plasmid bands on Agarose gel

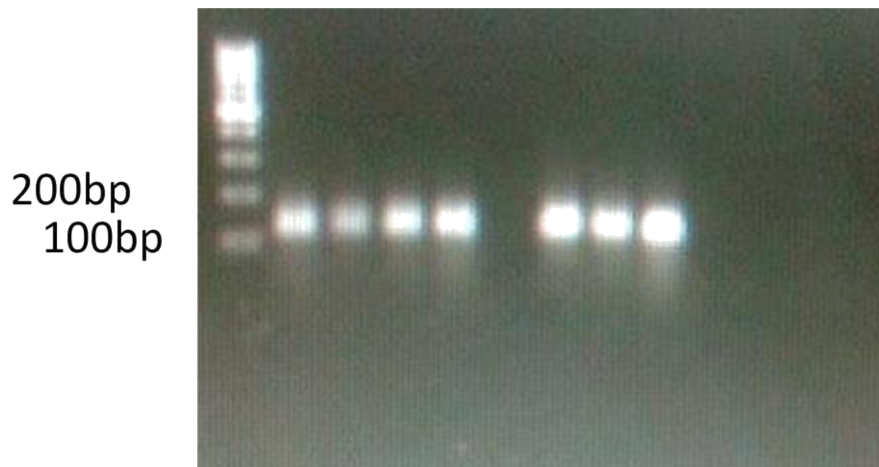

Figure 9 Electrophoresis of DNA derived from colony PCR

4.3 Yeast Transformation

The above isolated plasmid was transformed into yeast DTY4 cells and yeast complementation was studied. The positive growth shows that the MT gene was present in the *sibiricus* strain of *Suillus*. The growth was intense and fast in case of SsMT2 gene which may be inferred as a poor and good expression of SsMT1 and SsMT2 genes respectively.

4.4 Functional complementation of yeast cells

A drop assay of the transformants was done in which SsMT1 showed less resistance as compared to SsMT2 gene (fig 10). The drops were put on two plates one was SD URA^- media and other one was the same media supplemented with $CuSO_4$.

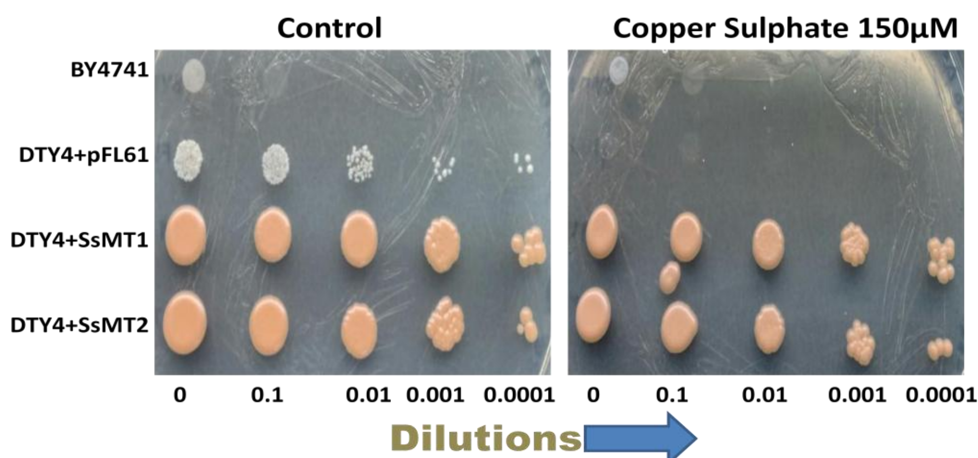

Figure 10 Analysis of yeast complementation assay, of MT genes of *Suillus sp*.

4.5 Liquid Assay

Following table shows the variation in overall growth of the mycelium and pH of the broth with time which can be compared to study the differential expression of the genes at different stress levels.

Table 4 Dry weight of *Suillus sibiricus* biomass that was exposed to different concentrations of $CuSO_4$

Fungal sample	$CuSO_4$ Concentration(µM)	Weight(mg/50mL)of biomass (±SE)
A	0	64.6±3.36a
B	100	50.76±1.86b
C	200	44.63±3.39c
D	300	37.6±1.53c
E	400	24.3±3.73c

The values followed by the same lower case letters are significant at P<0.5. The mean values were compared using Duncan's multiple range test.

Table 5 variation in pH with growth of the mycelium *Suillus sibiricus*

RNA Sample	Metal Concentration	Change in pH	pHafter Test
A	O	0.04	5.46
B	100	0.31	5.19
C	200	0.4	5.1
D	300	0.24	5.265

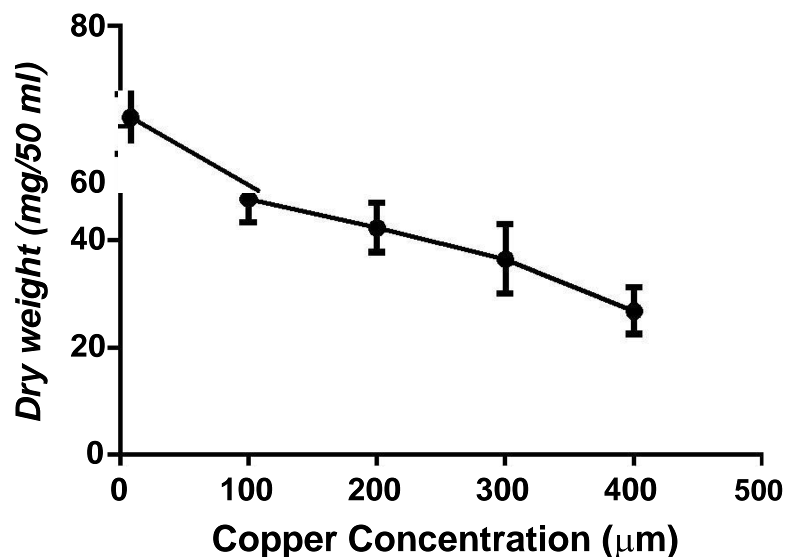

Figure 11 Effect of different Cu concentrations on growth of *Suillus sibiricus*

The graph so observed was analysed for various features of the ectomycorrhizal fungus *Suillus sibiricus*.The present graph shows that fungus was most tolerant towards the $CuSO_4$ at concentration between 100 and 200μM. It was observed that at concentration of $CuSO_4$ around

300μM the ectomycorrhizal biomass reduces to the half of the mycelium mass at 0μM of $CuSO_4$. Therefore we can conclude that in case of *Suillus sibiricus* the LD50 value is equal to or near about 350μM of $CuSO_4$.

Table 6 Growth variation in different strains of yeast under $CuSO_4$ stress at a concentration of 150μM

Time(hour)	pFL61	pFL61+Cu	BY4741+Cu	SsMT1+Cu	SsMT2+C
0	0.027±0.002	0.031±0.001	0.031±0.004	0.046±0.002	0.040±0.00
3	0.110±0.002	0.068±0.001	0.141±0.004	0.060±0.002	0.080±0.08
6	0.284±0.014	0.117±0.010	0.137±0.001	0.139±0.004	0.300±0.00
9	0.618±0.017	0.134±0.016	0.149±0.001	0.201±0.001	0.591±0.02
12	0.979±0.025	0.040±0.002	0.038±0.006	0.333±0.003	0.692±0.29
15	1.485±0.015	0.046±0.039	0.050±0.001	0.796±0.001	1.405±0.09

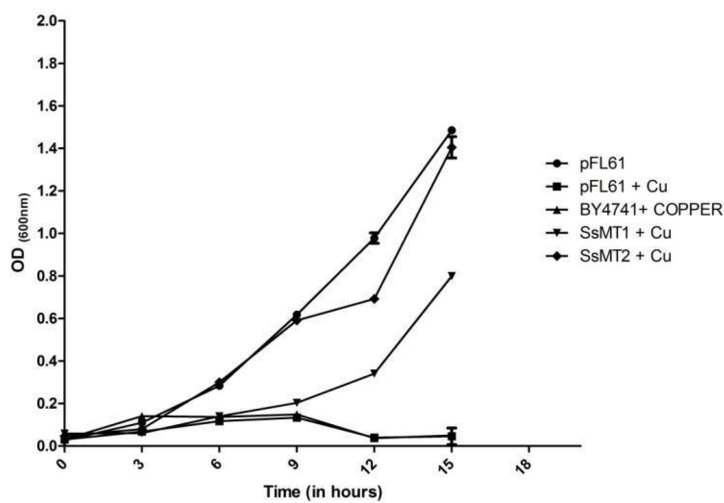

Figure 12 Growth variation indifferent strains of yeast cells under Cu stress

The present work was aimed to characterize and clone the MTs of the ectomycorrhizal fungus *Suillus sibiricus* and to study their specific induction to determine their role in detoxification of the heavy metals. Two MT genes were identified in the mycelium ssMT1 and SsMT2. SsMT1 gene is a slightly shorter fragment and SsMT2 is a slightly longer fragment. The peptide length and presence of C-x-C motifs shows a high similarity of the SsMTs with the metallothioneins of related species such as *S. luteus, S. indicus and S. himalayaensis.* Osobova et al. (2011) showed that no Cys residues in a sequence of 10-13 aa is a specific feature of metallothioneins isolated from ectomycorrhizal basidiomycetes. SsMT2 is composed of two domains which consists three C-x-C motifs which were reported in HcMT2 (Ramesh et al. 2009).

Ramesh et al. showed that HcMT1 responded to Cu and Cd but HcMT2 was induced only by the Cd, but here in this study with the help of yeast complementation assay and the growth kinetics study of the involved genes we could show that SsMT1 and SsMT2 the copper metal could induce both the genes however the level of expression and induction of SsMT1 under copper stress was low as compared to the SsMT2.

Capdevila and Atrian (2011) proposed the concept of MT evolution, based on the level of induction of MTs by different metals, which shows metal specificity of the MT peptide.

To demonstrate the effect of copper mediated gene induction the *Suillus sibiricus* was exposed to different concentrations of the $CuSO_4$ in liquid media which showed the level of gene induction with the change in biomass, the biomass decreased with an increase in the concentration of the metal and it was observed that the growth was most stable at metal concentration somewhere between 100 and 200µM of $CuSO_4$ (fig. 7).

Yeast complementation assay was performed to study the role of SsMT genes in metal detoxification, the assay showed that the genes were capable of providing an increased metal resistence to the mycelium therefore proving that SsMT1 and SsMT2 peptides are capable of developing defence against metal toxicity.

Conclusion

The present work was aimed at characterizing the Metallothinein gene of the *Suillus sibiricus***.**

Two SsMT genes were characterized in the mycelia they were observed to be of 115bp and 102bp in length. When translated it was observed that the protein have characteristic CXC residues concentrated at their N-terminal end. The metallothionein genes of the *Suillus* genera have same type of expression. Both the genes identified in *Suillus sibiricus* are functional and show same response against the heavy metal toxicity. But it was observed that SsMT2 gene have a higher expression as compared to SsMT1 under Copper stress (table 4, fig 8).

The dry weight of the biomass shows that SsMT1 and SsMT2 genes show maximum expression at $CuSO_4$ concentration of around 100-200μM above this concentration the mycelium starts showing a significantly slow growth (fig 7, table2). From the graph it was evident that for *Suillus sibiricus* the LD50 value got from ExPASy near about 300μM of $CuSO_4$

When drop assay was analysed it was observed that there was a very slow growth in BY4741 and DTY4 (without gene) cultures whereas DTY4 cells transformed with SsMT1 showed a very slow growth whereas SsMT2 showed a very fast expression (fig 5 and 6). If we compare the growth of the transformants with that of the wild strain and DTY4+pFL61 SsMT1 and SsMT2 show proves the MT evolution concept.

Thus, I conclude my work on “Characterisation and cloning of the Metallothionein genes of

Suillus sibiricus in metal-sensitive yeast mutants” by performing all the characterization and cloning procedures and studying various factors that influence the expression of the genes.

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Appendix I

Composition for SD Ura$^-$ media pH(5.9) for 1L

Yeast Nitrogen Base	6.7g
Glucose	20g
1% Adenine	1 mL
1% Tyrosine	5 mL
AA mixture	0.77g

For SDUra$^-$ $CuSO_4^+$ added the metal sulphate according to the required concentration.

LB amp$^+$

Added LB media to the distilled water in a concentration of 2%(w/v). Mix well and autoclave before use.

LA amp$^+$

Added LB media in the same concentration as in LB broth and added agar in the concentration of 1.5% (w/v). Autoclaved at 121°C for 15 mins at 15lbs.

Composition of YPD for 1L

Peptone	20g/L
Yeast extract	10g/L
Agar	20g/L

ME media

Added ME to distilled water in a concentration of 2%(w/v). Maintained a pHof 5.5 autoclved at 121°C for 15mins at 15lbs. For solid media added agar in the concentration of 1.5%(w/v).

Appendix II

Plasmid Isolation solution I (10X)

Tris-HCl	25mM(pH8)
Glucose	50mM
Na_2EDTA	10mM

Plasmid Isolation solution II (10X)

NaOH	5M
SDS	10%

Plasmid Isolation solution III (10X)

5M Potassium acetate (pH
4.5).

TBE (10X)

Tris-HCl	0.09M (pH8)
Boric acid	0.9M
EDTA	0.02M 9pH 8)

Agarose Gel Dye (6X)

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%

PEG 40% **TE buffer:** 1X **Lithium Acetate:** 1X