Cloning and Characterization of Metallothionein Genes of Ectomycorrhizal Fungus *Hebeloma cylindrosporum*

A Thesis

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IN

BIOTECHNOLOGY

RAMESH.G

Roll No. 9031004

Department of Biotechnology and Environmental Sciences

THAPAR UNIVERSITY

PATIALA-147004, INDIA

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CERTIFICATE

Certified that the thesis titled, **"Cloning and characterization of metallothionein genes of ectomycorrhizal fungus *Hebeloma cylindrosporum*"**, which is submitted by **Mr. G. RAMESH,** in fulfillment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY** in Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

(Dr. M. Sudhakara Reddy)
Supervisor
Associate Professor
Dept. of Biotech & Env. Sciences,
Thapar University
Patiala

(Dr. N. Das)
Head
Dept. of Biotech & Env. Sciences,
Thapar University
Patiala

DECLARATION

I hereby declare that the work which is being presented in this thesis **"Cloning and characterization of metallothionein genes of ectomycorrhizal fungus *Hebeloma cylindrosporum*"** submitted by the undersigned for the award of the degree of Doctor of Philosophy in Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. M. Sudhakara Reddy**, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Date: 28/11/08
Place: Patiala

(G.RAMESH)

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(G. RAMESH)

Dedicated to my father…

CONTENTS

List of Abbreviations

%	Percent
℃	Degree centigrade
bp	Base pair
Cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
Cu	Copper
Cys	Cystein
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
EST	Expressed sequence tags
g	Gram
IPTG	Isopropyl-β-thiogalactoside
kb	Kilo base
M	Molar
mg	Milligram
ml	Mililitre
mm	Millimeter
mM	Millimolar
MOPS	3-(*N*-morpholino)propane sulfonic acid
mRNA	Messenger RNA
Ni	Nickel
ORF	Open reading frame
p.I	Isoeletric point
Pb	Lead
PCR	Polymerase chain reaction
ppm	Parts per million
RNA	Ribonucleic acid
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
SE	Standard error
Tris	Tris-(hydroxymethyl-) aminomethane
U.V	Ultraviolet
w/v	Weight by volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
Zn	Zinc
µg	Microgram
µl	Microlitre
µM	Micromolar

Chapter I

Introduction

1.1. Mycorrhiza

Mycorrhiza refers to an association or symbiosis between plants and fungi that colonize the cortical tissue of roots during periods of active plant growth. The term "mycorrhiza", which literally means "fungus root", describes an intimate mutualistic relationship between fungi and plant roots. It was introduced by Frank (1885) to describe the long-lived association between plant roots and fungal mycelium. The vast majority of land plants form symbiotic associations with fungi, an estimated 95% of all plant species belong to different genera that characteristically form mycorrhiza. The mycorrhizal condition is the rule among plants, not the exception and these associations facilitated their growth in a new, nutrient poor and dry environments (Malloch *et al.,* 1980; Simon *et al.,* 1993). Mycorrhizas serve as the main organs for nutrient uptake in terrestrial ecosystems (Smith and Read, 1997). These symbioses are characterized by bi-directional movement of nutrients, where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil (Smith and Read, 1997). Mycorrhizal fungi usually proliferate both in the root and in the soil. The soilborne or extramatrical hyphae take up nutrients from the soil solution and transport them to the root. By this mechanism, mycorrhizas increase the effective absorptive surface area of the plant. In nutrient-poor or moisture-deficient soils, nutrients taken up

by the extramatrical hyphae can lead to improved plant growth and reproduction. As a result, mycorrhizal plants are often more competitive and better able to tolerate environmental stresses than are nonmycorrhizal plants. Mycorrhizal fungi are well known for improving the phosphate status of their hosts. Some mycorrhizal fungi are also able to mobilize nitrogen and phosphate from organic substrates and to provide plants with improved micronutrient and water acquisition, pathogen resistance, and a variety of other benefits (Smith and Read, 1997). One of these additional benefits is the amelioration of toxicity in metalliferous soils (Bellion *et al.,* 2006).

The majority of vascular plants are obligately or facultatively mycorrhizal in nature. The hosts comprise most species of angiosperms, all gymnosperms, pteridophytes and some bryophytes (Newman and Reddell, 1987). All the major taxonomic groups of fungi (Ascomycotina, Basidiomycotina and Zygomycotina) form mycorrhizas. Some of these are obligate symbionts that cannot survive without the host plant. Mycorrhizal fungi have wide host range and usually do not show strict symbiotic relationships. The species composition of mycorrhizal fungi is dependent on host plant age and environmental conditions (Wilcox, 1996).

The mycorrhizas have been classified into different types. The classification is based on fungal associates and structural characteristics of mycorrhizas at maturity (Isaac, 1992). The most ancient, widespread, and studied mycorrhizal class is arbuscular mycorrhiza (Smith and Read, 1997). Ectomycorrhiza is a common form of symbiosis in forest trees. Other forms of mycorrhizas are arbutoid, monotropoid, ericoid, and orchid mycorrhizas

(Peterson and Farquhar, 1994). Both the fungus and the plant may affect the type of mycorrhiza formed. Thus the mycorrhizal classification has to be considered mainly descriptive (Smith and Read, 1997). Besides, many fungi are able to form different types of mycorrhizas depending on the host species, and the species of the genera *Salix, Prunus* and *Acacia* form both ectomycorrhizas and arbuscular mycorrhizas (Isaac, 1992).

1.1.1 Ectomycorrhizas

Ectomycorrhizas (ECMs), the dominating mycorrhizal symbiosis in boreal, temperate and some tropical forests, are formed by 5000-6000 species of ascomycetes and basidiomycetes (Buscot *et al.,* 2000) and hosts that can be either gymnosperms or woody angiosperms, especially members of the families Betulaceae, Pinaceae, Fagaceae, Salicaceae and Dipterocarpaceae (Smith and Read, 1997). Fossil records and molecular clock dating suggest that ECMs evolved about 200 million years ago (Cairney, 2000). The hyphae are wholly intercellular and form a dense sheath (mantle) around lateral roots. From this mantle, individual hyphae or organized hyphal aggregates called rhizomorphs grow out and explore the soil. The mantle can vary widely in thickness, color, and texture depending on the particular plant-fungus combination. The mantle increases the surface area of absorbing roots and often affects fine-root morphology, resulting in root bifurcation and clustering. At first, a very loose network is formed around the root but then hyphae from the inner zone of the mantle penetrate between the epidermal cells of the root and extend between the outer cortical cells forming a network called the Hartig net, where nutrients between the symbiotic partners are exchanged (Fig. 1) (Smith and Read, 1997; Barker *et al.,* 1998). Ectomycorrhizal fungi can influence the host plant in numerous ways. Decades ago, it was demonstrated that ectomycorrhizal

fungi improve plant nutrition by enhancing nitrogen, Phosphate and potassium uptake, amongst others (Harley and Smith, 1983). Nutrients, particularly nitrogen and phosphate, are taken up by ectomycorrhizal mycelium through active absorption and specific cell membrane transporters following degradation by exoenzymes (e.g. phosphatases) (Smith and Read, 1997). Nutrient exchange between fungus and plant occurs using cell-to-cell contact in ectomycorrhizas, especially in the Hartig net. Ectomycorrhizal root tip, increase the plants access to soil water resources significantly. Ectomycorrhizas also protect host plant roots against root pathogens and root herbivorous soil microfauna (Smith and Read, 1997).

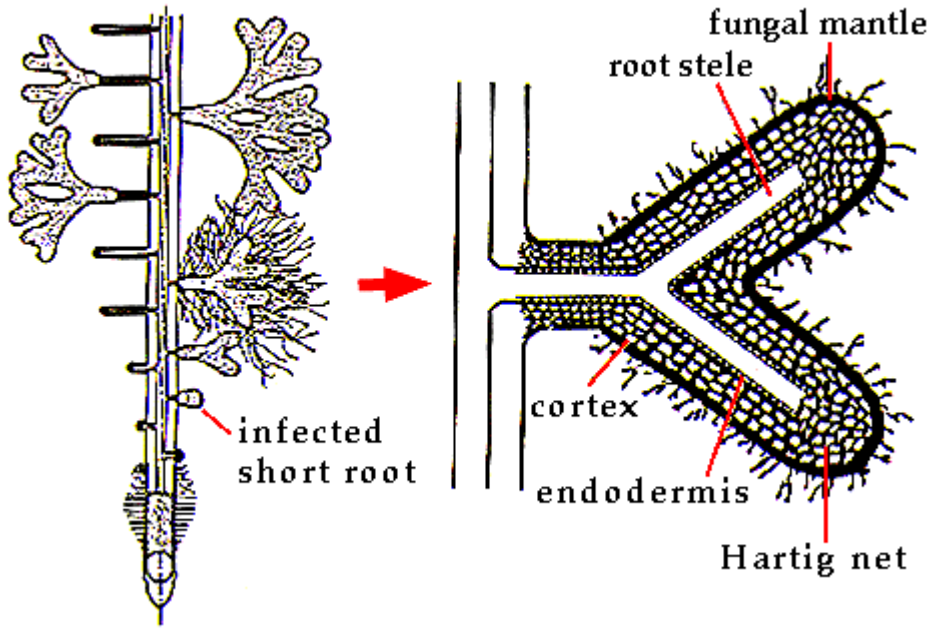


Figure 1: Ectomycorrhiza formation of plant root (Adapted from Ruehle and Marx, 1979)

1.1.1.1 *Hebeloma cylindrosporum*: Taxonomy, distribution and host range

The genus *Hebeloma* belongs to the agaric family Cortinariaceae (Basidiomycotina, Homobasidiomycetes, Agaricomycetideae, Cortinariales), which includes several other species-rich, ECM genera. *H. cylindrosporum* Romagnesi was described in 1965 (Romagnesi, 1965). This species can easily identified by its cylindrical basidiospores. *H. cylindrosporum* has been reported to occur in Europe, from Finland or Norway in the North, to Spain in the south (Courtecuisse and Duhem, 2000).

Figure 2: *In vitro* fruiting under controlled conditions of *Hebeloma cylindrosporum* associated with *Pinus pinaster* (Adapted from Debaud and Gay, 1987).

H. cylindrosporum occurs in sandy soils with no or very little organic matter, essentially in the dune ecosystems along the Atlantic coast (Contu, 1991). In these ecosystem it is found associated with *Pinus* species, such as *P. pinaster* in south west France or *P. sylvestris* in the Netherlands (Jensen, 1982). However, it is able to form ectomycorrhizas with a wide range of species from different gymnosperm and dicot families (Giltrap, 1982). *H. cylindrosporum* can be qualified as a pioneer species, which thrives in newly established forests where accumulation is low, or in disturbed habitats (Guidot et al., 2002). The basidiomycete *H. cylindrosporum* has been extensively studied with respect to mycorrhiza differentiation and metabolism. Its life cycle can be reproduced *in vitro* and it can be genetically transformed. *H. cylindrosporum* also proved to be a remarkable model species to uncover the dynamics of natural populations of ectomycorrhizal fungi and the way in which they respond and adapt to anthropogenic disturbance of the forest ecosystem. Both in field and on laboratory culture media, *H. cylindrosporum* forms mycorrhizas with taxonomically diverse plant species (Debaud and Gay, 1987) (Fig. 2).

1.1.2 Endomycorrhizas

The general term for all mycorrhizal types where the fungus grows within cortical cells (intracellularly) is endomycorrhiza. The diagnostic feature of endo mycorrhizas are the development of a highly branched arbuscule or vesicles within root cortical cells. The fungus initially grows between cortical cells, but soon penetrates the host cell wall and grows within the cell and form particular structures (vesicles, arbuscules). Reproductive spores can be formed either in the root or more commonly in the soil (Smith and Read, 1997). Spores produced by fungi forming endomycorrhizal associations are asexual,

forming by the differentiation of vegetative hyphae. The vesicular – arbuscular forms are most common and widely occurring of all the mycorrhizal associations with plant. However, only several fungal species are know to be implicated from the Zygomycetes including the genera *Glomus, Acaulospora, Gigaspora* and *Sclerocytsis* (Harley, 1989).

1.1.3 Ericaceous mycorrhizas

The term ericaeous is applied to mycorrhizal associations found on plants in the order Ericales. These are often showed characteristics of both ectomycorrhizas and endomycorrhizas. Intracellular penetration of cortical tissues can occur, a mantle forms, and a Hartig net. However, no arbuscules or vesicles are formed. Three major forms of ericaceous mycorrhiza have been described, Ericoid, Arbutoid and Monotropoid mycorrhizas (Smith and Read, 1997). The ericoid mycorrhizae are found on plants such as *Calluna* (heather*), Rhododendron* (azaleas and rhododendrons) and *Vaccinium* (blueberries) that have very fine root systems and typically grow in acid, peaty soils (Harley and Smith, 1983).

1.1.4 Orchidaceous mycorrhizas

Mycorrhizal fungi have a unique role in the life cycle of plants in the Orchidaceae. Orchids colonized shortly after germination, and the mycorrhizal fungus supplies carbon and vitamins to the developing embryo. For achlorophyllous species, the plant depends on the fungal partner to supply carbon throughout its life. The fungus grows into the plant cell, invaginating the cell membrane and forming hyphal coils within the cell. The fungi

participating in the symbiosis are basidiomycetes similar to those involved in decaying wood (e.g. *Coriolus, Fomes, Marasmius*) and pathogenesis (e.g. *Armillaria* and *Rhizoctonia*). In mature orchids, mycorrhizae also have roles in nutrient uptake and translocation (Harley and Smith, 1983).

1.2 Heavy metals: occurrence, chemical and physical properties and mode of their action

Environmental pollution by metals became extensive as mining and industrial activities increased in the late 19^{th} and early 20^{th} centuries. Mineral rock weathering and anthropogenic sources provide two of the main types of metal inputs to soils. According to Ross (1994) the anthropogenic sources of metal contamination can be divided to five main groups: (1) metalliferous mining and smelting (arsenic, cadmium, lead and mercury); (2) industry (arsenic, cadmium, chromium, cobolt, copper, mercury, nickel, zinc); (3) atmospheric deposition (arsenic, cadmium, chromium, copper, lead, mercury, uranium); (4) agriculture (arsenic, cadmium, copper, lead, selenium uranium, zinc); and (5) waste disposal (arsenic, cadmium, chromium, copper, lead, mercury, zinc).

Heavy metals are natural components of the earth's crust. They cannot be degraded or destroyed. Heavy metals are defined as metals with a density higher than 5 g cm^{-3}. Fifty three of the 90 naturally occurring elements are heavy metals (Weast, 1984), but not all of them are of biological importance. The chemical form (speciation) of heavy metals in soil solution is greatly dependent on the metal element concerned, pH and presence of other ions. Based on their solubility under physiological conditions, 17 heavy metals may be

available for living cells and of importance for organism and ecosystems (Weast, 1984). Among these metals, Fe, Mo and Mn are important as micronutrients. Some metals, such as calcium, cobalt, chromium, copper, potassium, magnesium, sodium, nickel and zinc, are essential, serve as micronutrients and are used for redox-processes; to stabilize molecules through electrostatic interactions; as components of various enzymes; and for regulation of osmotic pressure (Bruins *et al.,* 2000). Many other metals have no biological role such as silver, aluminium, cadmium, gold, lead and mercury, are nonessential (Bruins *et al.,* 2000) and potentially toxic to microorganisms (Fig. 3).

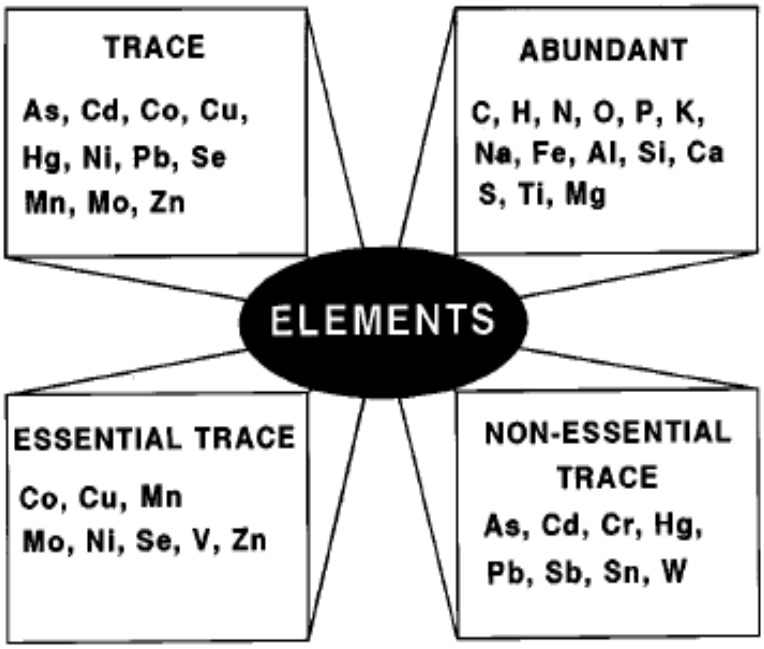


Figure 3: Metabolic and nutritional importance of elements and their classification (adapted from Prasad, 1998)

The toxicity symptoms seen in the presence of excess amount of heavy metals may be due to a range of interaction at the cellular/molecular level. Toxic metals can cause harmful effects in many ways, but principally as result of their strong co-ordinating abilities (Ochiali, 1987). Toxic effects include the blocking of functional groups of biologically important molecules (e.g. enzymes and transport system for essential nutrients and ions) the displacement and\or substitution of essential metal ions from biomolecules and functional cellular units, conformational modifications, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane integrity (Ochiali, 1987). In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, resulting in oxidative stress (Dietz *et al.,* 1999). Metals are directly or indirectly involved in all aspects of fungal growth, metabolism and differentiation (Gadd, 1986). This leads to expression of a detoxification mechanism for survival of organism. All organisms can achieve resistance to heavy metals by "avoidance" when the organism is able to restrict metal uptake, or by "tolerance" when the organism survives in the presence of high internal metal concentration (Joho *et al.,* 1985; Baker, 1987; Turnau *et al.,* 1996). The avoidance involves reducing the concentration of metal entering the cell by: extracellular precipitation, biosorption to cell walls, reduced uptake, or increased efflux. In the second situation, metals are chelated intracellularly through the synthesis of ligands such as metallothioenins, phytochelatins, polyphosphates and\or compartmentation within vacuoles.

1.3 Metallothionein

Metallothioneins (MTs) belong to super family of intracellular metal-binding proteins, present in virtually all-living organisms. Metallothionein was first isolated from horse kidney and characterized over 40 years ago by Margoshes and Vallee (1957). Typically, MTs have low molecular weight polypeptides (< 10 KDa), high metal content comprising predominantly Zn, Cu or Cd, highly conserved cysteine residues and no aromatic amino acids or histidine. They have characteristic of Cys-Cys and Cys-X-Cys cluster, where X is an amino acid other than cysteine. The predominant feature of MT is that one third of its amino acids are cystein, which are sulfydryl residues able to bind heavy metals such as Cu, Cd and Zn. MTs have been proposed to be involved in a number of cellular process including metal storage and detoxification, development, differentiation, control of metabolism, protection from free radical toxicity, and UV response (Kartin, 1985). MTs were widely distributed throughout living organisms, such as mammals, plants, fungi (Hamer *et al.,* 1985, Foulkes, 1982) and cynobacteria (Olafson, 1986; Olafson *et al.,* 1988).

Metallothioneins were shown to bind high concentrations of heavy metals (HMs) in metal thiolates and metal thiolate clusters (Kagi *et al.,* 1974; Hamer, 1986). According to Rauser (1990) Metallothioneins are subdivided into three classes. Class I MTs all exhibit sequence similarity to the equine renal MT, although first identified in mammals, Class I MTs are also found in certain fungi, such as *Neurospora crassa* (Lerch, 1980) and *Agaricus bisporus* (Munger and Lerch, 1985). Class II metallothioneins have been identified in cynobacteria, yeast (*Saccharomyces cerevisiae*) (Steffens, 1990) and nematode (*Caenorhabditis elegans*) (Kagi, 1991); they do not share extensive sequence

homology with Class I proteins. Class III metallothioneins were first detected in *Schizosaccharomyces pombe* (Murasugi *et al.,* 1984). These have the general structure poly (γ-glutamylcysteinyl)-glycine and, because they are commonly found in plants, they have been given the trivial name phytochelatins (Grill *et al.,* 1985). This class of metallothioneins is now known to be widespread in fungi (Kneer *et al.,* 1992). Later the second classification was performed by Binz and Kagi in 1999, and takes into account taxonomic parameters and the patterns of distribution of Cys residues along the MT sequence. It results in the classification of 15 MT families.

1.4 Phytochelatins

In addition to cysteine rich polypeptides of metallothioneins, another group of metal binding molecules such as phytochelatins (PCs) are synthesized by fungi to be metal resistant. One potential strategy to efficiently remove heavy metals from contaminated sites is the use of organisms with high heavy metal resistance and accumulation capacity (Salt *et al.,* 1995; Dhankher *et al.,* 2002). Phytochelatins consist of just three amino acids; Cysteine, Glycine, and Glutamic acid, arranged generally in a $(\gamma\text{-GluCys})_n\text{-Gly}$ conformation. This conformation proves to be significant in the identification of the origin of PCs. The fact that PCs are arranged in a γ -carboxylamide bond suggests that the phytochelatins are not a direct result of expression of a metal tolerance gene, but rather a product of a biosynthetic pathway, with glutathione, a detoxifying agent, most likely the substrate on which the pathway begins (Murphy *et al.,* 1997). Phytochelatins are synthesized inductively by exposure not only to Cd, but also to other heavy metals including toxic and essential ones such as Hg, Cu, Zn, and Ni. (Devars, 1998;

Vatamaniuk *et al.,* 1999; 2001). These PCs are the main metal detoxification mechanism in algae and plants (Grill *et al.,* 1988) as well as filamentous fungi and yeasts (Mewes *et al.,* 1997, Clemens and Simm, 2003).

1.5 Origin of the problem

The ultimate goal of successful revegetation strategy for metal polluted soils is the establishment of sustainable ecosystems, whether it is grass land or woodland, in which transfer of metals to above ground plant parts is restricted, unless phytoextraction of metals is required. The stability of such a managed ecosystem will depend on the colonization potential, the survival and performance of metal tolerant representatives of the major functional groups that are characteristic for a terrestrial ecosystem and that are suppose to be essential for its functioning. Ectomycorrhizal fungi are certainly such an essential group in forest ecosystems and therefore it is important to know how these fungi respond to elevated heavy metal concentrations in the soil solution (Meharg, 2003).

Ectomycorrhizal symbioses can play a crucial role in protecting plants from toxic metals. The response of ectomycorrhizal fungi to toxic metals is important, since these organisms are present at polluted sites, participate in crucial symbiotic relationships with trees that grow at these sites, and alleviate metal toxicity in the host plants (Courbot *et al.,* 2004). Potential amelioration of metal toxicity to plants by ectomycorrhizal (ECM) fungi has been proposed by a number of authors (Brown and Wilkins, 1985a,b; Colpaert and Van Assche, 1992b, 1993; Denny and Wilkins, 1987a; Jones and Hutchinson, 1986; Dixon, 1988; Dixon and Buschena, 1988; Van Tichelen *et al.,* 2001; Galli *et al.,* 1994; Tam,

1995; Jentschke and Goldbold, 2000; Schutzendubel and Polle, 2002). The efficiency of protection, however, differs between distinct isolates of mycorrhizal fungi and different toxic metals and protective effects cannot be demonstrated for all associations in all circumstances (Meharg, 2003). The mechanisms by which they are able to deal with these metals are numerous and varied in their action (Gadd, 1993). Metal resistance is a result of different mechanisms such as restriction of metal uptake, increased efflux, extracellular or intracellular complexation. However, these tolerance mechanisms are not well understood in mycorrhizal fungi and most data obtained so far concern yeasts and saprotrophic fungi (Ortiz *et al.,* 1992, 1995; Gadd, 1993). When considering the mechanism of intracellular chelation of metals, it must be considered that chelation by metallothioneins and phytochelatins. This process maintains homeostasis of essential trace metals within the cytoplasam.

Concerning fungi, *Candida glabrata,* like higher eukaryotes, employs different mechanisms to detoxify cadmium and copper. Cadmium stimulates the production of phytochelatins, whereas copper induces the synthesis of a family of metallothioneins (Mehra *et al.,* 1988, 1989, Zhou and Goldsbrough, 1995). In the baker's yeast *Saccharomyces cerevisiae,* resistance to the toxicity of copper is achieved by two distinct metallothioneins, which is encoded by *CUP1* and *CRS5* loci (Hamer *et al.,* 1985; Ecker *et al.,* 1986, Jensen *et al.,* 1996) but no PC synthase gene identified (Mewes *et al.,* 1997). *Schizosaccharomyces pombe* synthesize small cadmium-binding peptides cadystin, structure of which is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, in response to cadmium and one sequence annotated as MT like protein. (Clemens *et al.,* 1999; Ha *et al.,* 1999; Clemens and Simm, 2003). MTs were identified from different filamentous fungi, such as the ascomycete

Podospora anserina (Averbeck *et al.,* 2001), the discomycete *Pyrenopeziza brassicae* (Singh and Ashby, 1998). Lanfranco *et al.,* (2002) identified a gene encoding a MT like protein in *Gigaspora margarita*, designated *GmarMT1* and observed the role of this gene in symbiosis with a host plant. Despite an abounded number of studies on molecular regulation of metallothioneins in yeast, filamentous fungi, plants and animals (Kagi, 1974; Mehra, 1994; Murphy *et al.,* 1997) the number on ectomycorrhizal fungi is limited. Morselt *et al.,* (1986) first reported metallothionein- like proteins in pure culture of *Pisolithus tinctorius* in the presence of Cu and Cd using specific histochemical staining methods. Later copper-binding cysteine rich MTs were isolated from the ectomycorrhizal fungi, *Laccaria laccata* and characterized by its molecular mass and spectroscopic features (Howe *et al.,* 1994). Courbot et al. (2004) found as increased concentration of glutathione, γ-glutamylcysteine as well as MT like proteins in *Paxillus involutus* under Cd exposure. Using two-dimensional electrophoresis, Pierleoni *et al.,* (2004) have identified metallothionein protein under fruit body formation in the ascomycetes of *Tuber borchii*. However, the metal sequestration capacity of polypeptides and their actual MT-like nature has not yet been determined. The molecular regulation of MTs and PCs has been investigated, to a limited extent in ectomycorrhizal fungi. Putative MTs have been identified and described within the collection of Expressed Sequences Tags (ESTs) simply only on the basis of sequences similarity. This is the case for MT like sequences found in mycorrhizal fungus *Pisolithus tinctorius* (Voiblet *et al.,* 2001); the ericoid fungus *Oidiodendron maius* (Vallino *et al.,* 2005); and the arbuscular mycorrhizal fungi *Gigaspora rosea* (Stommel *et al.,* 2001), *Gigaspora margarita* (Lanfranco *et al.,* 2002) and *Glomus intraradices* (Gonzalez-Guerrero *et al.,* 2006). Jacob et al. (2004) showed

that complexation of Cd by metallothioneins (MTs) is a key mechanism for Cd tolerance in the ectomycorrhizal fungus *Paxillus involutus* and they also observed up regulation of laccase and aconitase in response to Cd. More recently, Bellion *et al.,* (2007) identified a metallothionein-coding gene (*PiMT1*) from *P. involutus,* and characterized functionally in terms of transcript profiles, heterologous complementation and transformation assays. The results highlight the key role of the gene in Cu and Cd tolerance. MTs are organized as small multigene families in fungal genome. Each of them probably has a particular role or at least a specific regulation. In ectomycorrhizal fungi, there are no reports available on multiple MT sequences. An EST collection of *P. involutus* revealed the existence of a second MT like sequence, but the exact regulation and function is unknown (Bellion *et al.,* 2007). However, the species studied to date represent a relatively small number of the ectomycorrhizal fungi and provide only a narrow picture of ectomycorrhizal fungi on molecular metal tolerance.

1.6 Aim of the study

Mycorrhizal fungi are a direct link between plants and soils and are often needed to ensure plant survival in heavily polluted areas. To use mycorrhizal fungi for bioremediation and soil protection purposes, we need to improve the understanding of the molecular mechanisms that underlie the metal detoxification processes in these fungi. This would allow better exploitation of the mycorrhizal symbiosis. The ectomycorrhizal fungus, *Hebeloma cylindrosporum* was used for this study. *Hebeloma cylindrosporum* was screened for their tolerance of different heavy metals such as Cu, Cd, Zn, Ni and Pb. Two different types of metal induced metallothionein genes were identified.

Metallothionein genes were cloned, characterized and its structure studied. The expression of metallothionein genes in the presence of different heavy metals and oxidative stresses were studied using competitive RT-PCR to document the specificity of their induction. Both metallothioneins genes were functionally characterized by means of complementation assays in metal hypersensitive yeast mutants.

Chapter II

Review of literature

2.1 Definition and classification of metals

Metals are defined chemically as “elements which conduct electricity, form cations, and have basic oxides” (Atkins and Jones, 1997). Metals account for a quarter of the Earth's mass. Heavy metals are metals with a density beyond 5 g/cm^3, thus, the transition elements from V (but not Sc and Ti) to the half-metal As, from Zr (but not Y) to Sb, from La to Po, the Lanthanides and the Actinides can be referred to as "heavy metals". Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (Weast, 1984). Most heavy metals are transition elements with incompletely filled d-orbitals (Hughes, 1990). These d-orbitals provide heavy metal cations with the ability to form complex compounds which may be redox active or not. Thus, heavy metal cations play an important role in sophisticated biochemical reactions such as nitrogen fixation, water cleavage during oxygenic photosynthesis, respiration with oxygen or nitrate, one-electron catalysis, re-arrangement of C-C bonds, hydrogen assimilation, cleavage of urea, transcription of genes into mRNA, and programmed development of a single cell to a human being. These are all based on the formation of biochemical heavy metal complex compounds.

Many attempts were made to define metals based on their physiological and biological properties in relation to biological effects. Metals and metalloids can be considered all the elements except the nobel gases and H, B, C, N, O, F, P, S, Cl, Br, I and At. Metalloids are Si, Ge, As, Se, Sb and Te. Metallic elements divide into four broad categories: S-block, P-block, D-block transition, and F-block (lanthanides and actinides). The S-block metals, Groups Ia and IIa of the Periodic table, form monovalent cations (Alkali metals) and divalent cations (Alkaline earth metals). P-block metals contain Group IIIb to VIb elements (Morgan and Stumm, 1991). The group IIIb metals have the III oxidation state only. The actinides and lanthanides comprise an "inner" transition series (Morgan and Stumm, 1991).

Metal ions were described as Class A, Class B, or borderline, depending on their observed affinity for different ligands. Fig. 4 shows the position of Classes A, B, and borderline in the periodic table. In general, there is a relatively sharp separation between Class A and borderline metal ions, but the difference between borderline and Class B is less clearly defined (Morgan and Stumm, 1991). Although alternative descriptions have evolved, notably the use of the term "hard acids" for Class A ions and "soft acids" for Class B ion (Pearson, 1963; Hughes and Poole, 1989).

Oxygen donating ligands are hard whereas sulphur donors are soft. Many essential metal ions, e.g. Fe^{3+}, Ca^{2+}, Mg^{2+} and K^{+}, are hard whereas inessential toxic metal ions, e.g. Ag^{+}, Cd^{2+}, Hg^{2+} and Sn^{2+}, are soft. However, biologically inessential hard metals include Rb^{+}, Sr^{2+} and Al^{3+} while borderline category includes Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} (Hughes and

Poole, 1991). The borderline categories reflect varying properties of metal ions rather than absolute distinctions (Gadd, 1993). The 'Type-A' metals ions are sharply separated from borderline metals, the distinction between 'Type-B' and borderline categories is not so clear. Metal ions compete with H^+ for sites on ligands and H^+ may, in fact, be regarded as borderline ions (Nieboer and Richardson, 1980).

Metals in soil are present as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides, or they may form part of the structure of silicate minerals (indigenous soil content). The toxicity of metals in soil depends on their bioavailability, defined as their ability to be transferred from a soil compartment to a living organism (Juste, 1988). According to Berthelin *et al.* (1995), metal bioavailability is a function not only of their total concentration but also of physico-chemical (e.g. pH, Eh, organic matter, clay content) and biological (e.g. biosorption, bioaccumulation and solubilization) factors.

Metals, radionuclides and other inorganic contaminants are among the most prevalent forms of environmental contaminants, and their remediation in soils and sediments is rather a difficult task (Cunningham *et al.,* 1997). Sources of anthropogenic metal contamination include smelting of metalliferous ore, electroplating, gas exhaust, energy and fuel production, the application of fertilizers and municipal sludge to land, and industrial manufacturing (Raskin *et al.,* 1994; Cunningham *et al.,* 1997; Blaylock and Huang, 2000). Heavy metal contamination of the biosphere has increased sharply since 1900 (Nriagu, 1979) and poses major environmental and human health problems

worldwide (Ensley, 2000). Unlike many organic contaminants, most metals and radionuclides cannot be eliminated from the environment by chemical or biological transformation (Cunningham and Ow, 1996; NRC, 1997). Although it may be possible to reduce the toxicity of certain metals by influencing their speciation, they do not degrade and are persistent in the environment (NRC, 1999). High metal concentrations in soil are toxic to microorganisms. Metal tolerance in soil microorganisms has been studied in the context of removing metals from polluted soils, but also to provide a biological understanding of the adaptation of living organisms to extreme environments.

2.2 Mode of action of toxic metals

To understand the mode of action leading to heavy metal toxicity in living cells, their chemical properties have to be considered. Most of the heavy metals are transition metals with an incompletely filled d-orbital present as cations under physiological conditions. The physiological redox range of aerobic cells stretches from -420 mV to +800 mV. Therefore, heavy metals of biological significance can be divided into two groups of redox active and inactive metals. Metals with lower redox potentials than those of biological molecules cannot participate in biological redox reactions. Auto-oxidation of redox active metals such as Fe^{2+} or Cu^{2+} results in O_2^- formation and subsequently in H_2O_2 and OH^+ production via Fenton-type reactions. Cellular injury by this type of mechanism is well documented for iron (Halliwell and Gutteridge, 1986; Imlay *et al.,* 1988), copper (Li and Trush, 1993a, b) as well as other metals (Jones *et al.,* 1991; Lund *et al.,* 1991; Shi and Dalal, 1993; Shi *et al.,* 1993).

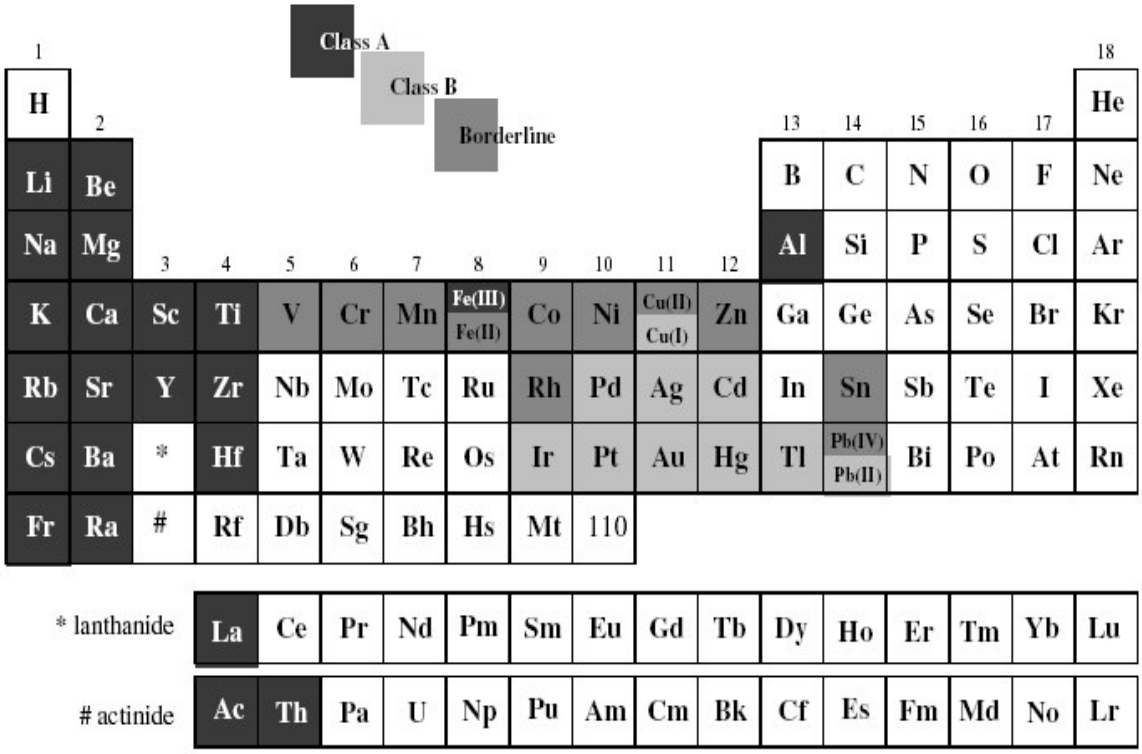


Figure 4: The periodic table showing those metals classified as: Class A: hard metals (dark gray); Class B: soft metals (light gray); intermediate metals (intermediate gray). Copper may be either Class B or borderline depending upon where it is Cu(I) or Cu(II), respectively; Lead may be either Class B or borderline depending upon where it is Pb(II) or Pb(IV), respectively; and iron may be either Class A or borderline depending upon it is Fe(III) or Fe(II), respectively.

Another important mechanism of heavy metal toxicity is their ability to bind strongly to oxygen, nitrogen and sulphur atoms (Nieboer and Richardson, 1980). This binding affinity is related to free enthalpy of the formation of the product of metal and ligand, shows a range of heavy metal cations with increasing affinity for sulphides and the low solubility of these products. Because of these features, heavy metals can inactivate enzymes by binding to cysteine residues. Direct effects of cadmium on the sulphydryl homeostasis of cells and inhibition of enzymes have been reported for mammalian and animal cells (Canesi *et al.,* 1998; Chrestensen *et al.,* 2000).

Many enzymes contain metals in positions important for their activity. The displacement of one metal by another will normally also lead to inhibition or loss of enzyme activities. Divalent cations such as Co^{2+}, Ni^{2+}, and Zn^{2+} were found to displace Mg^{2+} in ribulose-1,5-bisphosphate-carboxylase/oxygenase and resulted in loss of activity (Wildner and Henkel, 1979; Van Assche and Clijsters, 1986). Displacement of Ca^{2+} by Cd^{2+} in the protein calmodulin, important in cellular signaling, led to an inhibition in the calmodulin-dependent phosphodiesterase activity in radish (Rivetta *et al.,* 1997).

According to their chemical and physical properties, three different molecular mechanisms of metal toxicity can be distinguished:

(a) Production of reactive oxygen species by auto-oxidation and Fenton reaction

(b) Blocking of essential functional groups in biomolecules

(c) Displacement of essential metal ions from biomolecules

At higher concentrations, heavy metal ions form unspecific complex compounds in the cell, which lead to toxic effects. Some heavy metal cations, e g. Hg^{2+}, Cd^{2+} and Ag^{+}, are such toxic complex-formers that they are too dangerous for any biological function. Even highly reputable trace elements like Zn^{2+} or Ni^{2+} and especially Cu^{2+} are toxic at higher concentrations. Thus, the intracellular concentration of heavy metal ions has to be tightly controlled, and heavy metal resistance is just a specific case of the general demand of every living cell for some heavy metal homoeostasis system.

2.3 Mycorrhizal interaction with toxic metals

Mycorrhizal fungi play an essential role in providing access to mineral nutrients at all stages of plant development. This is achieved largely through their ability to mobilize key nutrients such as phosphorus and nitrogen (Harley and Smith, 1983). Consequently mycorrhizal fungi influence plant fitness and survival and the development of plant community structure (Read, 1991). While enhancement of nutrient uptake, both essential and non-essential elements may be toxic at high concentrations and in some circumstances enhanced uptake could be deleterious for both hosts and symbionts (Brown and Wilkins, 1985a,b). Moreover, accumulation of heavy metals in soils could adversely affect formation and development of ectomycorrhizas of tree species growing in contaminated soils (McCreight and Schroeder, 1982). There is considerable interest in the interaction between mycorrhizas and pollution. Studies of ECM sensitivity to potentially toxic metals have focused on:

(i) Sensitivity of ECM fungi in culture in the absence of a host

(ii) Sensitivity of ECM fungi in symbiosis with a host in artificial substrates.

Despite knowledge of the ubiquity of ectomycorrhizal fungi on sites highly contaminated with metals, their co-evolution with host species to potentially toxic metals has not been studied in detail. Metal exposure studies on ECM symbioses have concentrated on enhanced plant fitness in association with ECM fungi.

In relation to the role of ectomycorrhizas in metal tolerance by the host plant, most mechanisms that have been proposed involve various exclusion processes that restrict metal movement to the host roots. These have been extensively reviewed and assessed (Jentschke and Godbold, 2000) and include absorption of metals by the hyphal sheath, reduced access to the apoplast due to the hydrophobicity of the fungal sheath, chelation by fungal exudates, and adsorption onto the external mycelium (Jones and Hutchinson, 1986; Dixon, 1988; Galli *et al.,* 1994; Tam, 1995; Marschner and Dell, 1994). However, not all mycorrhizal associations provide increased levels of metal tolerance for the host plants (Jones *et al.,* 1988a,b). The mechanisms involved in conferring this increase in tolerance have proved difficult to resolve; they may be quite diverse and show considerable species and metal specificity since large differences in response to metals have been observed, both between fungal species and to different metals within a species (Hartley *et al.,* 1997; Hüttermann *et al.,* 1999).

A number of investigations of amelioration of metal toxicity have been carried out using ectomycorrhizal tree seedlings and these investigations shows that in a number of cases mycorrhizal colonization of roots increased the tolerance of host plants to metals.

Marx and Artman (1979) showed that inoculation of conifer roots with *Pisolithus tinctorius* greatly improved seedling survival and growth on mine spoils containing high levels of metals and extreme acidity.

Jones and Hutchinson (1986) found that colonization by *Laccaria proxima* or *Lactarius hibbardae* alleviated Ni toxicity in *Betula papyrifera* seedlings at 32 mM Ni but not at 64 mM and they concluded that alleviation of Ni toxicity does not necessarily imply tolerance to other metals (e.g. Cu). None of the fungal strains, which successfully reduced the sensitivity of the host to Ni, showed tolerance to copper.

Wilkins (1991) concluded that mycorrhizae can reduce metal concentrations in shoot tissues, although some fungi are inefficient and others affect growth or uptake alone. This general feature has been found for Zn, Ni and Cu. Jentschke et al. (1999) showed similar results in mycorrhizal Norway spruce seedlings for Cd. In the experiment with Cd, the fungus may have been affected by high Cd treatment (5 mM), thus losing its ability to alleviate Cd toxicity. However, indirect evidence suggested that the fungus was still viable at 5 mM Cd, indicating that the mechanism of amelioration does operate up to a certain threshold of metal exposure only.

Colpaert and Van Assche (1992a) showed that the ectomycorrhizal fungus *P. involutus* retained Zn and reduced the Zn content of *Pinus sylvestris*, whereas another species *Thelephora terrestris* retained little Zn and even increased the Zn content of the host.

Tam (1995) tested the ability of five ectomycorrhizal fungi to grow in culture with a range of nine different heavy metals and observed considerable variation among the ectomycorrhizal fungi. He concluded that the fungus *Pisolithus tinctorius* tolerance to Cu and Zn was achieved by binding to extrahyphal slime. Similar results were found by Blaudez et al. (2000a) screened thirty nine ectomycorrhizal isolates of *P. involutus*, *P. tinctorius*, *S. bovinus*, *S. luteus* and *S. variegatus* on cadmium, copper, nickel and zinc amended media to determine their *in vitro* tolerance, measured as inhibition of biomass production. There was a strong interspecific variation in metal tolerance. *S. luteus*, *S. variegatus* and *P. tinctorius* were more tolerant of Cu, Cd and Zn when compared with *P. involutus*, whereas the reverse was true for Ni.

Van Tichelen et al. (2001) studied the copper toxicity in Scots pine infected with *Suillus bovinus* and *Thelephora terrestris*. They concluded that mycorrhizal infection protects pine from copper toxicity. They observed greater reduction in root biomass in non-infected plants and enhanced copper uptake in the shoots. While the ECM fungi have deferentially affected root biomass and metal uptake, it had no effect on shoot biomass.

Adriaensen et al. (2005) described the population of the ectomycorrhizal fungus *Suillus luteus* adaptation to contaminated soils in field populations, which colonized a toxic Cu mine spoil in Norway. They hypothesized that this population had developed adaptive Cu

tolerance and was able to protect pine trees against Cu toxicity. They also tested for the existence of cotolerance to Cu and Zn in *S. luteus* and found that the Cu mine isolates exhibited high Cu tolerance, whereas the Zn-tolerant isolates were shown to be Cu sensitive, and vice versa. This indicates the evolution of metal-specific tolerance mechanisms is strongly triggered by the pollution in the local environment. The Cu-adapted *S. luteus* isolate provided protection against Cu toxicity in pine seedlings exposed to elevated Cu levels.

2.4. Mechanisms of resistance to metal toxicity in fungi

All microorganisms, including fungi, can achieve resistance to heavy metals by "avoidance" when the organism is able to restrict metal uptake, or by "tolerance" when the organism survives in the presence of high internal metal concentrations (Baker, 1987; Turnau *et al.,* 1996). The first mechanism involves reduced uptake or increased efflux, formation of complexes outside cells, biosorption to cell walls and organic acid release. In the second situation, metals are chelated intracellularly through the synthesis of ligands such as metallothioneins, phytochelatins, polyphosphates, and/or compartmentation within vacuoles (Gadd, 1993). Extracellular mechanisms are mainly implied in avoidance of metal entry, whereas intracellular systems aim to reduce metal burden in the cytosol (Fig. 5). Additional antioxidative detoxification systems, which allow the fungus to counteract the accumulation of reactive-oxygen species directly or indirectly, initiated by metals, may be part of tolerance mechanisms. The significance of these processes may vary as a function of the metal involved, its concentration, and the location of the primary lesion caused by the metal.

2.4.1 Extracellular chelation and cell-wall binding

2.4.1.1 Chelation by organic acids

Mycorrhizal fungi, like certain plant hosts, can excrete organic acids into the rhizosphere. The benefits of organic acid excretion might be to liberate base cations from soil minerals (Landeweert *et al.,* 2001), to mobilize phosphate from insoluble iron and aluminium phosphates, to counteract aluminium and iron toxicity by complexing their ions in soil solution, to mobilize trace metal cations by complexing them and to acidify the rhizosphere. Thus, organic acid exudation may either mobilize metal toxicants in soil, or they may immobilize, through precipitation with organic acids, or detoxify them by complexing them. Organic acids such as citric acid, malic acid and oxalic acid are readily utilizable organic substrates, making the dynamics of these acids in the rhizospheres complex (Jones, 1998). Their affinities for particular metal ions are also important in their cycling and in their ability to mobilise-immobilise ions. These properties will also be pH dependent. Oxalic acid excretion into the rhizosphere was higher in mycorrhizal Scots pine compared to non-mycorrhizal controls when no toxic metals were present for a range of fungal isolates from contaminated sites. When exposed to aluminium there was a much greater rise in oxalic acid excretion in mycorrhizal plants compared to non-mycorrhizal plants for *Suillus variegatus* and *Rhizopogon roseolus* isolates, but not for *Paxillus involutus* isolates. Nickel and cadmium exposure did not result in organic acid production. Copper exposure enhanced oxalic acid production in both mycorrhizal and non-mycorrhizal trees (Ahonen-Jonnarth *et al.,* 2000). Mycorrhizal species/strains also directed in their organic acid excretion in symbiosis in the absence of metal exposure. So

organic acid exudation in ectomycorrhizal fungal associations is isolate dependent, and also different metals elicited different excretion responses.

Different organic molecules particularly di- and tricarboxylic acids that do not belong to the matrix of the cell wall, are excreted by fungal cells to chelate metal ions, among other functions. In particular, citrate has been shown to be the most important Al^{3+} complex-former in soil solution from podzolized forest soils (Landeweert *et al.,* 2001; Van Hees *et al.,* 2001). The induction of oxalic acid efflux correlated closely with Cu tolerance in brown rot fungi (Green and Clausen, 2003), and over excretion of oxalic acid probably contributed to the metal tolerance exhibited by *Beauveria caledonica* (Fomina *et al.,* 2005a). Similarly, ectomycorrhizal fungi also often respond to metal exposure by increased oxalate exudation (Ahonen-Jonnarth *et al.,* 2000; Cumming *et al.,* 2001). Using ^{109}Cd uptake experiments with *P. involutus*, Jacob et al. (2001) found that oxalic acid reduced Cd uptake by more than 85%. Therefore, an increased oxalate exudation inducing a decreased Cd availability would be an efficient mechanism to avoid Cd entry into living cells of ectomycorrhizal fungi. Exudation of organic acids may provide a source of protons for metal solubilization from metal-containing minerals, often resulting in soil acidification (Devevre *et al.,* 1996; Fomina *et al.,* 2005b). The recent finding that metal-tolerant ectomycorrhizal fungi grew and solubilized metal-containing minerals better than nontolerant species (Fomina *et al.,* 2005b) confirm a possible relationship between tolerance to metals and extracellular chelation by extruded ligands. Isolates from copper mine spoils show enhanced resistance to copper and decreased free copper in solution to a greater extent. It is postulated that this enhanced precipitation is a tolerance mechanism (Adriaensen *et al.,* 2006).

Organic acid exudation does appear to have a clear role in mycorrhizal adaptation to metal contaminated sites. This adaptation may either reduce mobilization of toxic metals, through decreased organic exudation, or immobilizing toxic metals through precipitation with organic acids (Meharg, 2003)

2.4.1.2 Heavy metal binding in the extramatrical mycelium

Fungi can effectively bind metals to cell walls or extracellular polysaccharides may have been suggested as a tolerance mechanism in fungi (Ross, 1993; Gadd, 1993). Similar mechanisms have been suggested to protect mycorrhizal roots from metal toxicity. It was suggested that sorption of metals to fungal tissues or intracellular uptake and detoxification in fungal vacuoles subsequently reduced metal uptake into the host plant (Brown and Wilkins 1985b, Jones and Hutchinson 1986). Marschner et al. (1998) measured Pb sorption on the mycelium of the ectomycorrhizal fungi *Paxillus inolutus* and *Laccaria bicolor*. They found that Pb was probably bound to cation exchange sites, although dependent on the fungal species, some Pb may also be immobilized by precipitation.

At a gross level, the abundant production of extramatrical mycelium of ectomycorrhizal fungi has been suggested as providing increased protection from metal toxicity to the host. Dense extramatrical mycelium, however, will also provide an increased nutrient supply to the plant and will be more successful at infecting new plant roots due to its larger biomass. These two factors could also confer additional benefits to the plant in the presence of potentially toxic metals. Denny and Wilkins (1987b) used X-ray

microanalysis on freeze substituted samples to map Zn location in *P. involutus-Betula* mycorrhizal roots. They reported high Zn concentrations in extramatrical hyphae, located in hyphal cell walls and extrahyphal polysaccharide slime, but not in the fungal mantle

Colpaert and Van Assche (1992b, 1993) concluded that those ECM fungal species, which produce a dense extramatrical mycelium, have the most beneficial effect on host sensitivity. They suggest that a dense extramatrical mycelium provides an increased capacity for metal retention. Jones and Hutchinson, (1986) studied the sensitivity of Cu and Ni sensitivity of different ectomycorrhizal fungi. The ECM fungal infection increased the metal tolerance of *Betula papyrifera*, but sensitivity varies widely between fungal species. They concluded that the protection against metals in successful symbiosis was an ability to produce a densely infected and large root system. Colpaert and Van Assche (1992a) observed an increased density of mycelium upon exposure to high Cd concentrations.

Tam (1995) and Denny and Wilkins (1987a) have suggested that ECM fungi complex toxic metal ions on extracellular slime on the external surfaces of hyphae. They proposed that zinc binds to electronegative sites on cell walls and extra-hyphal, polysaccharide slime. The fungal slime theory was further propagated by Denny and Ridge (1995) for ericoid mycorrhizal fungi. They showed that four isolates differed greatly in their ability to absorb zinc (a four fold range) and tried to link this to slime production.

Frey et al. (2000) investigated zinc and cadmium localization in *Hebeloma crustuliniforme–Picea abies* root tips. They found that the cadmium was predominantly

bound extracellularly in the Hartig net, while zinc accumulated mainly in cell walls of mantle hyphae, Hartig net hyphae and in cortical cells. They proposed that zinc accumulated in high quantities in fungal tissues, protecting the host plant. A range of metal elements were shown to accumulate in the fungal mantle and rhizomorphs in *Suillus luteus–Pinus sylvestris* associations collected from polluted soils (Turnau *et al.,* 2001). Gonzalez-Chavez et al. (2002) showed that a number of *Glomus* isolates from copper mine spoil absorbed copper to their external hyphae. *Glomus species* have a high capacity to bind cadmium, and zinc. Isolates from metal contaminated sites that exhibited metal resistance bound more cadmium and zinc to their surfaces (Joiner *et al.,* 2000).

2.4.1.3 Heavy metal binding by cell wall components

The fungal cell wall has been suggested as the main barrier protecting fungal hyphae against uptake of potentially toxic metal species. Its composition implies glucan-, chitin- and galactosamine-containing polymers, and a minor amount of proteins. Thus a large number of potential-binding sites are exhibited by free carboxyl, amino, hydroxyl, phosphate and mercapto groups (Strandberg *et al.,* 1981). Binding to the wall, also called biosorption (Gadd, 1993), is a mechanism not depending on the metabolic activity of the fungus, whereas precipitation with excreted substances relies on the activity of the cells. Biosorption of heavy metals to fungal structures may reduce the intracellular accumulation of metals and their effect on cytoplasmic processes (Brown and Wilkins 1985).

Bayer and Kneifel (1972) found that *Amanita muscaria* accumulated extreme concentrations of Vd via specific metal binding proteins. Similar mechanisms are believed to account for high accumulation of Cd in *Rozites caperata*, Cs in *Cortinarius spp.,* Cd and Th in *A. muscaria* and Tl in *Tricholoma album* (Tyler 1980; Seeger and Schweinshaut 1981; Bakken and Olsen 1990). Mullen et al. (1992) could show that Ag^{+} was precipitated on to the outer surface of the cell walls of *Aspergillus niger* as colliedal silver.

Binding of Cd to cell walls was shown to represent a substantial fraction of the metal accumulation by *P. involutus* and may also be part of the mechanisms by which mycorrhizal fungi tolerate high amounts of metals (Blaudez *et al.,* 2000b). In *P. involutus,* biosynthesis of polyamines, involved in the maintenance of membrane integrity, was increased by Pb exposure but was little affected by Zn exposure (Zarb and Walters 1995, 1996). Lanfranco et al. (2002) showed that changes in hyphal morphology occur when an ericoid mycorrhiza-forming ascomycete is treated with millimolar concentrations of Zn. This led to apical swellings and increased branching in the subapical parts as well as a significant increase in the amount of chitin in metal-treated hyphae. Interestingly, glomalin, a protein synthesized and excreted by arbuscular mycorrhizal fungi (Wright and Upadhyaya, 1998; Gonzalez-Chavez *et al.,* 2004) was shown to be able to sequester metal ions, especially Cu, Pb and Cd, found at high concentrations in polluted soils. Bhanoori and Venkateswerlu, (2000) have shown the formation of a complex between the Cd and chitin in *Neurospora crassa* cell walls and proposed a structure for the chitin–Cd complex based on the results of ^{13}C-NMR spectroscopy, X-ray diffraction and infrared spectroscopy.

Melanins obtained from different fungi were also shown to be efficient biosorbants for heavy metals (Senesi *et al.,* 1987, Gadd and De Rome, 1988). These heterogeneous compounds, which are located in or exterior to the cell walls are important fungal pigments, which enhance the survival of many species in response to environmental stress (Gadd, 1993).

Jacob et al. (2004) found an induction of laccase and polyphenolic compounds under Cd exposure, which may be an important determinant of the cellular response to excess metals in *P. involutus*. It has been previously shown that Cu can induce laccase isozymes in *Pleurotus ostreatus* (Palmieri *et al.,* 2000; Faraco *et al.,* 2003). In the ericoid mycorrhizal fungus *Oidiodendron maius,* the activity of polygalacturonase, an extracellular enzyme that hydrolyses the pectin component of the plant cell walls, increased under Cd or Zn exposure, a mechanism that may be considered as a preadaptive factor for the colonization of polluted soils by *O. maius* (Martino *et al.,* 2000).

2.4.1.4 Transport mechanisms involved in metal tolerance

The plasma and vacuolar membranes are the main transport membranes of the fungi. There has been little work on the transport of metals in ectomycorrhizal fungi. Metal transport proteins may be involved in metal tolerance either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into intracellular compartments (Williams *et al.,* 2000; Hall, 2002). Gast et al. (1988) suggested a transport/regulation system at the cell membrane for essential elements such as Cu and

Zn, and an exclusion mechanism for Cd for some ectomycorrhizal species such as *P. involutus*. Using radiotracer flux analyses, the significant accumulation of Cd found in the vacuolar compartment has been suggested as an essential Cd detoxification mechanism in the ectomycorrhizal fungus *P. involutus* (Blaudez *et al.,* 2000b). A crucial step in Cd detoxification, certainly in fission yeasts and probably in higher plants, involves the accumulation of Cd-conjugated glutathione or Cd-conjugated phytochelatins in the vacuole. This process appears to be mediated by the ATP-binding cassette transporter *Hmt1* located at the tonoplast (Ortiz *et al.,* 1992), which would be of no significance in ectomycorrhizal fungi, given the lack of phytochelatin synthesis.

The yeast cadmium factor (Ycf1) gene encodes a MgATP energized glutathione S-conjugate transporter responsible for the vacuolar sequestration of bis (glutathionate) cadmium (Li *et al.,* 1997) as well as bis (glutathionate) mercury (Gueldry *et al.,* 2003). The presence of this specific permease in the tonoplast of *P. involutus* could explain the high Cd content in the vacuole (Blaudez *et al.,* 2000b). This hypothesis was further supported by X-ray microanalysis, which revealed that the accumulation of Cd correlated tightly with the accumulation of sulphur in electron-dense bodies in the vacuolar compartment (Ott *et al.,* 2002). However, the chemical nature of these sulphur components involved in Cd complexation was not confirmed in this study. With a similar approach, it was recently found that an enhanced Zn efflux may act as a potential tolerance mechanism in the ectomycorrhizal fungus *Suillus bovinus* (Adriaensen, 2005).

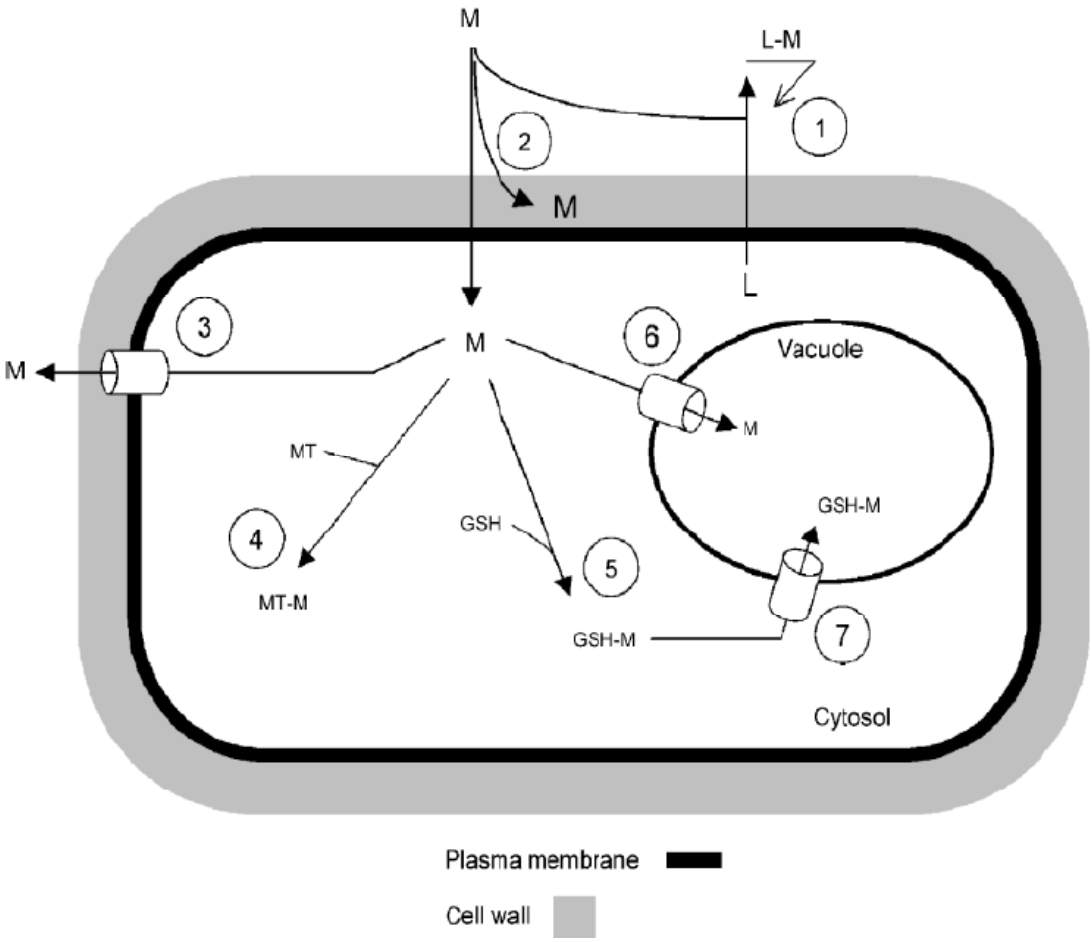


Figure 5: Schematic representation of cellular mechanisms potentially involved in metal tolerance in ectomycorrhizal fungi (Adapted from Bellion *et al*., 2006)

M, metal-ion; 1, extracellular chelation by excreted ligands (L); 2, cell wall binding; 3, enhanced efflux; 4, intracellular chelation by metallothionein (MT); 5, intracellular chelation by gluthathione (GSH); 6, Subcellular compartmentation (vacuole or other internal compartments); 7, vacuolar compartmentation of GSH-Mcomplex (i.e. ycf1).

Alternatively, down regulation of transporter genes involved in the uptake of metal at the plasma membrane may also be part of tolerance mechanisms, as described in other fungi (Eide, 2003) and plants (Clemens, 2001; Hall, 2002). Interestingly, an EST sequence showed a high similarity with the yeast transcription factor Zap1, involved in the regulation of numerous metal transporters in yeast (Zhao *et al.,* 1998). Zap1 plays a direct role in controlling Zn-responsive gene expression in yeast by binding to Zn responsive elements in the promoters of genes that it regulates. It thus constitutes an interesting target for Zn tolerance studies in ectomycorrhizal fungi. However, it is clear that the molecular mechanisms underlying metal transfer in intracellular compartments are still ignored in ectomycorrhizal fungi and more generally, nothing has been published in relation to the genes encoding proteins mediating intracellular metal transport in ectomycorrhizal fungi. A search for EST sequences encoding metal transporters promisingly indicates the presence of potential genes belonging to the ATP-binding cassette (the Ycf1 Cd-conjugate ABC transporter), cation diffusion facilitator, natural resistance-associated macrophage protein (Smf1 Mn transporter) or P-type ATPase families. Members of these transporter families have been shown to actively participate in metal detoxification of cells in a broad range of organisms (Williams *et al.,* 2000), and therefore they could also play a crucial role in metal protection in ectomycorrhizal fungi.

2.4.2 Intracellular complexation by peptides

Despite extracellular chelation and cell wall binding capacities of fungi described above, large amounts of metal may enter into the cells. Chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of heavy-metal

detoxification and tolerance. Potential ligands include amino acids and organic acids, and two classes of peptides, the metallothioneins and phytochelatins (Rauser, 1999; Clemens, 2001).

2.4.2.1 Metallothionein

The discovery of a cadmium (Cd)-binding, cysteine-rich protein from horse kidney by Margoshes and Vallee (1957) was the seminal finding that marked the birth of a field of research focused on the study of a low-molecular-weight polypeptide superfamily, the metallothioneins (MTs). Metallothioneins (MTs) is a family of Cys-rich, low molecular weight (3500 to 14000 Da) proteins. MTs have the capacity to bind both physiological (Zn, Cu, Se) and xenobiotic (Cd, Hg, Ag) heavy metals through the thiol group of its cysteine residues, which represents nearly the 30% of its amino acidic residues. Aromatic amino acids are usually absent. All Cysteines occur in the reduced form and are coordinated to the metal ions through mercaptide bonds, giving rise to spectroscopic features characteristic of metal-thiolate clusters (Hamer, 1986; Kagi, 1993).

2.4.2.1.1 Classification

MTs have been found in a vast range of taxonomic groups includes animal kingdom, higher plants, eukaryotic microorganisms, and in many prokaryotes (Hamer, 1986, Kagi, 1993). From their primary structure**,** MTs have been classified by different methods. The first one dates from 1987, when Fowler *et al.,* (1987) established three classes of MTs based on their structural similarities. Class I resemble the equine kidney protein and have been isolated from *Neurospora crassa* (Lerch, 1980) and *Agaricus bisporus* (Munger and

Lerch, 1985). Class II metallothioneins are present in *Saccharomyces cerevisiae* (Steaens, 1990); they do not share extensive sequence homology with Class I proteins. Class III metallothioneins were first detected in *Schizosaccharomyces pombe* (Murasugi *et al.,* 1984). These have the general structure poly (γ-glutamylcysteinyl)-glycine and they are commonly found in plants. They have been given the trivial name phytochelatins (Grill *et al.,* 1985). This class of metallothioneins is now known to be widespread in fungi (Kneer *et al.,* 1992). Class III, which includes Cadystins and Phytochelatins, Cys-rich enzymatically synthesised peptides which are no longer considered MTs. The second classification was performed based on taxonomic parameters and the patterns of distribution of Cys residues along the MT sequence (Binz and Kagi, 1999). MTs were classified into 15 families, which include vertebrate MTs, fungal MTs and plant MTs. The 15th family contains the plant MTs, further 15th family have been classified by Cobbet and Goldsbrough (2002) into 4 subfamilies (subfamily 1, 2, 3 and 4) depending on the distribution of their Cys residues and Cys-devoid regions (Table. 1).

2.4.2.1.2 Function

MTs are thought to play roles in the intracellular fixation of the essential trace elements zinc and copper, in controlling the concentrations of the free ions of these elements, in regulating their flow to their cellular destinations, in neutralizing the harmful influences of exposure to toxic elements such as cadmium and mercury and in the protection from of a variety of stress conditions. The biosynthesis of many MTs is greatly enhanced *in vivo* by certain hormones, cytokines, growth factors, tumor promoters and many other chemicals. A massive accretion of MT is also observed in the livers of animals submitted

to physical stress. Physiological MT-synthesis and MT concentrations are increased transiently several fold during cell proliferation. MT interchanges its zinc with zinc-finger proteins *in vitro* and hence, may imply a contributory role of MT to zinc-dependent processes involved in gene expression (Kagi and Vallee 1960; Lerch, 1980; Winge and Miklossy, 1982; Kagi and Kojima, 1987). It is also proposed that MT localized in the cytosol may be involved in protection from oxidative stress, whereas MT localized in the nucleus may provide protection against DNA-damaging electrophiles (Woo and Lazo, 1997). Although the physiological functions of MT are still elusive, and a number of functions have been attributed to it, they are all subject to debate. On the contrary, the only function of MT that has almost been unequivocally established (that has not been contradicted) is its role in protection against metal toxicity.

2.4.2.1.3 Metallothioneins and fungi

Fungal metallothioneins have been characterized almost exclusively in yeasts. In yeasts, several resistance mechanism are known that are activated on exposure to toxic metals, and molecular genetic analysis has been used to identify some specific genes involved in heavy metal detoxification pathways. In baker's *Saccharomyces cerevisiae,* two distinct metallothioneins have been identified, encoded by *CUP1* and *CRS5* loci. The metallothionein gene *CUP1* encodes a low-molecular-weight (6.6 kDa), cysteine-rich metal-binding protein, which play a predominant role in copper detoxification (Fogel and Welch, 1982) and the gene can be amplified up to 20 times or more (Butt *et al.,* 1984; Hamer *et al.,* 1985).

Table 1: Classification of metallothioneins according to Binz and Kagi, (1999)

Family	Sequence pattern	Example
1.Vertebrate	K-x(1,2)-C-C-x-C-C-P-x(2)-C	*M.musculus* MT1 MDPNCSCTTGGSCACAGSCKCKECKCTSCKKCCSCCPVGCAKC AQGCVCKGSSEKCRCCA
2.Molluscan	C-x-C-x(3)-C-T-G-x(3)-C-x-C-x(3)-C-x-C-K	*M.edulis* 10MTIV MPAPCNCIETNVCICDTGCSGEGCRCGDACKCSGADCKCSGCK VVCKCSGSCACEGGCTGPSTCKCAPGCSCK
3.Crustacean	P-[GD)-P-C-C-x(3,4)-C-x-C	*H.americanus* MTH MPGPCCKDKCECAEGGCKTGCKCTSCRCAPCEKCTSGCKCPSK DECAKTCSKPCKCCP
4.Echinoderms	P-D-x-K-C-[V,F)-C-C-x(5)-C-x-C-x(4)- C-C- x(4)-C-C-x(4,6)-C-C	*S.purpuratus* SpMTA MPDVKCVCCKEGKECACFGQDCCKTGECCKDGTCCGICTNAA CKCANGCKCGSGCSCTEGNCAC
5.Diptera	C-G-x(2)-C-x-C-x(2)-Q-x(5)-C-x-C-x(2)D-C-x-C	*D.melanogaster* MTNB MVCKGCGTNCQCSAQKCGDNCACNKDCQCVCKNGPKDQCCS NK
6.Nematoda	K-C-C-x(3)-C-C	*C.elegans* MT1 MACKCDCKNKQCKCGDKCECSGDKCCEKYCCEEASEKKCCPA GCKGDCKCANCHCAEQKQCGDKTHQHQGTAAAH
7.Ciliate	x-C-C-C-x	*T.termophila* MTT1 MDKVNSCCCGVNAKPCCTDPNSGCCCVSKTDNCCKSDTKECC TGTGEGCKCVNCKCCKPQANCCCGVNAKPCCFDPNSGCCCVS KTNNCCKSD TKECCTGTGEGCKCTSCQCCKPVQQGCCCGDKAKACCTDPNSG CCCSNKANKCCDATSKQECQTCQCCK
8.Fungal 1	C-G-C-S-x(4)-C-x-C-x(3,4)-C-x-C-S-x-C	*N.crassa* MT MGDCGCSGASSCNCGSGCSCSNCGSK
9.Fungal 2	---	*C.glabrata* MT2 MANDCKCPNGCSCPNCANGGCQCGDKCECKKQSCHGCGEQCK

		CGSHGSSCHGSCGCGDKCECK
10.Fungal 3	---	*C.glabrata* MT2 MPEQVNCQYDCHCSNCACENTCNCCAKPACACTNSASNECSC QTCKCQTCKC
11.Fungal 4	C-X-K-C-x-C-x(2)-C-K-C	*Y.lipolitica* MT3 MEFTTAMLGASLISTTSTQSKHNLVNNCCCSSSTSESSMPASCAC TKCGCKTCKC
12.Fungal 5	---	*S.cerevisiae* CUP1 MFSELINFQNEGHECQCQCGSCKNNEQCQKSCSCPTGCNSDDK CPCGNKSEETKKSCCSGK
13.Fungal 6	---	*S.cerevisiae* CRS5 TVKICDCEGECCKDSCHCGSTCLPSCSGGEKCKCDHSTGSPQCK SCGEKCKCETTCTCEKSKCNCEKC
14.Procaryota	K-C-A-C-x(2)-C-L-C	Synechococcus sp SmtA MTTVTQMKCACPHCLCIVSLNDAIMVDGKPYCSEVCANGTCKE NSGCGHAGCGCGSA
15.Plant		
15.1.Plant MTs Type 1	C-X-C-X(3)- C-X-C-X(3)- C-X-C-X(3)- espaiador- C-X-C-X(3)- C-X-C-X(3)- C-X-C- X(3)	*Pisum sativum* MT MSGCGCGSSCNCGDSCKCNKRSSGLSYSEMETTETVILGVGPAK IQFEGAEMSAASEDGGCKCGDNCTCDPCNCK
15.2.Plant MTs Type 2	C-C-X(3)-C-X-C-X(3)- C-X-C-X(3)- C-X-C- X(3)-spacer- C-X-C-X(3)- C-X-C-X(3)- C-X-C- X(3)	*L.esculetum* MT MSCCGGNCGCGSSCKCGNGCGGCKMYPDMSYTESSTTTETLVL GVGPEKTSFGAMEMGESPVAENGCKCGSDCKCNPCTCSK
15.3.Plant MTs Type 3	---	*A.thaliana* MT3 MSSNCGSCDCADKTQCVKKGTSYTFDIVETQESYKEAMIMDVG AEENNANCKCKCGSSCSCVNCTCCPN
15.4.Plant MTs Type 4 or Ec	C-x(4)-C-X-C-X(3)-C-X(5)-C-X-C-X(9,11)- HTTCGCGEHC- X-C-X(20)-SCGAXCNCASC- X(3,5)	*T.aestium* MT MGCNDKCGCAVPCPGGTGCRCTSARSDAAAGEHTTCGCGEHC GCNPCACGREGTPSGRANRRANCSCGAACNCASCGSTTA

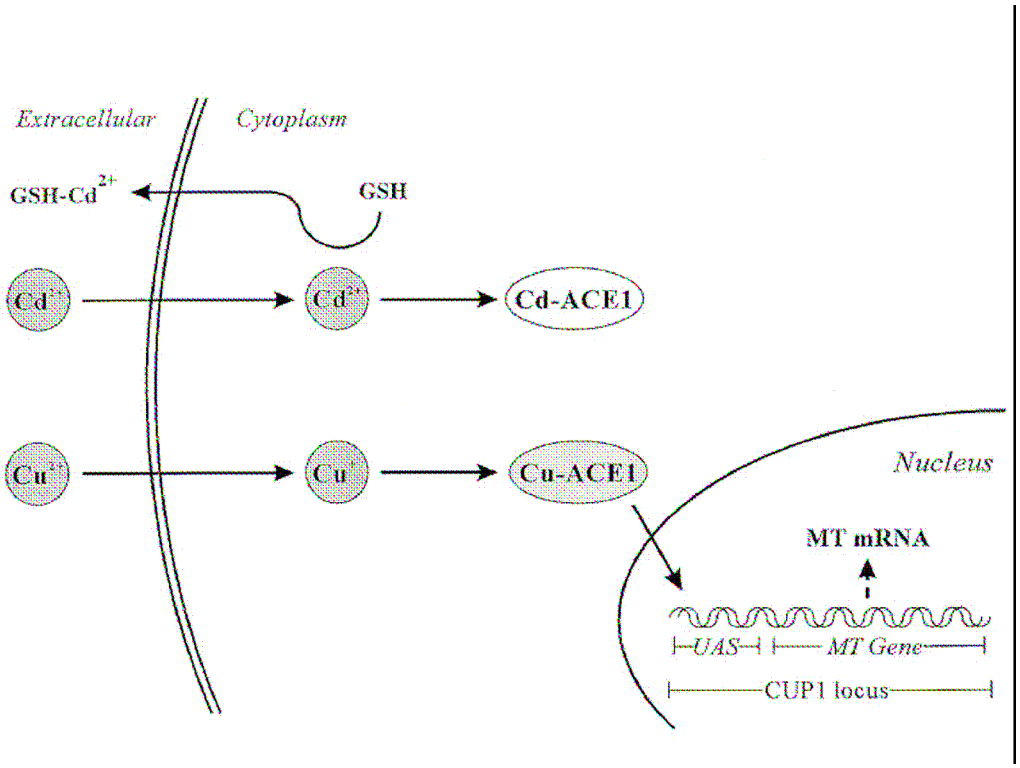


Figure 6: Metal detoxification pathways in yeast. *Saccharomyces cerevisiae* and *Candida glabrata* detoxify copper by sequestering it with the metal-binding protein metallothionein (MT). The synthesis of MT in *S. cerevisiae* and *C. glabrata* is transcriptionally regulated via the binding of Cu^{+}, respectively, to the transcription factors ACE1 and AMT. The binding of copper as Cu^{+} to ACE1 produces a conformational change that increases the protein's affinity for the upstream activation sequence (UAS). The binding of cadmium by ACE1 does not lead to an active conformation. *S. cerevisia*e limited resistance to Cd^{2+} is due to its limited ability to export some Cd^{2+} as a glutathione (GSH) complex and to synthesize phytochelatins. Cadmium is detoxified in *C. glabrata* through the robust synthesis of phytochelatins and sulfide (Adapted from Mehra *et al.*, 1991)

The multiple copy of gene allows *CUP1*-amplified strains to grow in medium with high levels of copper (Karin *et al.,* 1984), when over expressed it can also protect against Cd ions. However, it does not protect against the toxicity of Ni, Co, or Zn ions (Ecker *et al.,*

1986). Therefore, this defense mechanism is relevant to only a limited range of toxic metal ions. In contrast, the *CRS5* gene encodes a second MT, which is present as a single copy gene in yeast, provides minimal resistance to copper ion toxicity (Culotta *et al.,* 1994). In yeast, the metal-regulated transcription factor ACE1 (activator of CUP expression) regulates the transcription of the *CUP1* locus through an upstream activation sequence (UAS). Complexing of Cu^{+} or Ag^{+} produces a conformational change in ACE1 that increases its affinity for an UAS in the promoter of the metallothionein gene, leading to increased synthesis of metallothionein (Dameron *et al.,* 1993). ACE1 has little affinity for the UAS when Cd^{2+} ions are bound (Dameron *et al.,* 1993) (Fig. 6). The metallothionein induction in *S. cerevisiae* is thus specific for Cu^{2+} ions. Although the *S. cerevisiae* metallothionein will bind a variety of transition metal ions *in vitro*, its synthesis is only induced *in vivo* by copper. The limited tolerance to cadmium of *S. cerevisiae* is postulated to be provided through a glutathione export mechanism (Mewes *et al.,* 1997).

Tamai et al. (1993) demonstrated that the yeast *CUP1* gene was transcriptionally activated when cells are grown in the presence of high oxygen tensions or during respiration, two conditions known to generate oxidative stress. Expression of the *CUPl*-encoded MT suppresses a number of oxidative stress-induced growth defects of yeast strains lacking Cu, Zn superoxide dismutase. These observations demonstrate that yeast MT proteins are an important line of defense against oxidative stress. Later, Liu and Thiele, (1997) studied the *CUP1* gene transcription in response to a direct oxidative stress, they exposed cells to menadione, a derivative of vitamin K 3 that generates

superoxide anion through redox cycling. Primer extension analysis showed that treatment of *S. cerevisiae* cells with increasing concentrations of menadione results in a large induction of *CUP1* mRNA. The potent activation of *CUP1* transcription is mediated by the heat shock factor (HSF).

Uniquely, *Candida glabrata* expresses both metallothionein and the γ-glutamyl peptides for metal detoxification, and each system is regulated in a metal-specific manner. Exposure of *C. glabrata* to copper salts stimulates the formation of different metallothioneins, whereas exposure to cadmium leads to the formation of phytochelatins (Mehra *et al.,* 1989). Copper detoxification in the yeast, *C. glabrata,* is carried out in large part by a family of metallothionein (MT) genes: a unique MT-I gene, a tendemly amplified MT-IIa gene, and a single unlinked MT-IIb gene. In response to elevated environmental copper levels, members of this MT gene family are transcriptionally activated by a copper-dependent, sequence-specific DNA-binding transcription factor, AMT1. AMT1 shares several structural and functional features with the *S. cerevisiae* copper metalloregulatory transcription factor ACE1, which is constitutively expressed and poised for rapid transcriptional responses to the toxic metal copper (Mehra *et al.,* 1990; 1992).

Weissman et al. (2000) reported the isolation of a metallothionein, Ca*CUP1*, and a copper-transporting P-type ATPase in pathogenic yeast *Candida albicans.* Northern blot analysis revealed that the addition of $CuSO_4$ to an exponentially growing *C. albicans* culture leads to the induction of both genes and showed a higher resistance to elevated concentrations of copper.

Very few genetic analyses of metallothionein genes from filamentous fungi are available. The metallothionein gene (CuMT) of ascomycete *Neurospora crassa* has been cloned and nucleotide sequence determined; this gene codes a 26 amino acid protein and composed of 28% cysteine. The regulation of expression of the *N. crassa* metallothionein gene in response to different metal ions (Cu^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}) was studied by Northern analysis. Only copper led to the induction of metallothionein (Lerch, 1980). It was also postulated that *N. crassa* CuMT might serve a multifunctional biological role such as copper storage at low concentrations and copper detoxification at higher concentrations of this metal ion (Beltramini and Lerch, 1986).

Kumar et al. (2004) identified two metal response elements, flanking an antioxidant response element upstream (−3730 bp) to copper metallothionein (CuMT) gene of *N. crassa*. The CuMT gene expression was enhanced in the presence of copper, but not of pro-oxidants like H_2O_2 or menadione. Gel shift assays revealed the ability of nuclear extracts from copper induced cultures to bind PCR-amplified metal response or antioxidant response elements. Similar observations could not be made with cultures exposed either to pro-oxidants or antioxidants. These results differentiate between CuMT gene induction by copper from antioxidant functions associated with the identified upstream elements

Munger and Lerch (1985) performed the isolation and chemical characterization of the copper metallothionein from the basidiomycete *Agaricus bisporus* and presented the

complete amino acid sequence of the protein. It consists of 25 amino acids with characteristically high cysteine content (28%) and binds 6 mol of copper per molecule.

Averbeck et al. (2000) cloned and characterized a gene coding for metallothionein *PiMT* in *Podospora anserine,* involved in the control of copper homeostasis. This gene is single copy gene and encodes 26 amino acids, showing higher similarity to *N. crassa* and *A. bisporus*. Northern hybridization results showed that the expression levels of *PiMT* were increased in response of copper, not by cadmium, zinc and manganese and during aging of wild type cultures. They also studied the effect of H_2O_2 and paraquat on the expression of *PiMT,* results revealed no significant difference in transcript accumulation of *PiMT*.

2.4.2.1.3.1 Mycorrhizal fungi and metallothioneins

The occurrence of metallothioneins, phytochelatins, or both, in mycorrhizal fungi is still a matter of debate (Leyval *et al.,* 1997). There are very few reports on metallothionein like proteins in mycorrhizal fungi. Putative MTs have often been described within collections of expressed sequence tags (ESTs) simply on the basis of sequence similarity. This is the case for MT-like sequences found in mycorrhizal fungi, in particular in the ectomycorrhizal fungus *Pisolithus tinctorius* (Voiblet *et al.,* 2001) *H. cylindrosporum* (Lambiliotte *et al.,* 2004); the ericoid fungus *Oidiodendron maius* (Vallino *et al.,* 2005); and the arbuscular mycorrhizal fungi *Gigaspora rosea* (Stommel *et al.,* 2001), *Gigaspora margarita* (Lanfranco *et al.,* 2002) and *Glomus intraradices* (González-Guerrero *et al.,* 2006). However, their metallothioneins like nature have not yet been determined.

Lanfranco *et al.,* (2002) identified a full-length cDNA encoding a metallothionein like polypeptide, designated *GmarMT1*, in arbuscular mycorrhizal fungus *Gigaspora margarita.* As revealed by heterologous complementation assays in yeast, *GmarMT1* encodes a functional polypeptide capable of conferring increased tolerance against Cd and Cu. The *GmarMT1* RNA is expressed in both presymbiotic spores and symbiotic mycelia, even in the absence of metal exposure, but is significantly less abundant in the latter stage.

González-Guerrero et al. (2007) isolated a full-length metallothionein gene (*GintMT1*) from *Glomus intraradices* mycelium and studied its role in response to copper, cadmium and oxidative stress. Gene expression analyses revealed that the transcript levels of *GintMT1* were unregulated in response to Cu, Paraquat (Oxidative substance) but not to Cd. Functional analysis of *GintMT1* in a MT-defective yeast strain indicates that it encodes a functional MT. Induction of *GintMT1* expression by paraquat and Cu treatments that also produced an oxidative damage to the fungal membranes, suggests that *GintMT1* may play a role in the regulation of the redox status of the extraradical mycelium of *G. intraradices.*

Morselt et al. (1986) used specific histochemical staining to demonstrate the induction of metallothionein like peptides in *P. tinctorius* in the presence of Cu, Zn and Cd. They also observed the lacking of induction of the same peptides, in a metal sensitive strain of *Cenococcum geophilum* possibly due to lack of expression of metallothionein gene. However, this was not confirmed by molecular analysis.

In a study of ECM fungus *Laccaria laccata* in association with *Picea abies*, increased activity of 3'-phosphate 5'-phosposulphate sulphotranferase, responsible for sulphate reduction, and thiols were observed in response to Cd (Galli *et al.,* 1993). However, in this case, this was due to increased glutithionein (a precursor of phytochelatins and metallothioneins) levels in fungus. No phytochelatins or metallothioneins were detected in *L. laccata* exposed to cadmium, suggesting the Cd^{2+} is detoxified by complexation with glutathione and γ-glutamyl-cysteine.

Howe et al. (1997) purified copper binding proteins using a modified SDS-PAGE, produced in response to added copper and were examined in isolates of *L. laccata*, *P. involutus* and single isolate of *S. citrinum*. Copper binding proteins were found in *L. laccata* and *P.involutus*, but no such proteins were detected in *S. citrinum*. Copper binding protein molecular mass and spectral characteristics were consistent with metallothioneins.

Courbot et al. (2004) used an improved high-performance liquid chromatography method for the measurement of thiol-containing compounds from cysteine and its derivatives such as γ-glutamylcysteine, glutathione, and phytochelatins in ectomycorrhizal fungus *Paxillus involutus* in response to cadmium. They found that glutathione and γ-glutamylcysteine contents increased and lack of phytochelatins when *P. involutus* was exposed to cadmium. An additional compound with a 3-kDa molecular mass, most probably related to a metallothionein, increased drastically in mycelia exposed to

cadmium. The finding of this metallothionein (*PiMT1*) was supported by the presence of a metallothionein sequence, homologous to a known metallothionein from *Agaricus bisporus*, in the cDNA array analysis of *P. involutus* exposed to Cd (Jacob *et al.,* 2004). They also noticed the up regulation of laccase and aconitase genes.

More recently, Bellion et al. (2007) characterized the *PiMT1* gene, codes for a relatively short (34 amino acids) MT and contains just one domain bearing the classical C–X–C motifs. Transcripts were almost undetectable under control conditions, whereas Cu and Cd, but not Zn, strongly induced. Yeast complementation studies showed that *PiMT1* was able to complement the hypersensitivity of mutant strains to cadmium and copper, but not to zinc. In addition, the *PiMT1* gene has been functionally characterized *in vivo* using another heterologous system, *Hebeloma cylindrosporum.* Constitutive overexpression of *Pimt1* in *H. cylindrosporum* conferred a higher copper tolerance.

2.4.2.2 Phytochelatins

In addition to the cysteine rich peptides of the metallothionein family, another group of metal binding molecules synthesized by fungi are short, cysteine containing γ-glutamyl peptide, encompassed by the trivalent name 'phytochelatins' (Rauser, 1990). These are mainly synthesized by plants and algae as well as in a range of filamentous fungi and yeasts (Grill *et al.,* 1985). Phytochelatin (PC) was first discovered in 1981 in fission yeast, and was named cadystin (Murasugi *et al.,* 1981). It was then found in higher plants in 1985 and was named phytochelatin. In 1989 its enzyme, phytochelatin synthase, was discovered. Phytochelatins are oligomers of glutathione. PCs consisted of the three amino

acids: Glu, Cys and Gly with the Glu, and Cys residues linked through a γ-carboxylamide bond. PCs form a family of structures with increasing repetitions of the γ-Glu-Cys dipeptide followed by a terminal Gly; $(\gamma\text{-Glu-Cys})_n$-Gly, where *n* has been reported as being as high as 11, but is generally in the range of 2 to 5. They are structurally related to glutathione (GSH; γ-Glu-Cys-Gly) and were presumed to be the products of a biosynthetic pathway. Biosynthesis of the tripeptide GSH is catalyzed by the enzymes γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3). Synthesis of phytochelatins from GSH is catalyzed by the enzyme phytochelatin synthetase (Fig. 7) (Coblenz and Wolf, 1994). Numerous physiological, biochemical and genetic studies have confirmed that GSH is the substrate for PC biosynthesis. In addition, a number of structural variants of PCs, such as (γ-Glu-Cys)*n*– γ-Ala, (γ-Glu-Cys)*n*–Ser and (γ-Glu- Cys)*n*–Glu, have been identified (Rauser, 1995, 1999).

Numerous physiological, biochemical, and genetic studies have confirmed that GSH (or, in some cases, related compounds) is the substrate for PC. By far the most detailed characterization of the pathway of PC biosynthesis has come from studies in the fission yeast (*Schizosaccharomyces pombe)*, and in *Arabidopsis*. They are found in plants, fungi, nematodes and all groups of algae including cyanobacteria. Phytochelatins act as chelators, and are important for heavy metal detoxification, especially cadmium metal. Although both induction of PCs *in vivo* and activation of PC synthase *in vitro* are conferred by a range of metal ions, there is little evidence supporting a role for PCs in the detoxification of such a wide range of metal ions. For metals other than Cd there are few studies demonstrating the formation of PC-metal complexes either *in vitro* or *in vivo*. PCs

can form complexes with Pb, Ag, and Hg *in vitro* (Mehra *et al.,* 1996; Rauser, 1999). Phytochelatin seems to be transported into the vacuoles, so that the metal ions it carries re stored safely away from the proteins of the cytosol.

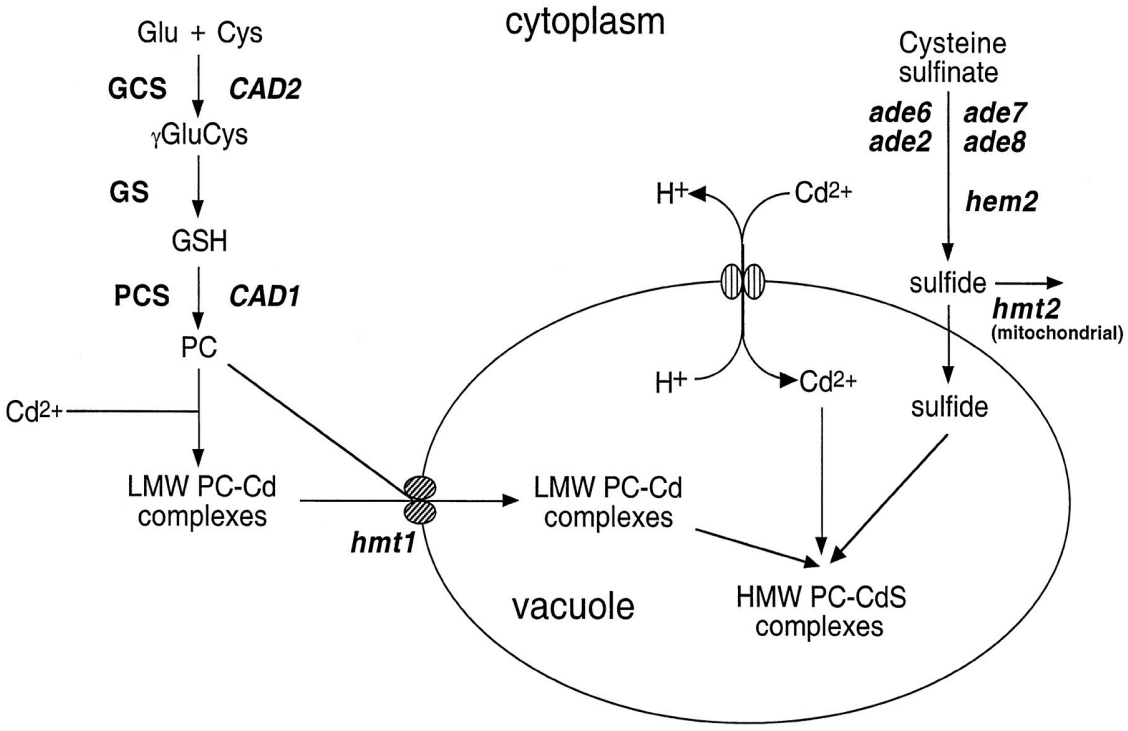


Figure 7: Genes and functions contributing to Cd detoxification in plants and fungi. The figure is a composite of different functions described in different organisms (Adapted from Cobbett, 2000). Enzyme abbreviations are shown in bold. GS, GSH synthetase; PCS, Phytochelatin synthase. Gene loci are shown in bold italics. *CAD1* and *CAD2* are in *Arabidopsis*; *hmt1*, *hmt2*, *ade2*, *ade6*, *ade7*, and *ade8* are in fission yeast; and *hem2* is in *Candida glabrata*

Murasugi et al. (1981) purified two Cd-PC complexes, a low molecular weight (LMW) and a high molecular weight (HMW) complex, in *Schizosaccharomyces pombe.* The two Cd-PC complexes were contain an identical amino acid composition, but differed in molecular size, charge properties and cadmium content. Mutoh and Hayashi, (1988) identified the importance of HMW in cadmium detoxification in *S. pombe.* They described that the HMW complex is essential for maximal detoxification of Cd, as the LMW complex alone is insufficient.

Ortiz *et al*. (1995) found that the protein HMT1 transported PCs and PC-Cd complex across the tonoplast of *S. pombe* .They proposed that as Cd entered the cell, LMW Cd-PC complex was synthesized in the cytosol, the complex was transferred to the vacuole by the transporter HMT1, where Cd and acid labile sulfide were added to form the HMW Cd-PC complex. Coblenz and Wolf (1994) identified the genes which are involved in the biosynthesis of phytochelatins in fission yeast *S. pombe* under cadmium stress. They found basically three genes to be involved in cadmium tolerance, namely the genes encoding γ-glutamylcysteine synthetase, glutathione synthetase and phytochelatin synthase. Complexes of thiolic peptides including GSH and phytochelatins which were associated with Cd (II) were identified from *S. pombe* (Li *et al*. 1997; Bae and Mehra, 1998).

C. glabrata employs different mechanisms to detoxify cadmium and copper salts (Liu and Thiele, 1997). Cadmium salts stimulate the production of (*c*-EC)nG peptides, whereas copper salts induce the synthesis of a family of MTs. In *C. glabrata,* Dameron et

al. (1989) demonstrated the biosynthesis of Cd^{2+} GSH complexes. The acquired Cd^{2+} resistance was due to an enhanced Cd^{2+} stimulated cytosolic fraction of thiol peptide coated Cd^{2+} sulfide crystallites (Mehra *et al.,* 1994). The production of PC2-like compounds, via a phytochelatin synthase independent pathway, has been documented in *S. cerevisiae, N. crassa* (Kneer *et al.,* 1992) and in an arsenate-hypertolerant *Aspergillus isolate* (Canovas *et al.,* 2004). Miersch et al. (2001) studied thiol production in an aquatic hyphomycete *Articulospora tetracladia* and a zygomycete *Mucor racemosus* and identified Cd-induced PC2 and PC3 in the *M. racemosus*. Collin-Hansen et al. (2007) first reported the presence phytochelatins and studied the induction of phytochelatins by cadmium in macromycete *Boletus edulis.* Jaeckel et al. (2005) was measured at the thiol peptide level in an aquatic hyphomycete *Heliscus lugdunensis* in response cadmium stress. The authors observed increase glutathione (GSH) content and induced the synthesis of additional thiol peptides. HPLC, electrospray ionization mass spectrometry, and Edman degradation confirmed that a novel small metallothionein as well as phytochelatins (PC2).

There are clear reports available on presence of phytochelatins in mycorrhizal fungi. Courbot et al. (2004) studies have highlighted the complete lack of phytochelatins (PCs) among the Cd-responsive thiols produced in *P. involutus* and in *Suillus bovinus* (J. V. Colpaert *et al.,* personal communication) and seem to confirm the general lack of phytochelatins in fungi. Under Cd exposure, an increase in sulphate assimilation and cysteine synthesis, and an increase of the nonprotein thiols glutathione and its precursor γ-glutamylcysteine were observed in *L. laccata*, although no metallothionein could be detected (Dameron *et al.,* 1989; Galli *et al.,* 1993). The role of glutathione as a metal

chelator in fungi is now clearly established (Pocsi *et al.,* 2004). Intracellular glutathione hinders the progression of heavy metal-initiated cell injuries by chelating and sequestering the metal ions themselves. Putative gene sequences encoding enzymes involved in the synthesis of glutathione and γ-glutamylcysteine have been identified in expression sequence tag (EST) databases obtained from the ectomycorrhizal fungi *H. cylindrosporum* and *P. involutus.*

2.4.2.3 Antioxidative mechanism

The formation of free radical species, which can be initiated directly or indirectly by metals, can cause severe damage to different cellular components (Bellion *et al.,* 2006). Formation of metal-induced reactive-oxygen species could occur via several mechanisms. The Fenton or Haber-Weiss reactions are catalysed by redox-active metals (e.g. Cu, Fe, Cr, V) and generate the highly reactive hydroxyl ($HO^{\bullet}$) radical from H_2O_2 and superoxide ($O_2^{\bullet-}$) substrates (Halliwell and Gutteridge, 1999). Redox-inactive metals such as Cd, Ni, Hg and Zn deplete glutathione and protein-bound sulphydryl groups, resulting in the production of reactive-oxygen species. The successive reduction of molecular oxygen to H_2O yields the intermediates $O_2^{\bullet-}$, $HO^{\bullet}$ and H_2O_2, which are potentially toxic, because they are relatively reactive compared with O_2. Reactive oxygen species may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA injury. As a consequence, tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins and malondialdehyde and show an increased production of ethylene (Dean *et al.,* 1993; Ames *et al.,* 1993). The control of oxidant levels is achieved by antioxidative systems. These defense systems are composed of

metabolites such as ascorbate, glutathione, tocopherol, etc., and enzymatic scavengers of activated oxygen such as superoxide dismutases (SODs), peroxidases and catalases (Noctor and Foyer, 1998; Asada, 1999). SODs have been categorized into three major families on the basis of metal cofactors at the active sites and their evolutionary homology; copper and zinc containing SOD (Cu/ZnSOD), manganese containing SOD (MnSOD) and iron containing SOD (FeSOD) (Bannister *et al.,* 1987). Evidence for a role of reactive-oxygen species in metal-induced damage to yeast includes increased metal tolerance during anaerobicity, protection exerted by certain free radical scavengers, and the many overlaps in the molecular mechanisms used by yeasts to cope with oxidative and metal stress (Avery, 2001). In yeast, heavy metals also caused oxidative stress (Mannazu *et al.,* 2000). Some scarce data also suggest that heavy metals affect antioxidative systems in mycorrhizal fungi. For example, an inhibition of Mn-SOD was found in pure cultures of *Rhizopogon roseolus* treated with 300 µM Cd (Miszalski *et al.,* 1996). The antioxidative systems of mycorrhizal fungi revealed important differences in comparison with plant tissues. For example, in pure cultures of *Laccaria laccata*, *Suillus bovinus*, and *Paxillus involutus* typical 'unspecific' peroxidase activities were not detected (Münzenberger *et al.,* 1997; Schützendübel *et al.,* 2001) neither was ascorbate peroxidase activity or ascorbate as a potential substrate (Ott e*t al.,* 2002). Much higher concentrations of glutathione were found in pure cultures of *Suillus bovinus* and *Paxillus involutus* than in pine roots (Schützendübel *et al.,* 2001). Cadmium indirectly contributes to oxidative stress by affecting the cellular thiol redox balance and found that Cu^{2+} and Cd^{2+} markedly induced *PiTrx1*, a gene encoding a thioredoxin in *P. involutus* (Bellion *et al.,* 2006). Thioredoxins are small heat-stable oxidoreductases, which contain two

conserved cysteine residues in their active sites (Holmgren, 1989). Up regulation of *PiTrx1* expression is a rapid response determinant in the handling of Cu^{2+} or Cd^{2+}, which might function as a first line of defense against intracellular metal ions. Similarly, thioredoxin was found to be induced upon exposure of yeast cells to Cd (Vido *et al.,* 2001). Ott et al. (2002), in a comprehensive study, analyzed the antioxidative systems in the ectomycorrhizal fungus *P. involutus* in response to Cd, which revealed the induction of superoxide dismutase (SOD) and the accumulation of glutathione, as well as the induction of glutathione-related systems at low Cd concentration (glutathione-dependent peroxidase, glutathione reductase). The authors concluded that *P. involutus* is able to detoxify high concentrations of Cd by a strong induction of glutathione synthesis accompanied by a rapid sulphur-dependent transport of Cd into the vacuole. Jacob et al. (2001) found an increase in Mn-SOD activity in Cd-treated *Paxillus involutus* cultures. The authors cloned the corresponding genes and studied up regulation of transcripts under cadmium stress. In addition, SOD could promote Cd resistance through its capacity to bind and buffer cellular Cd as demonstrated for Cu and yeast (Culotta *et al.,* 1994). The large number of ESTs found for SOD probably denotes a crucial function of these enzymes against oxidative stress in ectomycorrhizal fungi. It was found that a fast glutathione accumulation and maintenance of a relatively stable redox state prevented an accumulation of H_2O_2 in *P. involutus* (Ott *et al.,* 2002). The synthesis of cysteine-rich compounds may be efficiently reduced in *P. involutus*, thus redirecting cysteine to the manufacture of cysteine-enriched compounds needed for the chelation of Cd. It was also suggested that metallothioneins have antioxidant activity *in vivo,* which could be involved in the cellular response to oxidative stress (Tamai *et al.,* 1993).

Materials and Methods

3.1 Biological materials

3.1.1 Ectomycorrhizal fungal isolates and culture conditions

The monokaryotic strain h1 of the ectomycorrhizal fungus *Hebeloma cylindrosporum* was obtained from single spore germination (Debaud and Gay, 1987). *H. cylindrosporum* is a homobasidiomycete and predominant fungus occurring in the sand dune ecosystem of France. It was maintained at 25^0C in the dark on modified Melin's medium supplemented with Heller's micronutrients (Melin, 1953) (Appendix I).

3.1.2 Yeast and culture conditions

Two copper-sensitive strains DTY3 (*MATα, leu2-3, 112his3*$^{\Delta}$*1, trp1-1, ura3-50, gal1 CUP1*^{s}*)* and DTY4 (*MATα, leu2-3, 112his3*$^{\Delta}$*1, trp1-1, ura3-50, gal1, cup1::URA3)* referred as *cup1*^{s} and *cup1*$^{\Delta}$ and one cadmium sensitive *yap1* mutant (*MATα, his3D1, leu2D0, met15D0, ura3D0, YML:: kanMX4*) of *Saccharomyces cerevisiae* were used in this study. Strains DTY3 and DTY4 (wild type parental and *cup1*$^{\Delta}$, respectively) were kindly provided by Dr. Dennis Thiele, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Levine Science Research Center, Durham, North Carolina 27710, USA. These yeast stains were maintained on YPD agar medium

(Appendix I) or appropriate SD agar medium at 30^0C (Appendix I). For complementation studies, transformed cells were selected by their capacity to grow in complete synthetic medium (SD), without Trp (p424 vector selection marker) and Ura (*cup1*$^{\Delta}$ strain selection marker) (SD-Trp-Ura medium) and SD medium without Trp for *yap1* mutant.

3.2 *H. cylindrosporum* tolerance to different heavy metals

The response of *H. cylindrosporum* to various concentrations of copper, cadmium, nickel, lead and zinc was assessed by growing pure mycelial culture in liquid MMN medium with Heller's micronutrients (Appendix I). MMN liquid medium was prepared (pH 5.6), dispensed 50 ml in 250 ml flaks and autoclaved. Two mycelial discs (7 mm diameter) cut from actively growing colony of test fungus was inoculated and incubated in the dark at 25^0C for 3 days. To avoid immediate metal stress and also to allow fungus to initiate growth, the metals were added after three days of fungal inoculation The concentration of different metals used were: Cu^{2+} 0, 80, 160, 240, 320 µM as $CuSO_4.5H_2O$; Cd^{2+} 0, 7, 14, 21, 28 µM as $3CdSO_4 .8H_2O$; Zn^{2+} 0, 0.5, 1, 1.5, 2 mM as $ZnSO_4.7H_2O$; Ni^{2+} 0, 85, 170, 250, 350 µM as $NiSO_4.6H_2O$; Pb^{2+} 0, 25, 50, 75, and 100 µM as $PbCl_2$. The flasks were incubated at 25^0C in dark and were shaken manually once in a week for 20-30 seconds for uniform distribution of the medium. The mycelium was harvested after 21 days, washed with distilled water and dry biomass was measured.

3.4 MOLECULAR METHODS

3.4.1 Isolation of nucleic acids

3.4.1.1 Isolation of genomic DNA

H. cylindrosporum was grown in liquid MMN medium for 21 days, after that mycelia was harvested and rinsed with sterile distilled water. The harvested mycelia was placed in liquid nitrogen and ground into fine powder using a mortar and pestle. Genomic DNA was extracted from the ground mycelium by the method of Vankan et al. (1991). The pellet of DNA was dissolved in sterile MQ water and stored at -20^{0}C for future use.

Procedure for DNA isolation (Vankan *et al.*, 1991)

1. A 1.5 ml centrifuge tube was filled 1/3 rd with freeze-dried mycelium powder.
2. 0.5 ml of extraction buffer (Appendix II) was added, mixed well and kept for 15-20 minutes at 65^{0}C / or at room temperature.
3. Then 0.5 ml of equilibrated phenol was added and mixed well and incubated for 15 minutes at room temperature.
4. 0.5 ml of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. Then tubes were incubated at room temperature for 15 minutes and centrifuged for 20 minutes at 12, 000 g.
5. The upper aqueous layer was removed and transferred to new centrifuge tube.
6. 400 µl of chloroform: isoamyl alcohol (24:1) was added to aqueous layer and mixed by inverting gently and centrifuged for 10 minutes at 12,000 g and transferred supernatant to a new centrifuge tube.

7. Then 0.54 volumes of isopropanol was added to precipitate DNA and incubated for 15 minutes and centrifuged for 10 minutes at 12, 000 g.
8. The pellet was washed with 100 µl 70% (v/v) ethanol.
9. The DNA pellet was resuspended in 300 µl of 0.2M ammonium acetate and incubated overnight at 4^0C.
10. The DNA was precipitated by adding 600 µl of ethanol.
11. The pellet was dissolved in 50 µl of autoclaved water or TE buffer (pH. 8) (Appendix II).

3.4.1.2 Total RNA isolation

H. cylindrosporum was grown on liquid MMN medium for 21 days at 25^0C and mycelia was filtered aseptically and crushed into fine powder using liquid N_2 and stored at -80^0C. RNA was isolated from the mycelial powder using the TRIzol reagent (Invitrogen, Life Technologies, USA) method as per the manufacturer's instructions.

1. One ml of TRIzol Reagent was added to the samples and incubated for 5 minutes at 15^0C to permit the complete dissociation of nucleoprotein complexes and centrifuged at 12,000 g for 10 minutes at 4^0C.
2. 0.2 ml of chloroform was added and the tubes vigorously shook by hand for 15 seconds, incubated at 15^0C for 2 to 3 minutes and centrifuged the samples at 12,000 *g* for 15 minutes at 4^0C.

3. The upper aqueous phase was transferred into a fresh tube. Then the RNA was precipitated by mixing with 0.5 ml of isopropyl alcohol. Then samples were incubated at 15^0C for 10 minutes and centrifuged at 12,000 *g* for 10 minutes at 4^0C.
4. The supernatant was discarded and the RNA pellet washed with 75% ethanol, and the sample mixed by vortexing and centrifuged at 7,500 *g* for 5 minutes at 4^0C.
5. At the end of the procedure, the RNA pelletwas briefly dried and RNA dissolved in RNase-free water and stored at -70^0C.

3.4.2 Molecular analysis of nucleic acids

3.4.2.1 Electrophoresis of nucleic acids on non-denaturing agarose gels

Nucleic acids were loaded on agarose gels (0.7%- 2 %(w/v)) prepared in 0.5X TBE buffer pH 8.0 (Appendix II) using a 6X loading buffer (Appendix II). Ethidium bromide (EtBr) (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then migrated and visualized on a U.V. transilluminator (312 nm) and equipment used for RNA samples was washed in detergent and rinsed in autoclaved double distilled water to eliminate ribonuclease contamination prior to use.

3.4.2.2 Electrophoresis of RNA in denaturing conditions

RNA samples (20-40 µg) in 5.6 µl were denatured for 5 minutes at 65^0C in 0.5 x MOPS, 17.5% (v/v) formaldehyde and 50% (v/v) formamide in a final volume of 25 µl. Specially prepared 5x loading dye and ethidium bromide (1 mg/ml) were added to the samples prior to loading onto a 1% (w/v) agarose gel dissolved in 1x MOPS buffer

(Appendix II) and 9% (v/v) formaldehyde. Samples were fractionated in 1x MOPS buffer at 60 V. The RNA was then visualized under U.V. light.

3.4.3 Nucleic acid quantification

3.4.3.1 Ethidium bromide fluorescent quantification

DNA was migrated electrophoretically in an agarose gel containing ethidium bromide (0.5 µg/ ml). The quantity of DNA was visually determined with reference to a known λ phage DNA quantity by comparing the intensity of fluorescence after staining.

3.4.3.2 Spectrophotometric quantification

DNA and RNA were quantified by measuring the sample at $O.D._{260nm}$. One absorbance unit corresponds to approximately 40 µg/ml of RNA or single stranded DNA, and approximately 50 µg/ml of double stranded DNA (Sambrook *et al.,* 1989). The purity of the sample from contaminating polysaccharides and proteins was evaluated by the ratio between O.D 260/230 nm and O.D 260/280 nm respectively. Pure samples were indicated by a value closer to or higher than 1.8 for DNA and 2.0 for RNA.

3.4.4 DNA amplification by polymerase chain reaction (PCR)

3.4.4.1 Amplification of genomic DNA with gene specific primers

Genomic DNA was extracted from powdered mycelia and the amplification was performed using HcMT 1 F, 1R and HcMT 2F, 2R primers (Table. 2). The annealing temperature and PCR programs were the same for both set primers. PCR reactions were

carried out in a final volume of 25 µl containing 1x reaction buffer, 2 mM $MgCl_2$, 200 µM of each dNTPS, 0.5 µM of each primer, 10 ng of genomic DNA and 1.25 U of *Taq* DNA polymerase (Invitrogen, Life Technologies, USA). The PCR program was carried as follows: initial denaturation at 95^0C for 5 minutes, followed by 30 cycles for 1 minute at 92^0C, 1 minute at 55^0C annealing temperature, 1 minute at 72^0C and final a extension at 72^0C for 5 minutes. Amplified products were visualized on a 1.5% (w/v) agarose gel.

3.4.4.2 Amplification of mRNA by reverse transcription PCR (RT-PCR)

cDNA was synthesized from total RNA by reverse transcription PCR method (The Reverse AID^{TM} First Strand cDNA Synthesis Kit, Fermentas Life Sciences, USA). Two µg of total RNA was denatured at 70^0C for 5 minutes in the presence of 0.5 µg of oligo dT_{18} primer and immediately cooled on ice. The first strand cDNA was synthesized in the presence of 100U MMLV reverse transcriptase, 20U ribonuclease inhibitor, 10 mM of each dNTPs and 1X reaction buffer supplied by the manufacturer. The reaction was carried out at 42^0C for 1 hour. The enzyme was inactivated by incubating at 70^0C for 15 minutes and immediately placed on ice. cDNAs were verified by amplifying the tubulin gene (constitutive gene) with HcTub1 U and HcTub1 L primers. PCR reactions were carried out in a final volume of 25 µl containing 1x reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPS, 0.5 µM of each primer, 1 µl cDNA and 1.25 U of *Taq* DNA polymerase. The PCR program was as follows: initial denaturation at 95^0C for 5 minutes, followed by 30 cycles for 1 minute at 92^0C, 1 minute at 53^0C annealing temperature, 1 minute at 72^0C and final extension at 72^0C for 5 minutes. Amplified products were visualized on a 1.5% (w/v) agarose gel.

Table 2: PCR oligonuclotide primers used in this work

Primer	Sequence	Use
HcMT1 F	5'-AAGCTTGCGGTTCTGACAAT-3'	HcMT1 gene fragment
HcMT1 R	5'-GGCTGGACAAAATCGAACTC-3'	HcMT1 gene fragment
HcMR	5'- GACCGGGATGAATTGAGAGA- 3'	5' RACE for HcMT1 gene
HcMT2 F	5'-CTCTTCCTGCACCTGCCACTT-3'	HcMT2 gene fragment
HcMT2 R	5'-ACGAGGAAACTTGGCAGAAG-3'	HcMT2 gene fragment
HcMT1 specific primer	5'-TGGTAATGCGTACGAGCTGTTGCAG TTGCAG-3'	Genome walking of HcMT1
HcMT2 specific primer	5'-CACTCGCCAGCCTTGCAGGTGCAG CTAG- 3'	Genome walking of HcMT2
HcMT1	5'-TGGCAAGAGCCAGAGGAGCAGGTGCAACT-3'	Genome walking of HcMT1
HcMT2	5'-CCGCGAGAATGACGAACTACTCACGAAGTGC-3'	Genome walking of HcMT1
MT1 F	5'-CGGG ATCCATGCAATTCACTTCTATCCTCGTC-3'	Complementation studies
MT1 R	5'-CCGGAATTCTCA GTTGCAGTTGCAGTTGTTGG-3'	Complementation studies
MT2 F	5'-CGGGATCCATGCAGATCGTT CAAAACAGTCTCG-3'	Complementation studies
MT2 R	5'-CCGGAATTCTTAGCATTTGCACTCGC CAGCC-3'	Complementation studies
HcTub1U	5'-GGTCTCAGCCCTGATGGT-3'	Tubulin gene fragment
HcTub1L	5'-TGGAAAGACGCTCAAGGA-3'	Tubulin gene fragment
SP6	5'-ATTTAGGTGACACTATAG-3'	Sequencing
T7	5'-TAATACGACTCACTATAGGG-3'	Sequencing

3.4.4.3 Amplification of cDNA using specific primers

To induce the metallothionein gene, the *H. cylindrosporum* was grown in liquid MMN medium for 15 days then the fungus was transferred into fresh MMN medium containing 160 µM of copper and incubated for 24 hours. The mycelium was harvested and total RNA was isolated as mentioned earlier. Then, cDNA was prepared and used for the amplification of metallothionein gene. cDNA was amplified with both set of primers (HcMT 1F, 1R and HcMT 2F, 2R), which were used for the amplification of genomic DNA. The PCR program, reaction mixture and annealing temperature were same as mentioned earlier.

3.4.4.4 Rapid amplification of 5' cDNA ends (5' RACE) and 3' cDNA ends (3'RACE)

To find the complete sequence of *HcMT1* and *HcMT2*, RACE PCR method was used to isolate the 5' and 3' ends of cDNA according to the protocol provided by the manufacturer's (5'/3' RACE kit, Roche, USA) (Fig. 8). For 5'RACE, 5 µg of total RNA was reverse transcribed at 55^0C for 60 minutes in a 20 µl reaction, using HcMT1R primer (gene specific) and first strand cDNA was further purified. Homopolymeric (oligo dA) tailed cDNA was synthesized according to manufacture's instructions. 5 µl of oligo dA tailed cDNA was then amplified in the presence of 1X buffer, 1 mM $MgCl_2$, 2 mM of each dNTPs, 1.6 µm of primer (HCMR 5'- GACCGGGATGAATTGAGAGA- 3') and 1.6 mM oligo dT anchor primers (5'- GACCACGCGTATCGATGTCGACTTTTTTTTT TTTTTTTV- 3') for the first PCR. The first PCR product was gel purified and further amplified by anchor primer (5'- GACCACGCGTATCGATGTCGAC- 3') and HCMR

primer and the amplified products were cloned and sequenced as mentioned earlier. For 3' RACE, 5 µg of total RNA was reverse transcribed at 55^0C for 60 minutes in a 20 µl reaction, using HcMT1R primer by using oligo dT anchor primer and this first strand cDNA was amplified by HCMT1F and anchor primer. Amplified 3' RACE products were cloned and sequenced. In case of *HcMT2* gene, for 5' RACE, HcMT2R primer and for 3'RACE, HcMT2F primers were used. Amplified 5' RACE and 3' RACE products were cloned and sequenced.

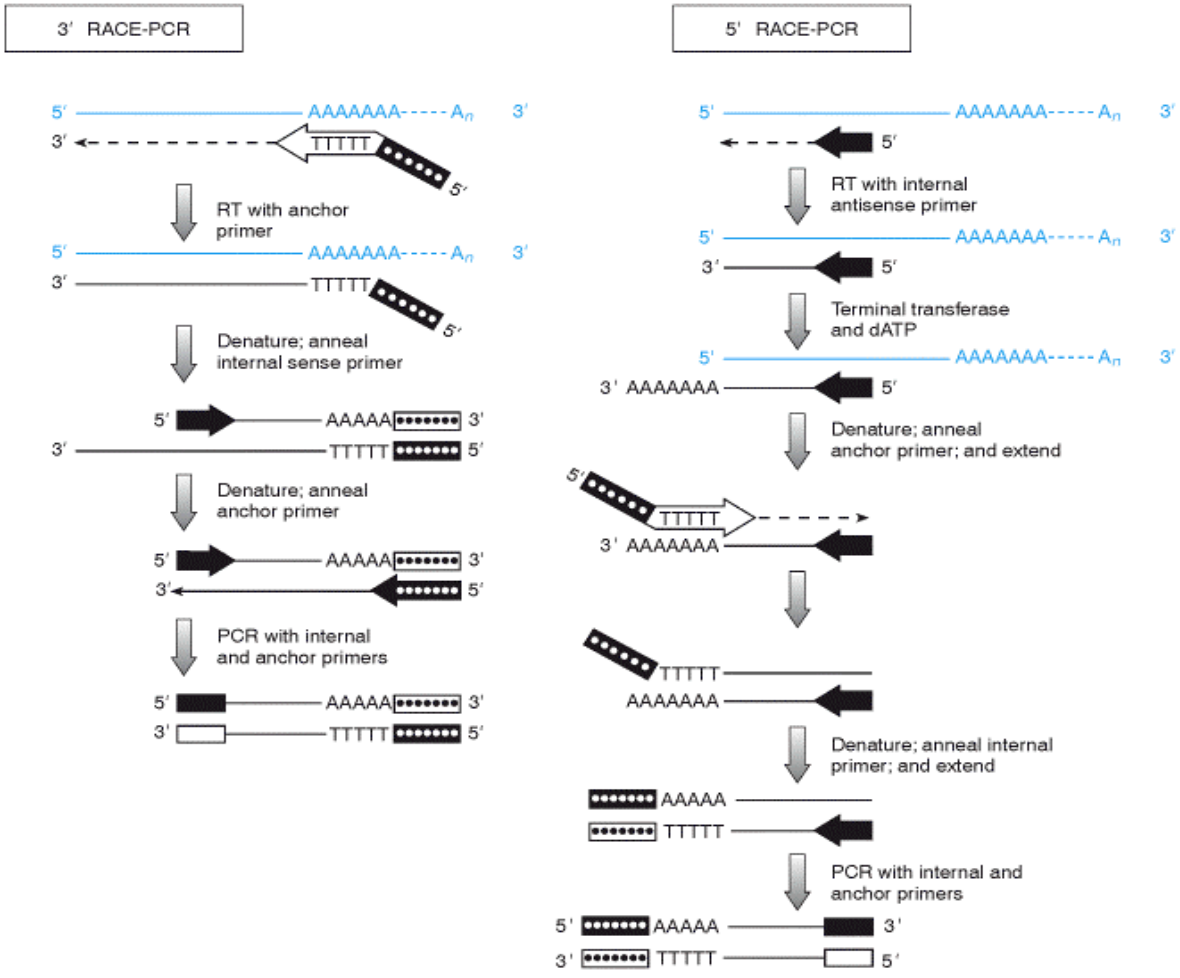


Figure 8: 5' RACE and 3' RACE facilitates the isolation of full length cDNA (Adapted form 5'/3' RACE kit manual, Roche, USA)

3.4.5 Identification of promoter regions of metallothionein gene

Universal Genome Walker Kit (Clontech Laboratories, Inc., Heidelberg, Germany) was used to determine the 5' upstream sequence (Promoter) of both metallothionein genes (Fig. 9). The genomic DNA was isolated from *H. cylindrosporum* as mentioned earlier. The quality of DNA was checked on a 0.8% (w/v) agarose gel and quantified spectrophotometrically as mentioned earlier.

3.4.5.1 Digestion of genomic DNA

The genomic DNA of *H. cylindrosporum* was digested by blunt end cutting enzymes such as *Dra* I, *Pvu* II and *Eco*R V, which were provided in the Universal Genome Walker kit.

1. Taken 3 labeled 1.5-ml tubes and added following reaction components for each reaction

   25 µl genomic DNA (0.1 µg/µl)

   8 µl restriction enzyme (10 units/µl)

   10 µl restriction enzyme buffer (10X)

   57 µl deionized H_2O
2. Mixed gently by inverting tube.
3. The samples were incubated at 37°C for 2 hours.
4. The tubes were spun down at slow speed for 5–10 sec and incubated again at 37°C overnight (16–18 hours).
5. From each reaction tube, removed 5 µl and ran on a 0.6% agarose/EtBr gel to determine complete digestion of genomic DNA.

3.4.5.2 Purification of DNA

1. To each reaction tube, 95 µl of phenol was added and the contents mixed by vortexing at slow speed for 5–10 seconds and spun briefly at room temperature to separate the aqueous and organic phases.
2. The upper aqueous layer was transferred into a new fresh tube.
3. 95 µl of chloroform was added to each tube and mixed the contents by vortexing at slow speed for 5–10 seconds and spundown briefly at room temperature to separate the aqueous and organic phases.
4. The upper aqueous layer was transferred into a fresh tube and 2 volumes (190 µl) of ice cold 95% ethanol, 1/10 volume (9.5 µl) of 3 M NaOAc (pH 4.5), and 20 µg of glycogen were added and precipitated at -20^{0}C for 2 hours.
5. DNA was pellleted by centrifugation at 14,000 g for 15 minutes at 4°C.
6. The supernatant was discarded and the pellet washed in 100 µl of ice cold 80% ethanol then centrifuged at 14,000 g for 10 minutes.
7. The supernatant was discarded and pellet was air dried.
8. Air dried pellets were dissolved in 20 µl of TE buffer.
9. One microlitre of purified digested DNA was run on a 0.6% (w/v) agarose gel to determine the approximate amount of DNA after purification.

3.4.5.3 Ligation of genomic DNA to GenomeWalker™ Adaptors

1. For each library construction, total of three ligation reactions were performed.
2. From each tube, 4 µl of digested and purified DNA was transferred to a fresh 0.5 ml tube.

To each, added the following:

1.9 µl GenomeWalker Adaptor (25 µM)

1.6 µl 10X Ligation Buffer

0.5 µl T4 DNA Ligase (6 units/µl)

3. The samples were incubated at 16°C for overnight.
4. To stop the reactions, samples were incubated at 70°C for 5 minutes and added 72 µl of TE buffer.

3.4.5.4 PCR-based DNA Walking in Genome Walker Libraries

Two PCR reactions were carried out using the AP1 (5'–GTAATACGACTCACTATAGG GC–3') and AP2 primers (5'–ACTATAGGGCACGCGTGGT–3') (Provided in the Universal Genome Walker Kit) and two metallothionein gene specific primers.

3.4.5.4.1 Primary PCR

For the primary PCR, a *HcMT1* specific primer (5'-GTGGTAATGCGTACGAGCTGTTG CAGTTGCAG-3') and a *HCMT2* specific primer (5'-CACTCGCCAGCCTTGCAGGTGCAGCTAG- 3') were designed and used in combination with the adopter specific primer AP1 to amplify large genomic segments adjacent to metallothionein genes.

1. Primary PCR reactions were carried out in a final volume of 50 µl containing 1x PCR reaction buffer, 2 mM $MgCl_2$, 200 µM of each dNTPS, 0.5 µM of AP1 primers, 0.5 µM of gene specific primers, 2.5 U of *Taq* DNA polymerase and 1 µl of adapter ligated library.

2. Briefly spun tubes in a microcentrifuge.
3. PCR was performed using the following two-step cycle parameters:
    - 7 cycles: 94°C 25 sec, 72°C 3 min
    - 32 cycles: 94°C 25 sec, 67°C 3 min
    - 67°C for an additional 7 min after the final cycle.
4. Analyzed 5 µl of the primary PCR products on a 1.5% agarose/EtBr gel along with 1 kb ladder DNA size markers.

3.4.5.4.2 Nested PCR

A nested PCR was performed with other primers for *HcMT1* (5'-TGGCAAGAGCCAGA GGAGCAGGTGCAACT-3') and *HcMT2* (5'-GTCCGAGTCGAACGATTTGTCGGCC CTCACGAC-3') designed upstream to the previous primers using adopter specific nested primer AP2.

1. 1 µl primary PCR reaction product was diluted in to 49 µl of water
2. The nested PCR was carried out in a final volume of 50 µl containing 1x PCR reaction buffer, 2 mM $MgCl_2$, 200 µM of each dNTPS, 0.5 µM of AP2 primers, 0.5 µM of gene specific primers, 2.5 U of *Taq* DNA polymerase and 1 µl of 50X diluted primary PCR product.
3. Briefly spun tubes in a microcentrifuge.
4. Performed PCR using the following two-step cycle parameters:
    - 5 cycles: 94°C 25 sec, 72°C 3 min
    - 20 cycles: 94°C 25 sec, 67°C 3 min
    - 67°C for an additional 7 min after the final cycle

5. Analyzed 5 µl of the primary PCR products on a 1.5% agarose/EtBr gel along with 1 kb ladder DNA size markers.

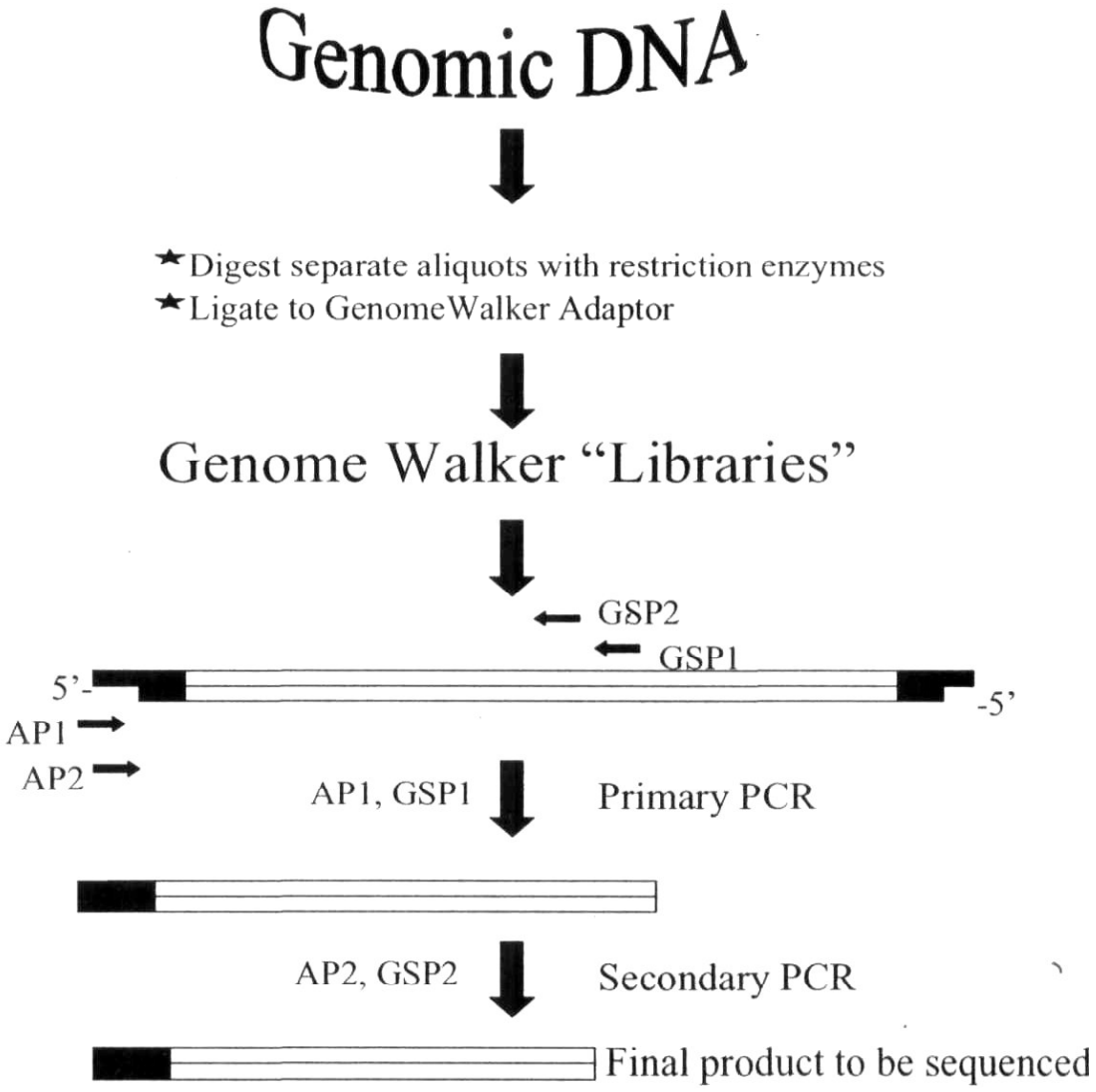


Figure 9: Flow chart of the *Genome Walker protocol*. AP1 and AP2 represent adaptor primers and GSP1 and GSP2 represent gene specific primers (Adapted form Universal Genome Walker Kit, Clonetech, Germany).

3.4.6 Cloning of metallothionein gene products

The PCR products amplified from genomic DNA and cDNA were extracted and purified from agarose gel by using QIAquick columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and cloned into the pGEM-T Easy vector system (Promega, USA). The ligated products were transformed into *E. coli* DH5α cells. For complementation studies, PCR products were cloned in to p424 vector and transformed in to yeast cells.

3.4.6.1 Transformation of DH5α by heat shock method

1. *E.coli* DH5α cells were taken from glycerol stock and streaked on Luria Agar (LA) plates and incubated at 37^0C overnight.
2. Single colonies were isolated and inoculated in Luria Broth (LB) (Appendix I) and incubated overnight at 37^0C.
3. 100 µl of the overnight grown culture was inoculated in 50 ml LB and incubated in a shaker at 180 rpm for 5 hours and then the culture was centrifuged at 7500 g for 15 minutes at 5^0C in autoclaved 30 ml tubes.
4. The supernatant was discarded and 10 ml of filter sterilized 0.1M $CaCl_2$ was added and the tubes were incubated in ice for 15 minutes.
5. The cells were again centrifuged and the supernatant was discarded and 1 ml of 0.1M $CaCl_2$ was added and the tubes were incubated in ice for 3 hours to make competent cells.

6. To 100 µl of competent cells, 5 µl of ligated product was added and mixed gently and kept in ice for 30 minutes for binding of the plasmid to the cells.
7. Then the cells were given a heat shock treatment for exactly 2 minutes at 42^0C in a still water bath.
8. The cells were rapidly transferred into ice and kept for 2-3 minutes. 1 ml of LB + Ampicillin (50 µg/ml) was added to each tube and the tubes were then kept at 37^0C for 1 hour for expression of Amp^r gene of the transformed cells.
9. 100 µl of the transformed cells were plated on LA + Amp + X-Gal + IPTG plates (Appendix I).
10. The plates were incubated at 37^0C for 16-20 hours and checked for appearance of white and blue colonies. The plates were kept in a refrigerator overnight to intensify the blue color of the colonies and differentiate between recombinants and non-recombinants.

3.4.6.2 Transformation of yeast cells by LiAc method

One copper sensitive strain DTY4 referred as *cup1*$^{\Delta}$ and one cadmium sensitive strain *yap1* mutant of *S. cerevisiae* were used for transformation of empty p424, p424-*HcMT1* and p424-*HcMT2* vector construct. The *cup1*$^{\Delta}$ containing empty p424 vector cells and *cup1*$^{\Delta}$ containing p424-*HcMT1* and p424-*HcMT2* cells were selected on SD without Trp and Ura (*cup1*$^{\Delta}$ strain selection marker) (SD-Trp-Ura medium). The *yap1* containing p424-*HcMT1* and p424-*HcMT2* cells were selected on SD medium without Trp.

1. In a test tube 1 ml of YPD medium was taken and inoculated a yeast colony picked from agar plate. The culture tubes were incubated at 30°C for 16–18 hr on shaker with shaking at 250 rpm.
2. The 50 µl yeast was transferred into a flask containing 50 ml of YPD.
3. Incubated at 30°C for 16–18 hr with shaking at 250 rpm to stationary phase (OD_{600}>1.5).
4. Transferred 30 ml of overnight culture to a flask containing 300 ml of YPD and checked the OD_{600} of the diluted culture.
5. The cells were further incubated at 30°C for 3 hours with shaking (230 rpm) and checked the OD_{600} (0.4–0.6).
6. The cells were taken in to 50-ml tubes and centrifuged at 1,000 g for 5 minutes at room temperature.
7. The supernatant was discarded and the cell pellets were resuspended in sterile distilled water and centrifuged at 1,000 g for 5 minutes at room temperature.
8. The supernatant was discarded and cell pellets were resuspended in sterile 1X TE buffer/LiAc (Appendix II). Finally the cells were pooled in one tube (final volume 25–50 ml) and centrifuged at 1,000 g for 5 minutes at room temperature. The supernatant was discarded.
9. The cells were resuspended in 250 µl of freshly prepared sterile 1X TE/LiAc
10. In a sterile prechilled 1.5 ml tube, 0.1 µg of carrier DNA (empty p424, p424-HcMT1 and p424-HcMT2 vector construct) and 3 µl of salmon sperm DNA was added and mixed gently.

11. 0.1 ml of yeast competent cells were added and mixed well by vortexing. After that 0.6 ml of sterile PEG/LiAc solution was added to each tube and vortexed at high speed for 10 seconds to mix.
12. The tubes were incubated at 30°C for 30 minutes with shaking at 200 rpm then 70 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) added and mixed well by gentle inversion.
13. The cells were given heat shock for 15 minutes at 42°C for 20 minutes.
14. The cells were transferred into ice and incubated for 1–2 minutes. The cells were centrifuged for 5 minute at 1,000 g at room temperature.
15. Supernatant was discarded and cells were resuspended in 0.5 ml of sterile 1X TE buffer.
16. 100 µl of transformed cells were plated on appropriate SD selection agar plate as mentioned earlier.
17. The plates were incubated at 30°C for 2-4 days until colonies appeared.

3.4.6.3 Plasmid isolation

3.4.6.3.1 Bacterial plasmid DNA isolation

1. The recombinant white colonies were picked up from the plates with the help of autoclaved toothpicks and inoculated in 2 ml of LB + Amp (Appendix I) and incubated overnight at 37^0C.
2. 1.5 ml of the culture was taken in a tube and centrifuged the cells and discarded the broth.

3. 100 µl of Solution 1 (Appendix II) was added and the cells were dissolved in the solution by vortexing and kept at room temperature for 5 minutes.
4. 200 µl of freshly prepared Solution 2 (Appendix II) was added to the cells and mixed the contents by inverting the tubes 10-12 times rapidly and at kept for 2 minutes.
5. 150 µl of Solution 3 (Appendix II) was added and the tubes were inverted slowly 10-12 times and kept in ice for 10-15 minutes.
6. 400 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added to the contents and inverted the tubes for 2 minutes and centrifuged at 12,000 g for 10 minutes.
7. The upper aqueous layer was transferred to a fresh tube and added 800 µl of 95% ethanol.
8. After proper vortex the tubes were kept at room temperature for 5 minutes and centrifuged at 12,000 g for 5 minutes for precipitation of DNA.
9. The supernatant was discarded and the pellet was washed with 400 µl of 70% ethanol and centrifuged 12,000 g for 5 minutes.
10. The supernatant was removed and the pellet was air-dried and dissolved in 50 µl of autoclaved MQ (Milli Q) water.

3.4.6.3.2 Yeast plasmid DNA isolation

1. The recombinant yeast colonies were picked up from the plates and inoculated in 1 ml of appropriate SD liquid medium. Then the cells were incubated at 30°C overnight with shaking at 230–250 rpm.

2. 10 µl of lyticase (5 unints/ µl) (Zymogen, USA) solution was added to tube and cells were mixed thoroughly by vortexing.
3. The cells were incubated at 37°C for 30–60 minutes with shaking at 200-250 rpm.
4. 10 µl of 20% SDS was added to the cells and vortexed vigorously for 1 minute to ensure complete lysis of the cells.
5. 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 12,000 g for 10 minutes.
6. The upper aqueous phase was transferred to a fresh tube and added 8 µl of 10 M ammonium acetate and 500 µl of 95% ethanol.
7. The tubes were incubated at –70°C for 1 hour and samples were centrifuged at 12,000 g for 10 minutes.
8. The supernatant was discarded and the pellet was air dried for 5 minutes.
9. The pellet was resuspended in 20 µl of MQ water.

3.4.6.4 Screening for positive clones

The plasmid DNAs of the recombinant bacterial cells and yeast cells were isolated to confirm the presence of the plasmid in the cells. These plasmids were used for PCR reaction by insert specific primes to know the plasmid DNA contained the insert.

3.4.7 Induction kinetic study of metallothionein genes

To study the MT induction by different heavy metals, the fungus was first grown in Melin's liquid medium for 15days. Then the mycelium was transferred to fresh medium

containing different concentrations of heavy metals. Mycelium was harvested and total RNA was isolated and cDNA was prepared as mentioned earlier. For dose responsive studies, the fungus was grown on Melin's medium with different concentrations of Cu (0, 80, 160, 240 and 320 µM) Cd (0, 7, 14, 21 and 28 µM) Zn (0, 0.15, 0.75 and 1.5 mM), Pb (0, 25, 50, 75 and 100 µM) and Ni (0, 85, 170, 250 and 350µM) for 24 hours. To study the optimum time for maximum induction of MT, the fungus was grown in Melin's medium with 320 µM of Cu and 21 µM of Cd concentration for different time intervals 0, 12, 24, 36, 48, 60 and 72 hours. Oxidative stress treatment was imposed by incubating the culture supplemented with 25mM H_2O_2 and harvesting the mycelium at different time intervals 0, 12, 24, 36, 48, 60 and 72 hours.

3.4.8 Quantification of RT-PCR

Reverse transcription, coupled with PCR, permits the detection of RNA transcripts of any gene, which is independent of the amount of the starting material or the relative abundance of the specific mRNA. In RT-PCR, an RNA template is copied into cDNA using reverse transcriptase. The cDNA sequence of interest is then amplified exponentially using PCR; RT-PCR is the most sensitive method for mRNA detection and can used for relative or absolute quantification. the reliability of quantitative PCR is governed by many factors including annealing efficiency of the primer to the target sequence, type of standards used and time when data is collected (e.g. during log phase), etc.

Use of internal and external standards in RT-PCR for quantification of specific mRNA transcript level.

a) Relative quantification using internal standards

Variation in mRNA isolation and analysis procedure can often lead to errors in to the analysis process. One of the foremost methods to minimize such errors is the use of internal standards, against which other RNA values can be normalized for relative quantification. The size of the reaction product should be significantly different from the exponential product in order for them to be easily resolved during electrophoresis but not so different that an incomplete extension of one product over the other changes the initial ratio of standard to target. Amplification of both molecules is performed with the same set of primers.

One of the main benefits of using internal standards, with the same amplimer binding sequence as the experimental target is that it is not necessary to obtain data through out the exponential phase of the reaction because the initial ratio of target to standard (competitor) remains the same even if the reaction reaches a plateau.

b) Absolute quantification using external standards

In absolute RT-PCR, accurate and reliable quantification of mRNA expression can be accomplished with an external standard using a competitive strategy. An external standard should be as close as possible to the experimental target DNA in terms of size and base composition. It should preferably utilize amplifiers of similar size and annealing

temperature to the experimental template. When using such standards it is very important that the PCR parameters are optimal for both reactions because small variations in amplification efficiency can result in considerable change in product yield.

Competitive RT-PCR, first described by Becker- Andre and Hahlbrock (1989); Gilland *et al.,* (1990), involves co-amplification of the target DNA and the internal standard or competitive DNA, which is similar to, but distinguishable in size or restriction sites from the target (Fig. 10). Competitor DNA contains same set of primers binding site and compete with the target templates during the amplification reactions. Estimation of the number of the target sequence is achieved by comparison of ratios between target and competitor sequence with those of the standard curve generated by the amplification of competitor DNA with the range of target DNA concentrations. A drawback of this method is that it requires substantial optimization. Also due to sensitivity of this technique it is required that samples be completely free of genomic DNA.

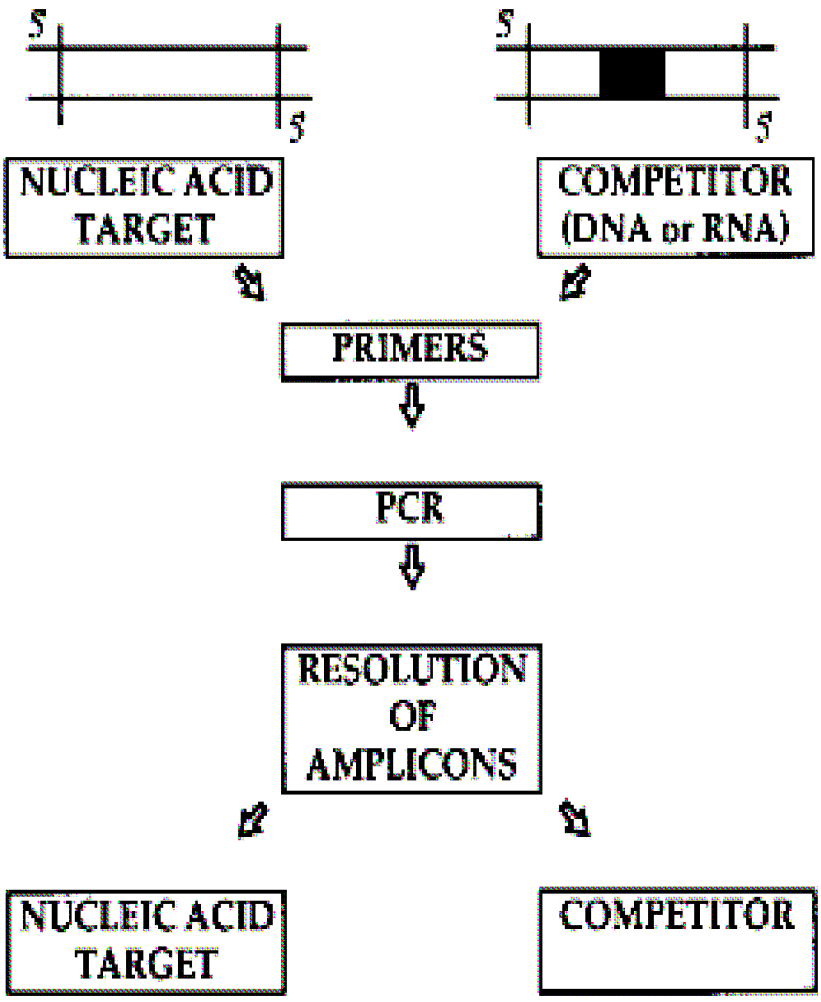


Figure 10: General principle of competitive PCR. The nucleic acid target (DNA or RNA) and the competitor compete for the primers during annealing and PCR amplification. After PCR the two species are separated by an analytical procedure and the ratio between target and competitor is evaluated. Their final ratio reflects the ratio between template and competitor in the initial sample.

3.4.8.1 Development of the competitor to study the expression levels of *HcMT1* and *HcMT2* gene by competitive RT-PCR

Genomic DNA of *H. cylindrosporum* was used as competitor for this technique. The genomic DNA was amplified by using HcMT1F and 1R and HcMT2F and 2R primer pairs and these products were cloned into pGEM-T Easy vector. The plasmid DNA containing cloned competitor was quantified spectophotometrically. In a PCR reaction containing a RT-cDNA sample and a known amount of plasmid-cloned competitor. The *HcMT1* primer pair amplifies simultaneously a 295 bp-long cDNA fragment and a 490 bp-long competitor; however, *HcMT2* primer pairs amplify a 214 bp long cDNA and a 295 bp long competitor. Owing to this size differences, both amplified sequences were easily separated by 1.5% agarose gels and their relative amounts were quantified using the Gel Doc system (UltraLum, Inc., USA). PCR reactions were performed in a final volume of 25 µl using 1 µl of RT-cDNA, 0.2 µM of each primers, 1 mM $MgCl_2$, 100 µM dNTPs, 1.25 U *Taq* polymerase and 1x reaction buffer plus an appropriate amount of competitor.

Amplification conditions consisted of 3 min at 94^0C, 25 cycles of 1 min at 94^0C, 1 min at 55^0C and 1 min at 72^0C followed by 5 min at 72^0C. Preliminary experiments showed that at 25 cycles, the PCR reaction was still in an exponential phase. A standard curve was constructed by co-amplifying different known concentrations of target DNA with a constant amount of competitor. A standard curve was obtained by plotting the log values of the amplification ratios of target DNA/ competitor against the log values of the target DNA (pg of target DNA) added to the PCR mix before amplification.

3.4.9 Hybridization of DNA by Southern blotting

3.4.9.1 Single labeling of probes with α ^{32}P dCTP by PCR

Plasmid containing cDNA fragments were used for the preparation of radioactive probes. The plasmids were amplified for the desired cDNA fragment using gene specific primers and fragment was checked in agarose gel electrophoresis. The PCR fragments were purified and radiolabelled with α ^{32}P dCTP by PCR. PCR reactions were carried out in a final volume of 100 µl containing 1x advantage Taq buffer, 200 µM of dGTP, dATP, dTTP and 20 µM cold dCTPS, 1 µM of gene specific forward and reverse primer, 1 µl purified PCR fragment, 5 U of Advantage *Taq* polymerase (Clonetech, Germany) and α ^{32}P dCTP. The PCR program was carried as follows: initial denaturation at 95^{0}C for 2 minutes, followed by 30 cycles 15 seconds at 95^{0}C, 30 seconds at 55^{0}C of annealing temperature, 2 minutes at 72^{0}C and final extension at 72^{0}C for 5 minutes. The radioactive labeled probes were purified using a Qiagen Nucleotide removal Kit. Probes were denatured at 100^{0}C before use.

3.4.9.2 Electrophoresis and Transfer of DNA

1. 30 µg of genomic DNA was digested with *Eco*R 1, *Bam*H 1, *Xho I* and *Pst I*
2. Digested samples were separated on a 0.7% (w/v) agarose gels.
3. The gels were rinsed with double distilled water and the nylon membrane was prewet with double distilled water.
4. Capillary blotting was setup and DNA was transferred on to a nylon membrane following partial hydrolysis in 0.2 N HCl (20 minutes) and denaturation in 0.4 M NaOH (30 minutes).

5. The transfer was performed in 0.4 M NaOH overnight.
6. After overnight transfer, membrane was rinsed in a solution of 2x SSC (Appendix II) and 0.5% (w/v) SDS, the membrane was dried.
7. Immobilized the DNA onto the membrane by U.V. crosslinking for 2 minutes.

3.4.9.3 Hybridization

1. Placed the membrane in a hybridization tube and prehybridized in 50 ml of hybridization solution (Appendix II) for 4 hours at 65^0C in a hybridization oven.
2. Prehybridization solution was discarded from the tube and added another 50 ml of hybridization solution.
3. Prepared the probe using PCR by single labeling with radiolabelled dCTP. The probe was denatured by boiling 10 minutes.
4. After that the probe was added to the blot and hybridized overnight at 65^0C.
5. Removed the probe and hybridization solution from the tube and blot was rinsed with washing solution I (Appendix II) once at room temperature.
6. The membrane was washed again twice with 50-100 ml of washing solution I for 10 min and once with washing solution II (Appendix II) for 10 minutes at 65^0C.
7. Then the membrane was sealed in Saran wrap and membranes were exposed to phosphorimage screen
8. The hybridization and quantification of hybridization signals was done by using Bio Rad phosphorimage system (Bio Rad, Foster City, CA, USA)

3.4.10 Sequencing and Sequence analysis

The inserts in the plasmids were sequenced by chain termination method (Sanger *et al.,* 1977) using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of biochemistry, South campus, Delhi university, New Delhi, India).

Comparison of clone sequences was performed using the BLAST program (Altschul *et al.,* 1997) to reported nucleotide and protein sequences in the database Gen Bank accessible through NCBI (National Centre for Biotechnology Information – http://www.ncbi.nlm.nih.gov). The sequenced genomic and cDNA sequences were submitted to Genbank. Multiple alignments were performed using *Multalin* program.

3.4.11 Yeast functional complementation assays

The ORF of *HcMT1* and *HcMT2* sequences were amplified using MT1F (5'-CGGG ATCCATGCAATTCACTTCTATCCTCGTC-3') and MT1R (5'-CCGGAATTCTCA GTTGCAGTTGCAGTTGTTGG-3') and MT2F (5'- CGGGATCCATGCAGATCGTT CAAAACAGTCTCG-3') and MT2R (5'-CCGGAATTCTTAGCATTTGCACTCGC CAGCC-3') primers introducing *Bam*HI and E*co*RI sites (sites underlined). PCR reactions were carried out in a final volume of 50 µl containing 1x reaction buffer, 2 mM $MgCl_2$, 200 µM of each dNTPS, 0.5 µM of each primer, 1 µl of cDNA and 5 U of Advantage *Taq* DNA polymerase (Clonetech, Germany). The PCR program was carried as follows: initial denaturation at 95^0C for 5 minutes, followed by 30 cycles 1 minute at 92^0C, 1 minute at 67^0C of annealing temperature, 1 minute at 72^0C and final extension at 72^0C for 5 minutes. Amplification products were visualized on 1.8% (w/v) agarose gels.

The amplified PCR products were digested with *Bam*H I and *Eco*R I restriction enzymes and digested PCR products were purified using Qiagen PCR purification kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The p424 shuttle vector was isolated from DH5α cells by an alkali lysis method as mentioned earlier. The plasmid DNA was digested with the same restriction enzymes (*Bam*H I and *Eco*R I), which were used to digest PCR products. The digested plasmids were gel purified using QIAquick columns (Qiagen, Valencia, CA, USA) according to manufactures instructions. The PCR products were ligated in yeast expression vector p424 (Mumberg *et al.,* 1995) under the transcriptional control of the yeast GPD (glyceraldehyde 3-phosphate dehydrogenase) promoter. The p424 vector also contains the CYC1 (cytochrome *c* oxidase) terminator, the 2μ replication origin and the TRP1 tryptophan marker (Fig. 11). Empty vector p424 and the construct p424-HcMT1 and p424-HcMT2 were introduced into *cup1*$^{\Delta}$ and *yap1* cells using a lithium acetate procedure as described earlier and transformed cells were selected by their capacity to grow in complete synthetic medium (SD), without Trp (p424 vector selection marker) and Ura (*cup1*$^{\Delta}$ strain selection marker) (SD-Trp-Ura medium) and SD medium without Trp for *yap1* mutant.

For functional complementation experiments, cultures of *cup1*$^{\Delta}$ and *yap1* yeast cells carrying either p424 or p424-HcMT1 and p424-HcMT2 were grown in each respective medium at 30^{o}C and 220 rpm. Yeast cultures were adjusted to OD_{600} =1.0 and 5 μl serial dilutions were spotted on SD plates and on SD supplemented with 150μM $CuSO_4$ and 40μM $CdSO_4$ plates. Plates were incubated for 3d at 30^{o}C and photographed. In parallel experiments, Falcon jars containing 20 ml of fresh SD-Trp-Ura and SD-Ura media were inoculated with mid-log precultures of *cup1*$^{\Delta}$ and *yap1* cells containing p424, p424-

HcMT1 and p424-HcMT2 to attain a starting optical density of 0.02 at 600nm. Cells were grown at 30^{o}C and 220 rpm and $CuSO_4$ (150µM) and $CdSO_4$ (40µM) were added 5 hours after inoculation. The optical densities of the cultures were measured at 2-3 hours interval for 42 hours.

3.4.12 Statistical analysis

All the data obtained were subjected to analysis of variance and the significant differences among the means were compared with Tukey's Test $p<0.05$.

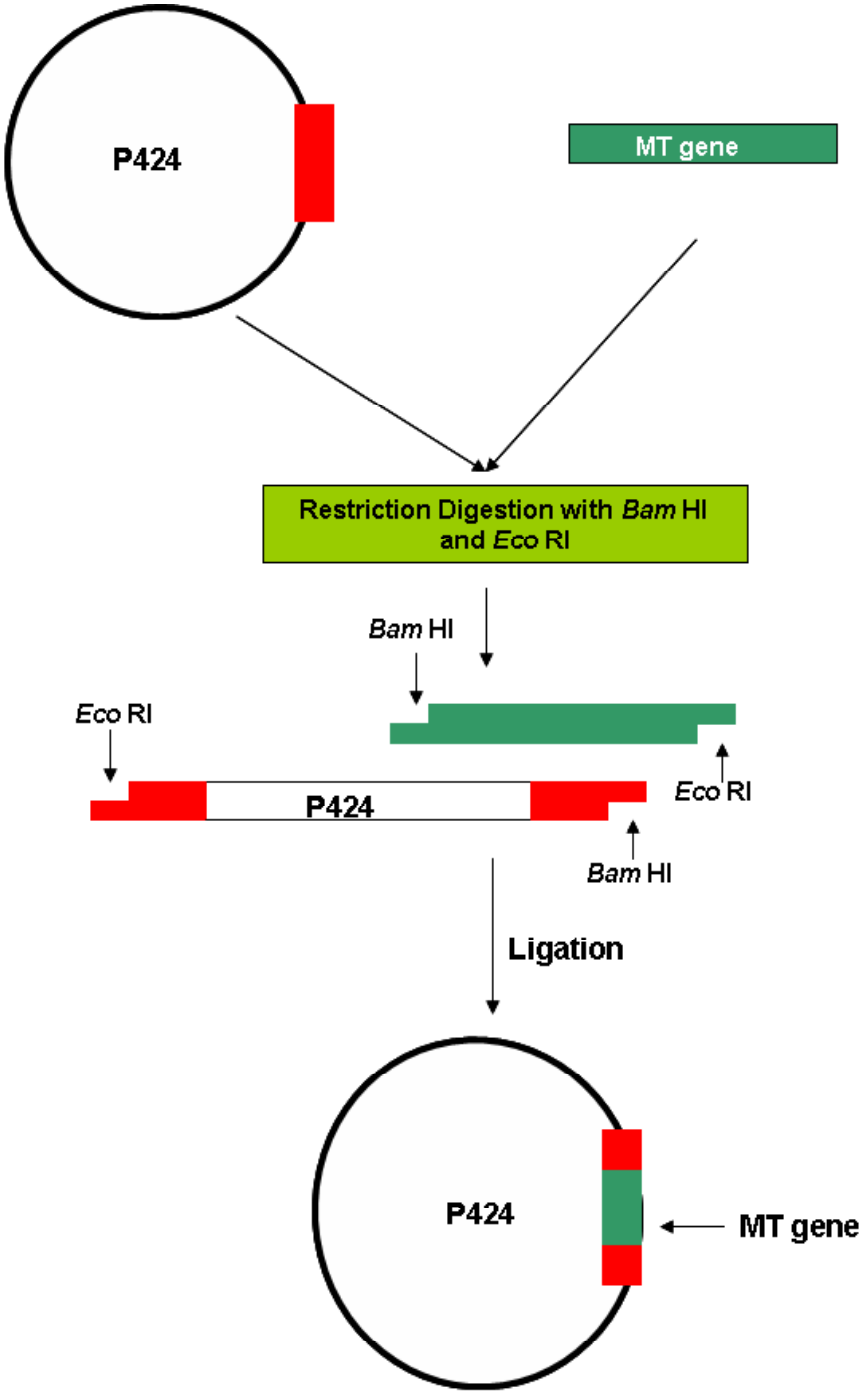


Figure 11: Flow chart for the construction of p424-MT vector. The MT genes and p424 vector were digested with *Bam H1* and *Eco R1* and both were ligated.

Results

4.1 Screening of *Hebeloma cylindrosporum* for their tolerance to different heavy metals

Axenic screening of ectomycorrhizal fungi for their tolerance to different metals is prerequisite for genetic analysis of metal tolerance as this provides the threshold toxic levels of fungi as well as physiological response. The response of *H. cylindrosporum* to various concentrations of copper, cadmium, zinc, nickel and lead was assessed by growing pure mycelial cultures in liquid MMN media and the biomass was harvested after 21 days of incubation and dry weight was recorded. The assessment of fungal biomass used as measurement of growth.

The growth of *H. cylindrosporum* was adversely affected with increasing concentrations of metals. Increasing concentration of copper in medium significantly decreased the growth of fungus. The fungus showed higher tolerance until 160 of µM copper and thereafter growth was significantly decreased. The growth was inhibited 50% at 160 µM of copper treatment and the growth was almost inhibited at 320 µM of copper, which could be toxic concentration to fungus, when compared with control (Table. 3; Fig. 12). In response to nickel, the growth of *H. cylindrosporum* was decreased with increasing concentration of nickel and 50% of growth reduction was recorded at 85 µM of nickel treatment. The growth was significantly decreased at 170

µM and completely inhibited at 250 µM of nickel treatment (Table. 4; Fig. 13). The *H. cylindrosporum* was very sensitive to cadmium as their 50% growth inhibition was recorded at cadmium lower concentration (21 µM of cadmium). Increasing concentration of cadmium was associated with significant decrease of growth. The growth was significantly affected in all concentrations of cadmium (Table. 5; Fig. 14). The growth of *H. cylindrosporum* decreased as function of lead concentration in the growth medium and 50% growth reduction was observed at 50 µM lead. The *H. cylindrosporum* showed significant reduction in the growth with increasing concentration of lead with respect to control (Table. 6; Fig. 15). The *H. cylindrosporum* showed higher tolerance to zinc and there was less growth reduction observed till 2 mM of zinc treatment with respect to control. Maximum 30% growth reduction was observed at 2 mM of zinc treatment (Table. 7; Fig. 16). Among all metals tested, *H. cylindrosporum* showed higher tolerance to zinc, copper and nickel respectively as zinc, copper and nickel are trace elements, which are required for fungal growth and *H. cylindrosporum* found to be more sensitive to cadmium and lead as these metals are nonessential toxic metals to fungi. These results indicated that *H. cylindrosporum* showed large variation in metal tolerance to all metals tested and confers that the fungus is more tolerant to one or more types of metals not to other metals.

Table 3: Effect of different copper concentrations on the mycelial growth of *H. cylindrosporum*

Copper concentration (µM)	Dry weight (mg/50ml)
0	50.6±3.2a
80	38.0±2.0b
160	23.0±2.0c
240	17.3±2.0d
320	8.3±1.5e

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

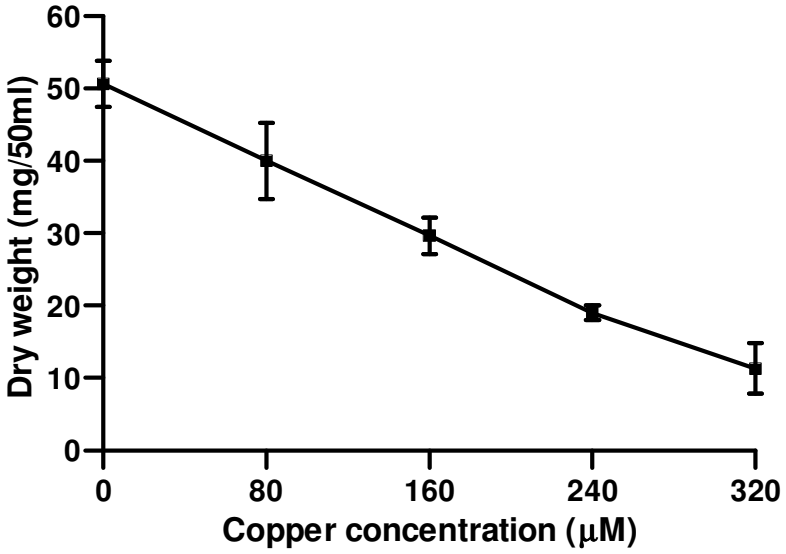


Figure 12: Influence of copper concentrations in the nutrient solution on the mycelial growth (mg/50ml) of *H. cylindrosporum.*

Table 4: Effect of different nickel concentrations on the mycelial growth of *H. cylindrosporum*

Nickel concentration (µM)	Dry weight (mg/50ml)
0	40.6±2.0a
85	22.6±2.5b
170	13.6±1.5c
250	3.6±1.5d
350	3.0±1.0d

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

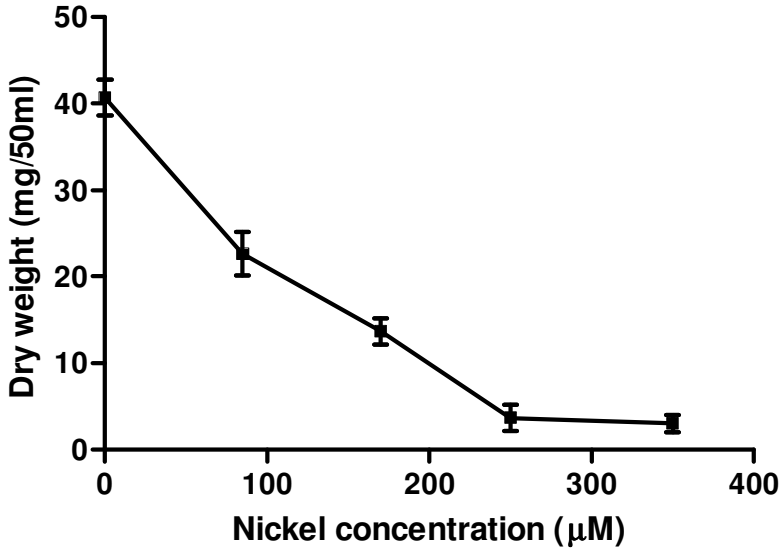


Figure 13: Influence of nickel concentrations in the nutrient solution on the mycelial growth (mg/50ml) of *H. cylindrosporum.*

Table 5: Effect of different cadmium concentrations on the mycelial growth of *H. cylindrosporum*

Cadmium concentration (µM)	Dry weight (mg/50ml)
0	51.3±1.7a
7	34.2±0.9b
14	29.4±0.4b
21	22.0±0.8c
28	15.6±2.0c

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

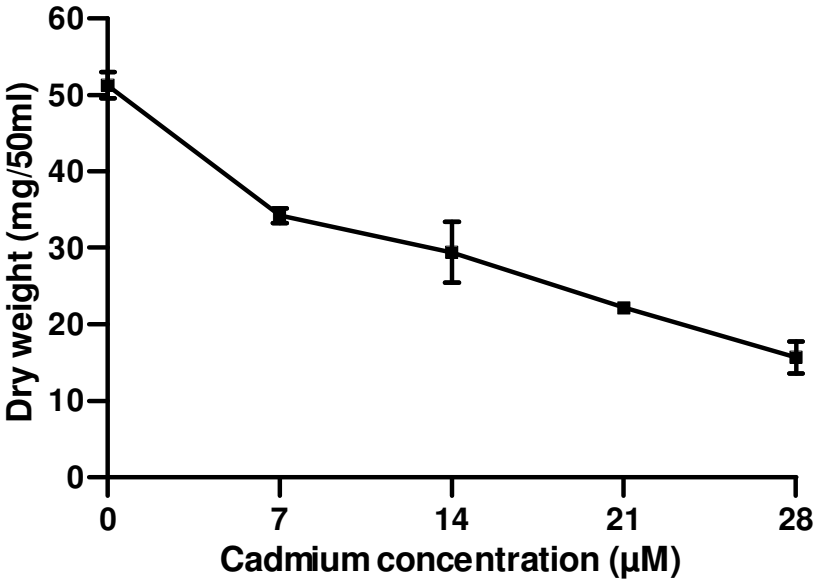


Figure 14: Influence of cadmium concentrations in the nutrient solution on the mycelial growth (mg/ 50ml) of *H. cylindrosporum.*

Table 6: Effect of different lead concentrations on the mycelial growth of *H. cylindrosporum*

Lead concentration (µM)	Dry weight (mg/50ml)
0	35.3±2.5a
25	30.0±2.0b
50	14.6±1.5c
75	8.3±1.5d
100	4.3±0.5d

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

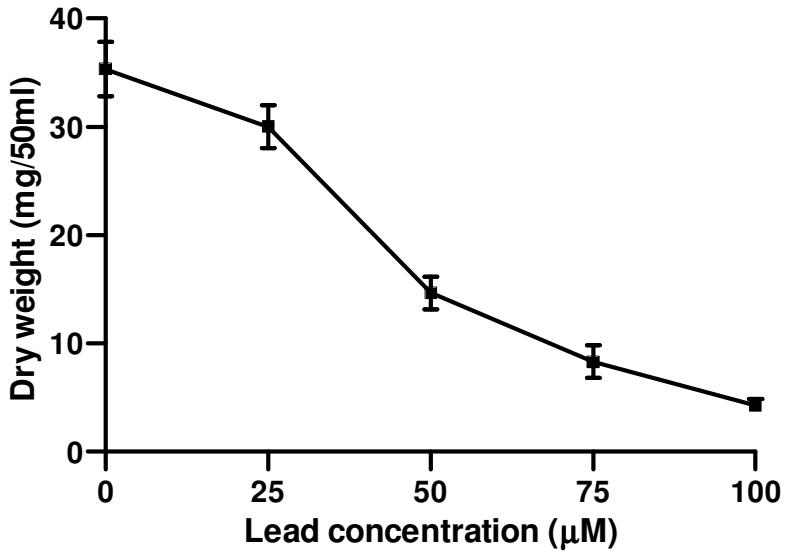


Figure 15: Influence of lead concentrations in the nutrient solution on the mycelial growth (mg/50ml) of *H. cylindrosporum.*

Table 7: Effect of different zinc concentrations on the mycelial growth of *H. cylindrosporum*

Zinc concentration (mM)	Dry weight (mg/50ml)
0.0	47.0±2.0a
0.5	40.3±1.5b
1.0	35.6±3.0bc
1.5	31.0±1.0cd
2.0	27.3±2.0d

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

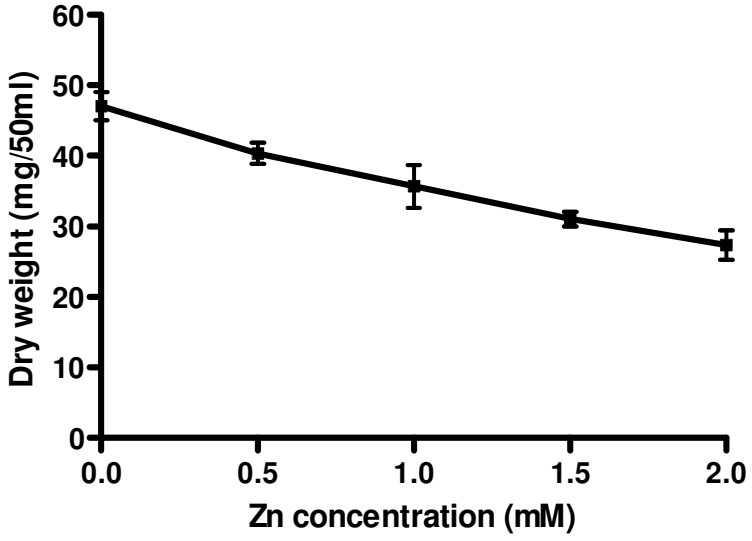


Figure 16: Influence of zinc concentrations in the nutrient solution on the mycelial growth (mg/50ml) of *H. cylindrosporum.*

4.2 Identification of metallothionein genes in *H. cylindrosporum*

Lambiliotte et al. (2004) constructed the EST library of *H. cylindrosporum* under potassium and phosphate free stress. Out of ca. 4500 ESTs (corresponding to ca. 3500 unigenes) of *H. cylindrosporum*, we identified six metallothionein like ESTs, which corresponding to the two different metallothionein like genes. Two partial cDNAs encoding metallothionein (MT) like polypeptides were designated as *HcMT1* (Accession numbers: CK992169) and *HcMT2* (Accession numbers: CK995302). We have designed two sets of primers (HcMT 1F and 1R, HcMT 2F and 2R) and used for the amplification of metallothionein genes from the genomic DNA and cDNAs of *H. cylindrosporum.*

4.2.1 DNA isolation and PCR

Genomic DNA was isolated from the *H. cylindrosporum* by the method of Vankan et al. (1991). Hundred nanograms of genomic DNA was used as template in PCR for the amplification of metallothionein genes by using HcMT 1F and 1R and HcMT 2F and 2R primers pair. The resultant PCR products were separated on a 1.5% (w/v) agarose gels and visualized by ethidium bromide staining under U.V light. The results showed that HcMT1F and HcMT1R primers amplified the 490 bp long *HcMT1* fragment of genomic DNA (Fig. 17). In case of HcMT2F and HcMT2R primers, 295 bp long *HcMT2* fragment of genomic DNA was obtained (Fig. 18).

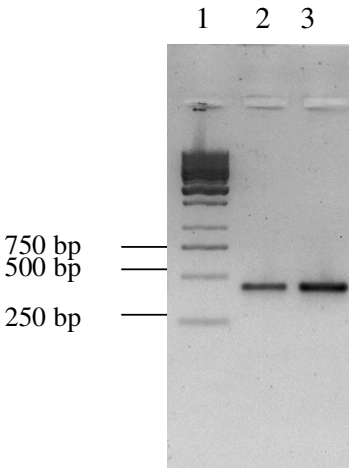


Figure 17: Amplification of genomic DNA of *H. cylindrosporum* with HcMT1F and 1R primers Lane 1: 1kb ladder, Lane 2 and 3: *H. cylindrosporum* genomic DNA amplified with HcMT1 primers.

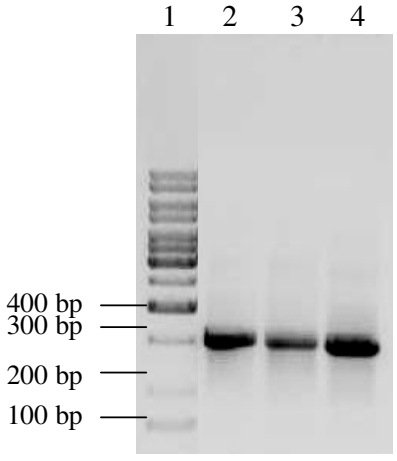


Figure 18: Amplification of genomic DNA of *H. cylindrosporum* with HcMT2 F and 1R primers Lane 1: 100 bp ladder, Lane 2,3 and 4: *H. cylindrosporum* genomic DNA amplified with HcMT2 primers.

4.2.1 RNA isolation, cDNA preparation by RT-PCR and PCR

To induce the metallothionein genes, *H. cylindrosporum* was first grown in MMN liquid medium for two weeks. Then the mycelium was transformed to fresh medium containing 160 µM copper and incubated for 24 hours. Total RNA was isolated from metal treated mycelium using the TRIzol method (Fig. 19). First strand cDNA was synthesized from 5 µg total RNA by RT-PCR method. To check the quality of cDNA preparation, 1 µl of reverse transcribed product was amplified with HcTub 1U and HcTub 1L (Tubulin gene) primers for amplification of Tubulin (constitute gene) and got an amplicon of 380 bp (Fig. 20). These cDNA were used for the amplification of metallothionein genes with HcMT 1F and 1R and HcMT 2F and 2R primers pair and got an amplicon of 295 bp with HcMT 1F and 1R primers pair (Fig. 21) and 210bp with HcMT 2F and 2R primers pair (Fig. 22).

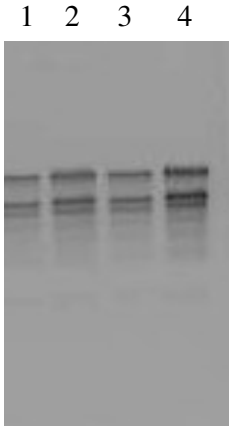


Figure 19: Total RNA isolation from metal treated *H. cylindrosporum*. Lane 1,2,3 and 4: Total RNA

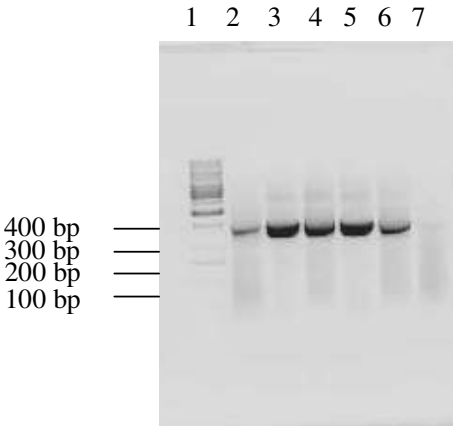


Figure 20: Amplification of cDNA of *H. cylindrosporum* with HcTub 1U and HcTub 1L primers Lane 1: 100 bp ladder, Lane 2,3,4,5 and 6: *H. cylindrosporum* cDNA amplified with Tubulin primers. Lane 7: Negative control

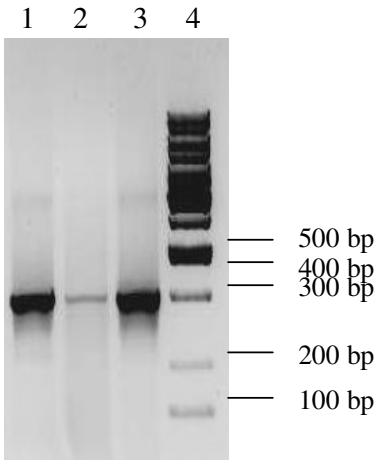


Figure 21: Amplification of cDNA of *H. cylindrosporum* with HcMT1F and 1R primers, Lane 1,2 and 3: *H. cylindrosporum* cDNA amplified with HcMT1 primer pair, Lane 4:100 bp ladder

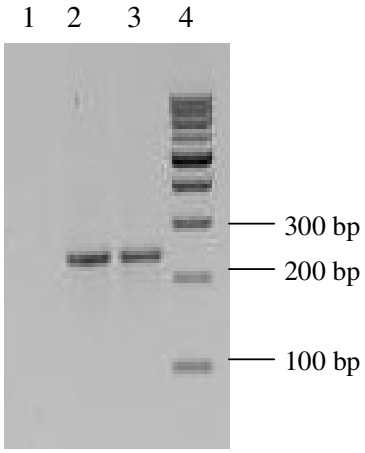


Figure 22: Amplification of cDNA of *H. cylindrosporum* with HcMT2F and 2R primers, Lane 1: negative control, Lane 2 and 3: *H. cylindrosporum* cDNA amplified with HcMT2 primers, Lane 4: 100 bp ladder

4.3 Cloning and sequencing of metallothionein genes

The PCR products amplified from genomic DNA and cDNA were excised and purified from agarose gel electrophoresis using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA) and cloned into pGEM- T Easy vector (Promega, USA). These ligated products were transformed into DH5α *E. coli* cells by a heat shock method. The positive clones were screened by α- complementation and the plasmid DNAs of the recombinant cells were isolated using the alkali lysis method (Fig. 23). Plasmid containing *HcMT1* and *HcMT2* inserts were confirmed by PCR with insert specific primers (Fig. 24a and 24b). The inserts in the plasmids were sequenced by chain termination method (Sanger *et al.,* 1977) using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South campus, Delhi university, New Delhi, India).

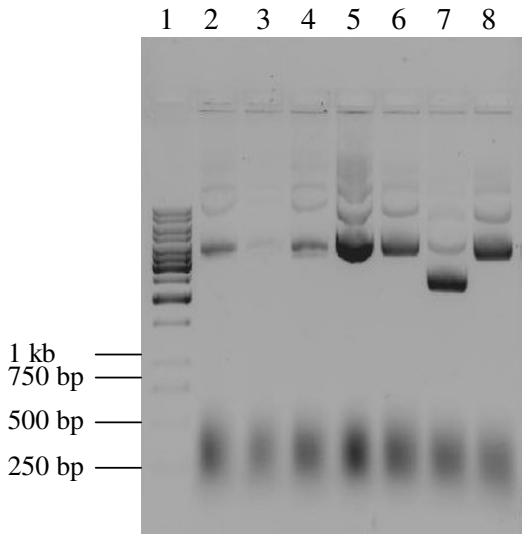


Figure 23: Plasmid DNA was isolation from DH5α *E. coli* using alkali lysis method. Lane 1: 1 kb Marker, Lane: 2,3,4 and 5: pGEM-T Easy vector containing *HcMT1* genomic DNA, Lane: 6,7 and 8: pGEM-T Easy vector containing *HcMT2* genomic DNA

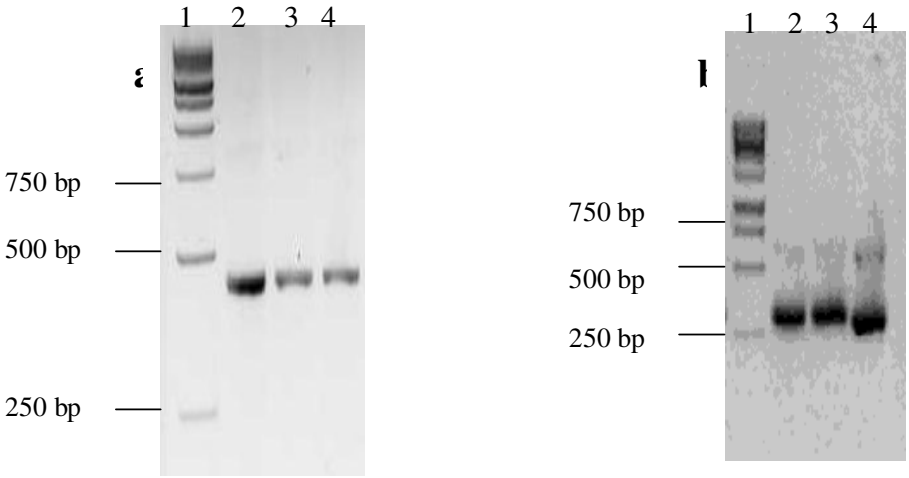


Figure 24: a) Amplification of plasmid DNA containing *HcMT1* insert with HcMT 1F and 1R primers, Lane 1: 1 kb ladder, Lane 2,3 and 4 : Amplified insert with HcMT1 primers. b) Amplification of plasmid DNA containing *HcMT2* insert with HcMT 2F and 2R primers, Lane 1: 1 kb ladder, Lane 2,3 and 4 : Amplified insert with HcMT 2 primers

4.4 Characterization of metallothionein genes

4.4.1 Rapid amplification of cDNA ends (RACE PCR)

To obtain corresponding full length cDNAs of *HcMT1* and *HcMT2* genes, RACE PCR was performed to isolate the 5' and 3' ends of cDNA according to the protocol provided by the manufacturer's (5'/3' RACE kit, Roche, USA). For 5'RACE of *HcMT1*, 5 µg of total RNA was reverse transcribed at 55^0C for 60 min. in a 20 µl reaction, using HcMT1R primer (gene specific) and first strand cDNA was further purified. Homopolymeric (oligo dA) tailed cDNA was synthesized according to manufacture's instructions. 5 µl of oligo dA tailed cDNA was amplified using HcMT1 R primer and oligo dT anchor primers pair for the first PCR and obtained 365 bp fragment. The first PCR product was further gel purified and used as a template for nested PCR using anchor primer and HCMR primer. A fragment of 262 bp was obtained in nested PCR (Fig. 25). The 262 bp of *HcMT1* 5' RACE product was cloned and sequenced. For 3' RACE of *HcMT1*, 5 µg of total RNA was transcribed by using oligo dT anchor primer and these first strand cDNAs were amplified by HcMT1F and anchor primer. The results showed a 390 bp long fragment of 3' RACE products obtained (Fig. 26). These products were cloned and sequenced as mentioned earlier. The 5' and 3' RACE sequences of *HcMT1* were aligned and overlapping sequences were removed. A total of 459 bp long full length *HcMT1* cDNA obtained by RACE PCR.

In case of *HcMT2* gene, for 5' RACE, the first strand cDNA was synthesized using 5 µg of total RNA by using HcMT2 R primer. These cDNAs were purified and homopolymeric (oligo dA) tailed cDNA was performed according to manufacturer's

instructions. 5 µl of homopolymeric tailed cDNA was amplified using a HcMT2 R primer and oligo dT anchor primers pair to get an amplicon of 385 bp long fragment (Fig. 27). The amplified PCR product was cloned and and sequenced. For 3' RACE, 5 µg of total RNA was transcribed by using oligo dT anchor primer and these cDNAs were amplified by HcMT2 F along with an anchor primer. A fragment of 272 bp product was obtained (Fig. 28). These products were cloned and sequenced as mentioned previously. The sequence alignment of 5' and 3' RACE sequences revealed 434 bp long full length *HcMT2* cDNA.

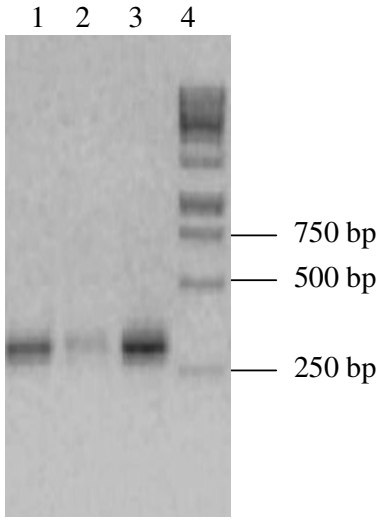


Figure 25: Nested PCR of 5' RACE of primary PCR of *HcMT1* product with anchor primer and HCMR primer. Lane 1,2 and 3: nested PCR product of *HcMT1* gene, Lane 4: 1 kb ladder

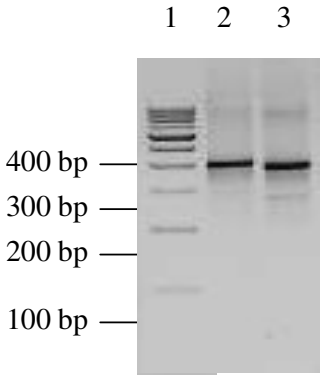


Figure 26: Amplification of 3' RACE of *HcMT1* product with anchor primer and a HcMT1 F primer. Lane 1: 100 bp ladder, Lane 2 and 3: 3' RACE PCR product of *HcMT1* gene

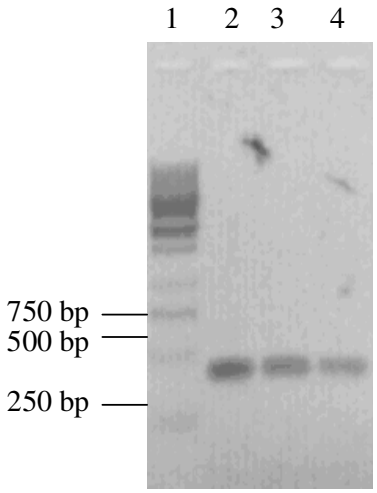


Figure 27: 5' RACE of *HcMT2* with anchor primer and a HcMT2R primer. Lane 1: 1 kb ladder, Lane2,3 and 4: 5' RACE PCR product of *HcMT2* gene

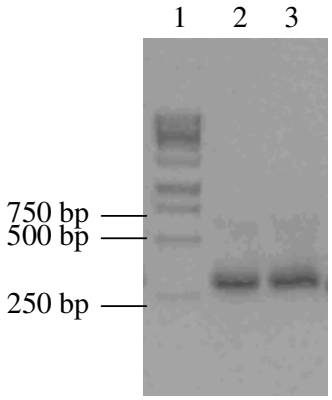


Figure 28: Amplification of 3' RACE of *HcMT2* product with anchor primer and HcMT2R primer. Lane 1: 1 kb ladder, Lane 2 and 3: 3' RACE PCR product of *HcMT2* gene

4.4.2 Sequence analysis of *HcMT1* and *HcMT2*

Full length cDNAs of *HcMT1* and *HcMT2* were obtained by RACE-PCR. Sequence analysis was performed with BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.,* 1997) using the nucleic acid and predicted amino acid sequences deposited in multiple data bases. The BLAST results showed that the *HcMT1* and *HcMT2* gene were showed similarity with other known fungal MTs such as *A. bisporus MT1, A. bisporus MT2, G. lucidum MT, Magnaporthe grisea MT* and mycorrhizal fungi *P. involutus MT and G. margarita MT* (Table 8, 9). The ORFs of *HcMT1* and *HcMT2* were searched using the ORF finder program through NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi).The physicochemical properties of the deduced amino acids of *HcMT1* and *HcMT2* were analyzed from the Expasy molecular biology site (http://www.expasy.org/cgi-bin/pi_tool). The *HcMT1* cDNA contains a 177 bp ORF encoding 59 amino acids (Fig. 29) with a predicted molecular mass of 5.99 KDa and PI of 4.22. The *HcMT1* cDNA possessed 5' and 3' UTRs of 41

bp and 238 bp respectively. The 3' UTR contained a polyadenylation signal with the sequence of AATAA upstream of poly A tail. Other characteristics of *HcMT1*, besides its small size, are the presence of 13 Cys residues (representing 22 % of the total amino acid content) with only one aromatic residue (Phe-3). Most of the Cys residues are part of the C-x-C (where x is any amino acid other than Cys) motif which is supposed to play a role in metal binding. *HcMT1* contains two C-x-C motifs at N-terminal part and three at their C-terminal portion as found in other fungal MTs and also some plant MTs.

HcMT2 cDNA has an ORF of 174 base pairs encoding a polypeptide containing 57 amino acids (Fig. 30) with a calculated molecular mass of 5.61 KDa and an isoelectric point of 7.95. The *HcMT2* sequence contains 22.8% of cystein with no aromatic residue and six C-x-C motifs. *HcMT2* contains 3 C-x-C motifs at N- terminal and 3 C-x-C at C-terminal part. *HcMT2* cDNA contained 43 bp of 5' UTR and 157 bp of 3' UTR. The deduced amino acid sequences and ORF nucleotide sequence of *HcMT1* and *HcMT2* were aligned pairwise by using *Multalin* program (http://www.toulouse.inra.fr/multalin.html) to see the similarity between them. The analysis showed that both the MTs differ in their size and the similarity in open reading frame of both MTs was 40% (Fig. 31) and *HcMT1* and *HcMT2* were 31% identical to each other (Fig. 32). The sequences were deposited in the GenBank of NCBI data library under accession numbers EU049884 and EU049885 for *HcMT1* and *HcMT2* respectively.

4.4.3 Putative amino acid sequence comparison

The alignment of deduced amino acid sequences of *HcMT1* and *HcMT2* with other known fungal MTs showed that fungal MTs bear the C-x-C-x(2,3)-C signature at the N-terminal part together with a conserved Cys residue at the C-terminal end (Fig. 33). This consensus sequence is far more restricted than the C-G-C-S-x(4)-C-x-C-x(3,4)-C-S-x-C consensus proposed as a signature of fungal MTs by Binz and Kägi (1999) (http://www.expasy.ch/cgi-bin/lists?metallo.txt). The alignment presented in Fig. 33 shows that this latter consensus sequence resembles the signature of basididomycete MTs which is C-x(3, 4)-C-x-C-x(3)-C-x-C at the N-terminal end together with C-x-C at the C-terminal end. *HcMT1* and *HcMT2* genes are most closely related to MT like polypeptides from *Agaricus bisporus, Paxillus involutus, Lentinula edodes* and also with the AM fungi *Gigaspora margarita*.

The putative amino acid sequences of *HcMT1* and *HcMT2* genes were compared with the closely related metallothioneins other fungal species from the GenBank using BLASTP program (Altschul *et al.,* 1997). The sequences were aligned using *Multalin* program. The evolutionary distance was calculated by Kimura 2 parameter and phylogenetic tree was constructed by a neighbor-joining method. Bootstrap analysis was based on 1500 resamplings using the MEGA 4.0 package (Tamura *et al.*, 2007). Phylogenetic analysis of metallothioneins of *HcMT* genes with that of the related fungal species was placed in 4 groups. Group A consisted of only *HcMT1* metallothionein of the present study. Group B consisted of five metallothioneins of *G. intraradices, G. margaritaMT1, G. margarita GmarMT1, N. crassa* and *P. anserine.* Group C consisted of six metallothioneins of *A. bisporus MT1* and *A. bisporus MT2, L. edodes, P. involutus, P. tinctorius* including *HcMT2* of current study. Multiple

sequence analysis of *HcMT2* showed 41, 44 and 54% similarity with metallothioneins of *L.edodes, P.involutus* and *P.tinctorius* respectively. Group D consisted of metallothioneins of *S. cerevisiae Crs5* and *CUP1* (Fig. 34).

Table 8: *HcMT1* sequence analysis performed using Blast program through NCBI.

Sequences producing significant alignments:	Score (Bits)	E Value
gi|7413495|emb|CAB85689.1| metallothionein [Agaricus bisporus]	42.2	7e-04
gi|18250469|emb|CAC85298.1| metallothionein [Agaricus bisporus]	42.2	7e-04
gi|116507292|gb|EAU90187.1| predicted protein [Coprinopsis ciner	39.7	0.004
gi|116498726|gb|EAU81621.1| predicted protein [Coprinopsis ciner	39.2	0.006
gi|6324605|ref|NP_014674.1| Copper-binding metallothionein, r...	36.3	0.044
gi|116207846|ref|XP_001229732.1| hypothetical protein CHGG_03...	34.6	0.14
gi|42541140|gb|AAS19463.1| metallothionein [Paxillus involutus]	34.1	0.19
gi|116503285|gb|EAU86180.1| predicted protein [Coprinopsis ciner	33.7	0.26
gi|46254401|gb|AAS86162.1| metallothionein MMT1 [Magnaporthe gri	33.7	0.26
gi|127375|sp|P15113|MT1_CANGA Metallothionein-1 (MT-1) (Metal...	32.9	0.46

Table 9: *HcMT2* sequence analysis performed using Blast program through NCBI.

Sequences producing significant alignments:	Score (Bits)	E Value
gi|116507292|gb|EAU90187.1| predicted protein [Coprinopsis ciner	84.2	2e-16
gi|116498726|gb|EAU81621.1| predicted protein [Coprinopsis ciner	68.9	7e-12
gi|42541140|gb|AAS19463.1| metallothionein [Paxillus involutus]	48.6	9e-06
gi|144686982|gb|ABP02008.1| metallothionein [Ganoderma lucidum]	48.1	1e-05
gi|7413495|emb|CAB85689.1| metallothionein [Agaricus bisporus]	46.0	5e-05
gi|18250469|emb|CAC85298.1| metallothionein [Agaricus bisporus]	46.0	5e-05
gi|116503285|gb|EAU86180.1| predicted protein [Coprinopsis ciner	45.2	1e-04
gi|46254401|gb|AAS86162.1| metallothionein MMT1 [Magnaporthe gri	37.5	0.019
gi|22138761|emb|CAD13456.1| metallothionein [Gigaspora margarita	37.5	0.019

5‘ taaatccaatccgagtaatttagcccaaacctcttacaaac

ATG CAA TTC ACT TCT ATC CTC GTC AAC CAA GCT TGC GGT TCT

M Q F T S I L V N Q A C G S

GAC AAT TGC CAG TGC GAC GCA GCT TGC ACC TGC TCC TCT GGC

D N C Q C D A A C T C S S G

TCT TGC CAC GCC CCC GTC AAC CGC GCT TGC GGT TCC AGC GAC

S C H A P V N R A C G S S D

TGC AAC TGC AAC AGC TCG TGC GGC TGC GAG TCC AAC AAC TGC

D C N C N S S C G C E S N N

AAC TGC AAC TGA

C N C *

agaatcaacgtgacgatgccctctctcaattcatcccggtctcactctcactacacgtttttttgaaaacccggcgaatgaaataacgtac
gaatatgtatcaacaatctcttcatgttacgcgagttcgattttgtccagccgtctaattggtacatacatcatgctgtaaacgacatccttgg
tcaaatcagatcactttcactgtcttgtaaaagcgacag***AATAA***aaaaaaaa **3’**

Figure 29: The Nucleotide and deduced amino acid sequence of *HcMT1* cDNA. The C-x-C motifs are underlined.

5‘ cgaactcccactactacaaccacatcacatctcatccacaaac

ATG CAG ATC GTT CAA AAC AGT CTC GTC TCC CAG TCT TCT GGG

M Q I V Q N S L V S Q S S G

TGC ACC TGT ACT TCC TGC AAG TGC GGC TCT AAC TGC ACC TGC

C T C T S C K C G S N C T C

GGA GCC CCT GTC AAC CAG TCC TCT GGA TGC GGA AGC TCT TCC

G A P V N Q S S G C G S S S

TGC ACC TGC ACT TCC TGC ACC TGC AAG GCT GGC GAG TGC AAA

C T C T S C T C K A G E C K

TGC TAA

C *

atgcgctgccgtgaaggacttaatgtcgtgagggccgacaaatcgttcgactcggactaatgaactgtactactacgatttctgggtcag
cttttgagaaacgtttcttcccttgacaaagaacatttaaatttgtaccttctgccaagt **3’**

Figure 30: The Nucleotide and deduced amino acid sequence of *HcMT2* cDNA. The C-x-C motifs are underlined.

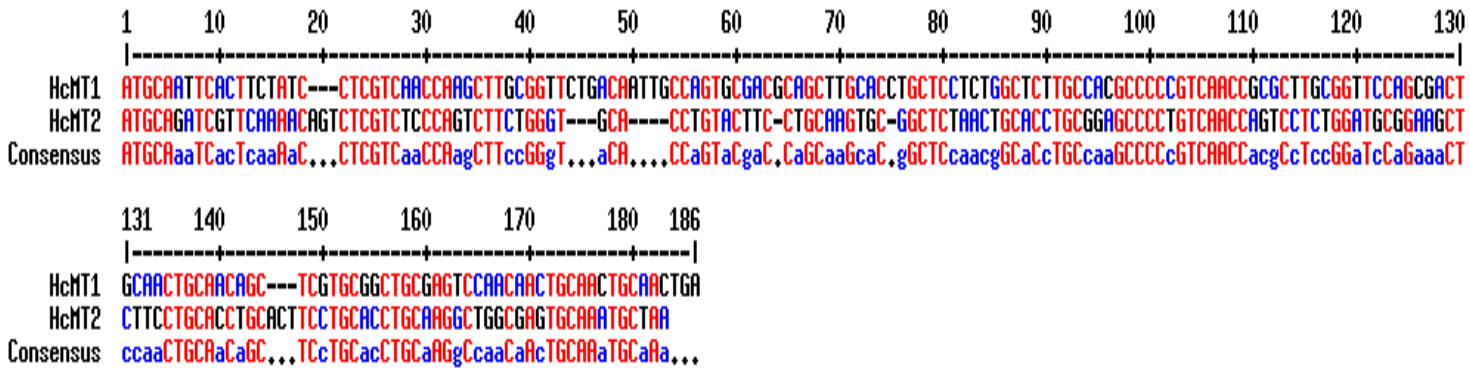


Figure 31: Sequence alignment of *HcMT1* and *HcMT2* ORF sequences of *H. cylindrosporum* using *Multalin* program

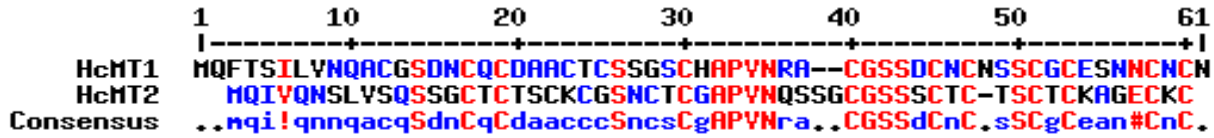


Figure 31: Sequence alignment of *HcMT1* and *HcMT2* deduced amino acid sequences of *H. cylindrosporum* using *Multalin* program

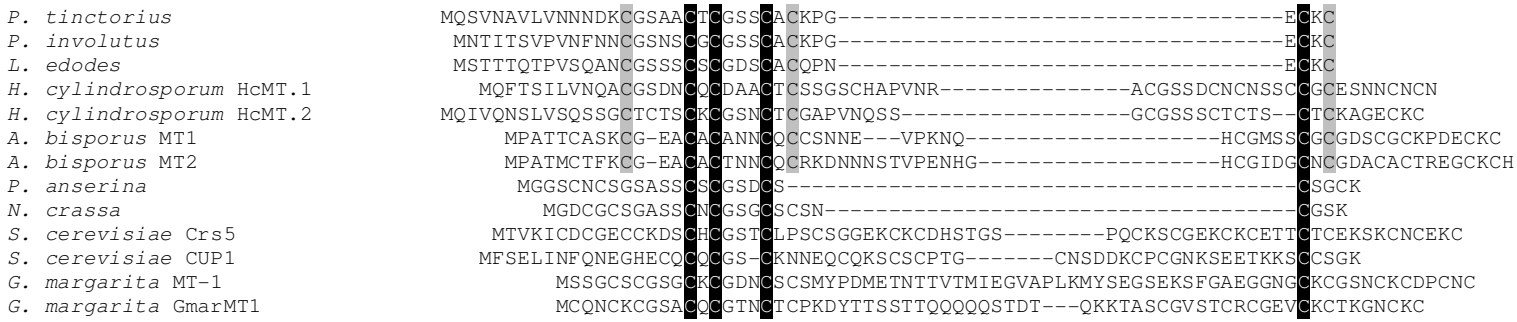


Figure 33: Multiple alignment of fungal metallothionein protein sequences. Gaps introduced to provide the best alignment are indicated by dashes. Fully conserved Cys residues are boxed in black background; Cys residues conserved in basidiomycetes are boxed in grey. Accession numbers are as follows: *Pisolithus tinctorius,* EST 11A2; *Paxillus involutus*, AAS19463; *Lentinula edodes*, CO501612, *Hebeloma cylindrosporum HcMT1*, EU049884; *Hebeloma cylindrosporum HcMT2*, EU049885; *Agaricus bisporus* MT1, CAC85298; *Agaricus bisporus* MT2, CAC85299; *Podospora anserina*, CAA06385; *Neurospora crassa*, P02807; *Saccharomyces cerevisiae* Crs5, YOR031W; *Saccharomyces cerevisiae* CUP1, YHR055C; *Gigaspora margarita* MT1, CAD13456; *Gigaspora margarita* GmarMT1, AJ421527.

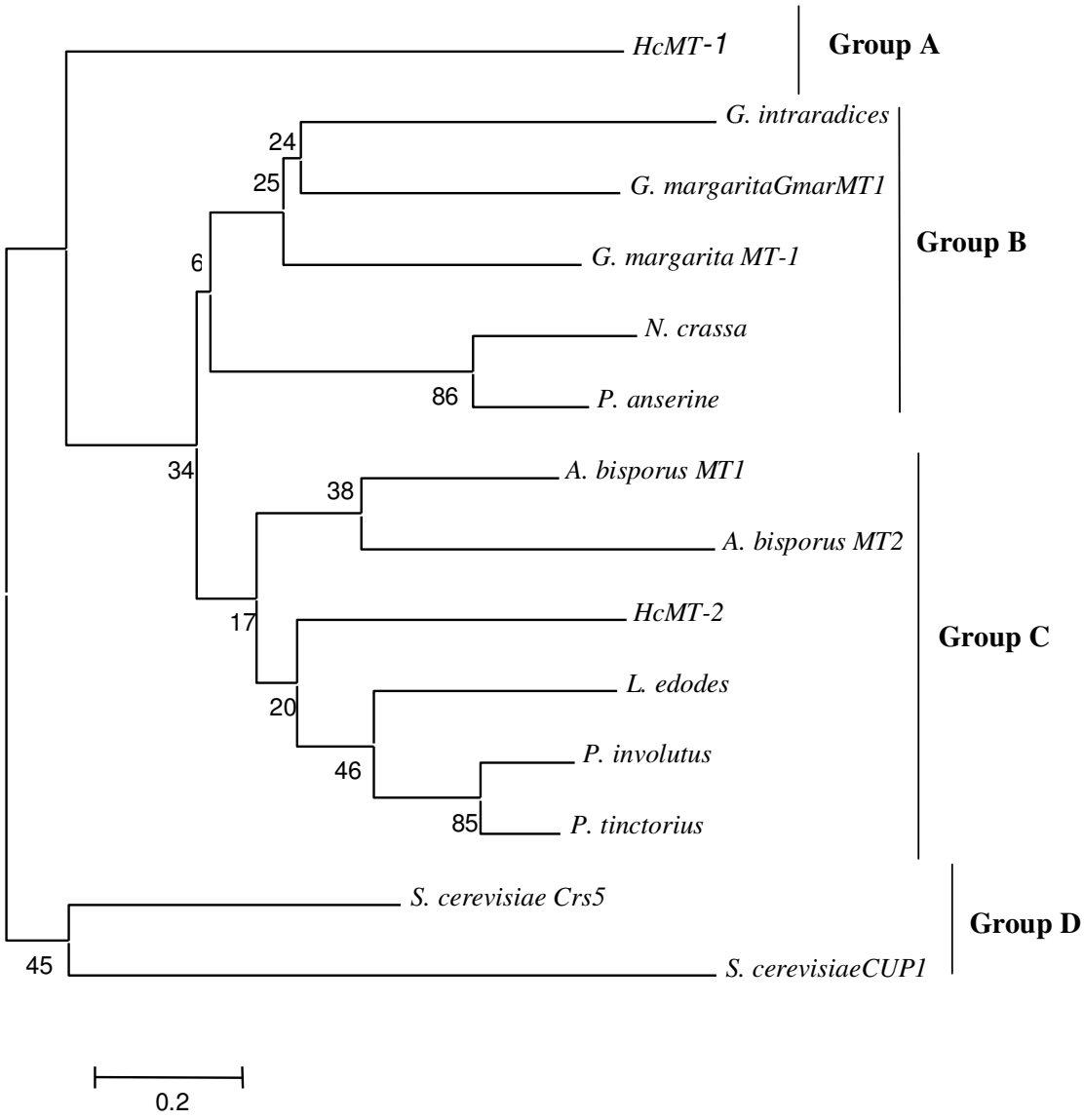


Figure 34: Neighbor-joining tree based on MT sequences of *H. cylindrosporum* of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1500 replicates.

4.5 Promoter analysis of *HcMT1* and *HcMT2*

The promoter is a regulatory region of DNA generally located upstream (towards the 5' region of the sense strand) of transcription start of the gene that allows transcription of the gene and regulates the timing and location of gene expression. An analysis of promoter provides possible insights into the regulation of genes. The adjacent DNA sequence upstream (Promoter sequence) from *HcMT* genes in the *H. cylindrosporum* genome were investigated with the Clontech Universal GenomeWalker kit (Clontech Laboratories, Inc., Heidelberg, Germany). Genome walking experiments allowed us to isolate a fragment of 1500 bp upstream from the start codon of *HcMT* genes.

Genomic DNA of *H. cylindrosporum* was digested with three different restriction enzymes such *Dra* I, *Pvu* II and *Eco*R V, which are provided in the Universal GenomeWalker kit (Clontech, Germany). The digested DNA was then purified and each batch of digested genomic DNA was then ligated separately with GenomeWalker Adaptor. After the libraries have been constructed, the first PCR was performed using the outer adaptor primer (AP1) provided in the kit and an outer gene-specific primer (GSP1). *HcMT1* specific primer used as gene specific primer (GSP1) for amplification of upstream sequence of *HcMT1* gene. In case of *HcMT2* gene, *HcMT2* specific primer was used to amplify large genomic segments adjacent to *HcMT2* gene. Out of three restriction enzymes used to digest genomic DNA, the major PCR products were obtained with *Eco*R V digested genomic DNA, approx. 1500 bp long fragment obtained for both *HcMT* genes. The primary PCR mixture was then diluted and used as a template for a secondary or "nested" PCR with the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2). HcMT1 and HcMT2 primers designed upstream to the previous primers and used in nested PCR with

adopter specific nested primer AP2. Fragments of approximately 1400 bp obtained for both genes in nested PCR (Fig. 35). These PCR products were gel purified and ligated in to pGEM-T Easy vector. Ligated products were transformed in to DH5α cells by heat shock method. Then the positive clones were selected and plasmid DNA was isolated. The plasmids were screened the appropriate insert by PCR using gene specific primers and sequenced.

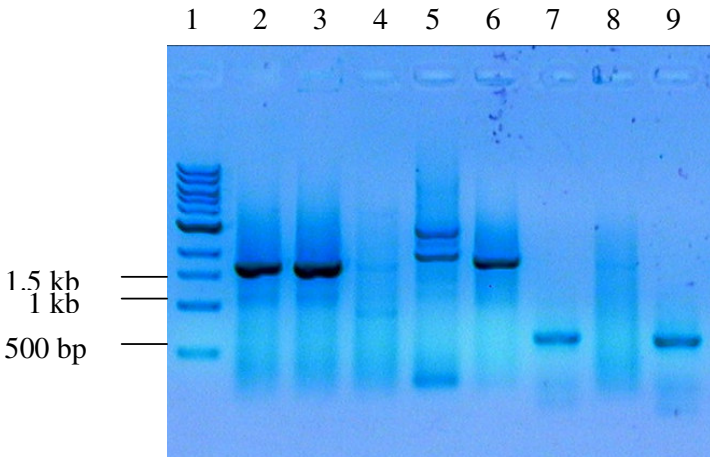


Figure 35: Nested PCR of a primary PCR product with the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2). Lane 1: 1 kb marker, Lane 2 and 3: amplified product *HcMT1* from *Eco*R V digested genomic DNA, Lane 4: amplified product of *HcMT1* from *Dra* I digested genomic DNA, Lane 5: amplified product of *HcMT1* from *Pvu* II digested genomic DNA, Lane 6: amplified product *HcMT2* from *Eco*R V digested genomic DNA, Lane 7 and 9: amplified product of *HcMT2* from *Pvu* II digested genomic DNA, Lane 8: amplified product of *HcMT2* from *Dra* I digested genomic DNA.

In order to explain whether the differential expression of *HcMT1* and *HcMT2* is due to differential regulation, we performed computational analysis of their respective upstream regions of the promoters. The upstream sequence of *HcMT1* and *HcMT2*

were analyzed using Genomatix software (Cartharius *et al.,* 2005) and TRANSFAC databases for potential transcription factor binding sites or elements (http://helixweb.nih.gov/transfac/). Both Genomatix and TransFac software provide high level of confidence in the prediction of regulatory elements based on multiple matches and conservation of key nucleotide sequences (Wingender *et al.,* 2001). For example, Genomatix scores of 0.9 or above indicate very high confidence level for the predicted regulatory elements. We have listed the elements that met the score of 0.9 or above and confirmed by high scoring values on Transfac (Fig. 36 and 37).

The computer analysis revealed that the number and variety of potential regulatory elements in *HcMT1* and *HcMT2* promoter regions were different (Fig. 38). *HcMT1* upstream sequence contain different standard stress response elements implicated in metal response such as metal response element (MRE), general stress response (GATA), response to nitrogen utilization (NIT), regulatory sequences for glycolytic genes (GCR), multiple stress response elements (STRE) and heat shock factors (HSF). The *HcMT1* promoter has a putative NIT (TATCAAA) at -184, a putative TATA box (TAAA) at -196, a putative STRE (CCAGGCGAA) at -508, two GATA box, at – 563 and -603 respectively and one GCN (GAATCATCG) at -662. The HSF (TTTCTGGAA), which mediates transcription induction by heat /oxidative stress, was found at position -907. The *HcMT1* contains one metal responsive element (TGCGCCCG) found at position -338, which is *cis* regulatory elements implicated in the induction of MT gene transcription (Fig. 38 and Fig. 39).

In addition to MRE, GATA, NIT, HSF and TATA box, the *HcMT2* promoter contained several additional regulatory elements such as drug resistant responsive

elements (DRE), metabolic regulatory elements (MCM), regulatory elements of purine and pyrimidine utilization (RPU), phosphate starvation responsive elements (PHO) and CAT box (CAAT). The *HcMT2* promoter contained one TATA box at -51, one HSF at -96, two RPU (CGAT) at -166 and 181, three PHOs (GCACGT) at -222, -784 and -937, GATA at -266, CAT box (CCAAT) at -493, two NITs (TATCAA) at -519 and -1240, one MCM (ACCCAAT) at -864, one GCR (GCTTC) at -1103 and one DRE (TCCACGG) at -1194. One MRE (TGGCTGCCA) was found at position -1045 in *HcMT2* promoter (Fig. 38 and Fig. 40). *HcMT1* contained response elements, which were not present in *HcMT2* promoter region such as STRE, which are known to be responsible for multiple stress response or enhance the response of other elements and GCN, which responds to amino acid and nitrogen starvation related stress. The core sequences of HSF, NIT, GATA were same in both promoters, but their position were different. Other responsive elements position and core sequences were different in both *HcMT1* and *HcMT2* promoter regions. The difference in the position and sequences of responsive elements in *HcMT1* and *HcMT2* promoter might be the reason for different pattern of gene regulation.

The *HcMT1* genomic DNA contained three introns; intron 1, 48 bp (51-98); intron 2, 65 bp (155-219); intron 3, 64 bp (298-362) and four exons; exon 1, 9 bp (42-50); exon 2, 56 bp (99-154); exon 3, 78 bp (220-297); exon 4, 37 bp (362-398) (Fig. 39). *HcMT2* contained 4 exons; exon 1, 6 bp (32-37); exon 2, 68 bp (98-165); exon 3, 65 bp (223-290); exon 4, 34 bp (349-382) and 3 introns; intron 1, 60 bp (38-97); intron 2, 57 bp (166-222); intron 3, 58 bp (291-348) (Fig. 40). The first exon is very small in both cases (6-9 nucleotides long) and all the 3 introns contained conserved intron junctions GT-AG.

Check transcription factor <-> matrix family assignment			Opt.	Position	Str.	Core sim.	Matrix sim.	Sequence (red: ci-value > 60 capitals: core sequence)
Family	Further Family Information	Matrix		from - to				
O$INRE	Core promoter initiator elements	O$DINR.01	0.94	20 - 30	(-)	0.968	0.953	ttTCATttttc
F$YCAT	Yeast CCAAT binding factors	F$HAP234.01	0.86	43 - 55	(-)	1.000	0.932	tactaCCAAttac
F$YGAL	Yeast GAL4 factor	F$LAC9.01	0.75	86 - 110	(-)	1.000	0.753	ctcCGGAgcgatgcaggtcgctgca
F$YGAL	Yeast GAL4 factor	F$GAL4.01	0.75	88 - 112	(+)	0.850	0.755	cagcgacctgcatcgctCCGGagac
F$AAAU	A. nidulans activator of acetate utilization genes	F$FACBCB.01	0.80	100 - 116	(-)	1.000	0.869	GCAGgtctccggagcga
F$ACPF	Aspergillus cell pattern formation	F$STUAP.01	0.95	136 - 146	(+)	1.000	0.951	tttCGCGcccg
F$YMAT	Yeast mating factors	F$MATA1.01	0.92	364 - 374	(-)	1.000	0.931	tGATGcaaaat
F$YGCR	Yeast activator of glycolytic genes	F$GCR1.01	0.92	386 - 402	(-)	1.000	0.921	gtCTTCcagaaacaaac
F$YHSF	Yeast heat shock factors	F$HSF.01	0.85	390 - 400	(-)	0.880	0.874	cTTCCagaaac
F$YHSF	Yeast heat shock factors	F$HSF.01	0.85	391 - 401	(+)	1.000	0.928	tTTCTggaaga
F$YGAL	Yeast GAL4 factor	F$GAL4.02	0.74	470 - 494	(-)	1.000	0.753	agagggagaatgattctCCGAtcct
F$YCTR	Centromer Dna Element I	F$CBF1.01	0.92	492 - 502	(+)	0.898	0.937	tctCACAtgaa
F$ACPF	Aspergillus cell pattern formation	F$STUAP.01	0.95	576 - 586	(-)	1.000	0.985	gaaCGCGagaa
F$YMCB	Yeast Mlu I cell cycle box	F$MCB.01	0.84	577 - 587	(+)	1.000	0.898	tctCGCGttcg
F$YCAT	Yeast CCAAT binding factors	F$HAP234.01	0.86	587 - 599	(-)	1.000	0.944	gacgtCCAAtaac
F$YGCN	Yeast GCN4 factor	F$GCN4.01	0.91	634 - 646	(+)	0.816	0.916	cattgaATCAtcg
F$SXBP	S.cerevisiae, XhoI site-binding protein I	F$XBP1.01	0.88	685 - 697	(+)	1.000	0.883	tccTCGAaaaagg
F$GATA	Fungal GATA binding factors	F$GATA.01	0.89	695 - 705	(+)	1.000	0.951	agGATAagcca
F$GATA	Fungal GATA binding factors	F$GATA.01	0.89	736 - 746	(+)	1.000	0.970	aaGATAagaat
F$YSTR	Yeast stress response elements	F$STRE.01	0.98	792 - 800	(+)	1.000	0.980	ccAGGGgaa
F$HPHO	Homeodomain protein Pho2	F$PHO2.01	0.84	834 - 848	(+)	1.000	0.865	tgaCTAAattaacca
F$HPHO	Homeodomain protein Pho2	F$PHO2.01	0.84	834 - 848	(-)	0.909	0.858	tggTTAAtttagtca
O$VTBP	Vertebrate TATA binding protein factor	O$ATATA.01	0.78	852 - 868	(+)	0.750	0.811	ctgtatgTAATttggct
F$YQA1	Neurospora crassa QA1 gene activator	F$QA1F.01	0.75	863 - 883	(+)	0.764	0.820	ttggctaaggctTCAAgcttg
O$INRE	Core promoter initiator elements	O$DINR.01	0.94	883 - 893	(-)	1.000	0.973	tgTCAGttcac
F$FBAS	Fungi Branched Amino acid bioSynthesis	F$LEU3.01	0.78	939 - 951	(-)	1.000	0.891	gtcggaACCGgga
F$YHSF	Yeast heat shock factors	F$HSF.01	0.85	1075 - 1085	(-)	1.000	0.929	tTTCTtgaagt
O$VTBP	Vertebrate TATA binding protein factor	O$MTATA.01	0.84	1096 - 1112	(+)	1.000	0.930	tggtaTAAAccctttggg
F$YNIT	Asperg./Neurospora-activ. of genes induced by nitrogen	F$NIT2.01	0.99	1118 - 1124	(+)	1.000	0.993	TATCaaa
F$ABAA	Aspergillus spore/developmental regulator	F$ABAA.01	0.93	1226 - 1240	(+)	1.000	0.960	cctttgCATTctagc
O$VTBP	Vertebrate TATA binding protein factor	O$ATATA.01	0.78	1260 - 1276	(-)	0.750	0.845	ctgtattTATGcatttg

Figure 36: Elemental analysis of *HcMT1* promoter using Genomatix softwares

Check transcription factor <-> matrix family assignment			Opt.	Position	Str.	Core sim.	Matrix sim.	Sequence (red: ci-value > 60 capitals: core sequence)
Family	Further Family Information	Matrix		from - to				
F$ABAA	Aspergillus spore/developmental regulator	F$ABAA.01	0.93	26 - 40	(-)	1.000	0.952	agtcagCATTccacg
F$GATA	Fungal GATA binding factors	F$GATA.01	0.89	72 - 82	(-)	1.000	0.988	ttGATAagcgg
F$YNIT	Asperg./Neurospora-activ. of genes induced by nitrogen	F$NIT2.01	0.99	77 - 83	(+)	1.000	0.993	TATCaag
F$PDRE	Pleiotropic drug resistance responsive elements	F$PDRE.01	0.87	120 - 128	(-)	1.000	0.973	TCCGtggaa
F$PDRE	Pleiotropic drug resistance responsive elements	F$PDRE.01	0.87	121 - 129	(+)	0.787	0.910	TCCAcggaa
F$CYTO	Activator of cytochrome C	F$HAP1.01	0.76	139 - 153	(+)	0.750	0.772	ttggaattGTCGcct
F$YGCR	Yeast activator of glycolytic genes	F$GCR1.01	0.92	157 - 173	(-)	1.000	0.931	tgCTTCctccgtggcga
F$YGCR	Yeast activator of glycolytic genes	F$GCR1.01	0.92	166 - 182	(-)	1.000	0.960	ggCTTCctgtgcttcct
F$MREF	Metal regulatory element factors	F$CUSE.01	0.88	265 - 279	(+)	1.000	0.925	aagttttgGCTGcca
F$YGCN	Yeast GCN4 factor	F$GCN4.02	0.90	276 - 288	(-)	1.000	0.923	caaTGACtttggc
F$GATA	Fungal GATA binding factors	F$GATA.01	0.89	332 - 342	(-)	1.000	0.896	atGATAaaaat
F$YPHO	Yeast regulator of phosphatase genes	F$PHO4.01	0.92	374 - 386	(+)	1.000	0.932	ctgCACGttgtca
F$MADS	Yeast MADS-Box factors	F$RLM1.01	0.78	390 - 408	(-)	1.000	0.783	tcctTCTAattgtcgcctc
F$YMAT	Yeast mating factors	F$MATA1.01	0.92	438 - 448	(+)	1.000	0.942	tGATGtgcacc
F$YMCM	Yeast cell cycle and metabolic regulator	F$MCM1.01	0.83	444 - 460	(+)	0.983	0.840	gcaCCCAatctggacgc
F$ACPF	Aspergillus cell pattern formation	F$STUAP.01	0.95	454 - 464	(-)	1.000	1.000	catCGCGtcca
F$YMCB	Yeast Mlu I cell cycle box	F$MCB.01	0.84	454 - 464	(-)	1.000	0.846	catCGCGtcca
F$YMCB	Yeast Mlu I cell cycle box	F$MCB.01	0.84	455 - 465	(+)	1.000	0.888	ggaCGCGatgt
F$YPHO	Yeast regulator of phosphatase genes	F$PHO4.01	0.92	527 - 539	(+)	1.000	0.930	aggCACGttgtac
F$SXBP	S.cerevisiae, XhoI site-binding protein I	F$XBP1.01	0.88	621 - 633	(+)	1.000	0.892	ggaTCGAggagta
F$YGCR	Yeast activator of glycolytic genes	F$GCR1.01	0.92	681 - 697	(-)	1.000	0.921	gaCTTCcagtcgcggta
F$YNIT	Asperg./Neurospora-activ. of genes induced by nitrogen	F$NIT2.01	0.99	698 - 704	(+)	1.000	0.993	TATCaat
F$CYTO	Activator of cytochrome C	F$HAP1.01	0.76	766 - 780	(+)	0.900	0.785	gtggaagtACCGact
F$YCAT	Yeast CCAAT binding factors	F$HAP234.01	0.86	818 - 830	(+)	1.000	0.930	cctggCCAAttaa
F$ARPU	Regulator of pyrimidine and purine utilization pathway	F$UAY.01	0.75	1010 - 1026	(+)	0.944	0.756	acCGCAgtcagctgcca
F$YNIT	Asperg./Neurospora-activ. of genes induced by nitrogen	F$NIT2.01	0.99	1046 - 1052	(-)	1.000	1.000	TATCtac
F$GATA	Fungal GATA binding factors	F$GATA.01	0.89	1047 - 1057	(+)	1.000	0.952	taGATAagtaa
F$CYTO	Activator of cytochrome C	F$HAP1.01	0.76	1052 - 1066	(+)	0.900	0.774	aagtaaatACCGtta
F$YPHO	Yeast regulator of phosphatase genes	F$PHO4.01	0.92	1093 - 1105	(-)	1.000	0.943	attCACGtgcggt
F$YCTR	Centromer Dna Element I	F$CBF1.01	0.92	1094 - 1104	(+)	1.000	0.920	ccgCACGtgaa
F$YPHO	Yeast regulator of phosphatase genes	F$PHO4.01	0.92	1094 - 1106	(+)	1.000	0.921	ccgCACGtgaatg
F$YCTR	Centromer Dna Element I	F$CBF1.01	0.92	1095 - 1105	(-)	1.000	0.946	attCACGtgcg
F$ARPU	Regulator of pyrimidine and purine utilization pathway	F$PPR1.01	0.73	1105 - 1121	(+)	0.750	0.751	tgaggcattttCTGAac
F$ARPU	Regulator of pyrimidine and purine utilization pathway	F$PPR1.01	0.73	1140 - 1156	(-)	0.750	0.835	atcggtaatcgTCGAtc
F$ARPU	Regulator of pyrimidine and purine utilization pathway	F$PPR1.01	0.73	1141 - 1157	(+)	1.000	0.797	atcgacgattaCCGAtc
F$YGCR	Yeast activator of glycolytic genes	F$CSRE.01	0.79	1161 - 1177	(-)	1.000	0.835	caagttgaTCCGccatt

Figure 37: Elemental analysis of *HcMT2* promoter using Genomatix softwares

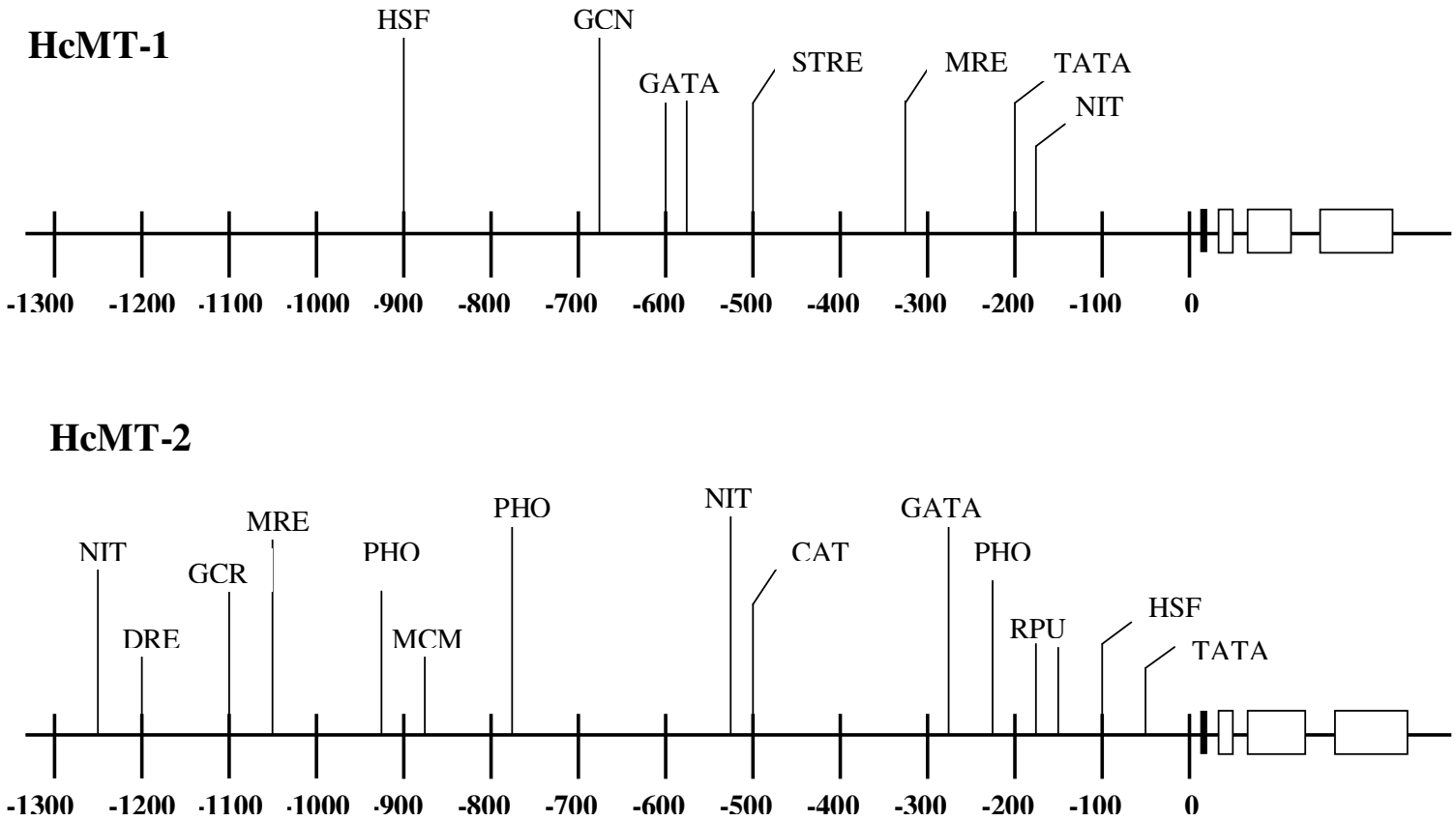


Figure 38: Gene structure and promoter analysis of *HcMT1* and *HcMT2* from *H. cylindrosporum.* Approximately 1250 bp upstream of transcription start point analyzed using MatInspector and Transfac software to identify various responsive elements. Both *HcMT1* and *HcMT2* promoters contain common responsive elements and also differences, which presumably responsible for differences in the regulation of expression of *HcMT1* and *HcMT2*.

-1308 TAAAAGTCAGACGAAGAAGGAAAAATGAAAGACCTGGAGAATGTAATTGGTAGTAAGGGTGGTAGTGGGATGCAGAGAAGGCATTTGCAGCGA -1216

-1215 CCTGCATCGCTCCGGAGACCTGCACAGTTTTTCGCAACAAGGTTTCGCGCCCGACACTGACACAAACAATCACCATCATTCTCGATCACTTTG -1123

-1122 CCTCCCTGCCAGCATTTACTCTGATTTTGTGCTAGGTTTCTCTTACCGAAACTAAAGAACGGTCGATACTCGGTTCTACAGAAAGTGGGCGTG -1030

-1029 GCGTGTACTCCTCAAGGGCTCCAGGTCTCGACTTGGCTACAGTAGCGGCTACATCTCTGGCTCAAAGTCTGGAACTCTCTTCGTATTTTGCAT -937

-936 CATGGGGGGGAAAGTTTG**tTTCTggaaga**CGAAGACAAGCTATCAGAAAGATCGTAGAAAGCTCCATCACCACCGAAACGTATACCCTCTCAG -844
HSF

-843 AAACAGGATCGGAGAATCATTCTCCCTCTCACATGAAGGTCGAAAACGTTCATGGTCGTCACGAAGTCGCTCTACGTGAATGTCATAGTGCAG -751

-750 CGATCTACCCTTCTGGATTCTCGCGTTCGTTATTGGACGTCGGCGACCATGAACGTACTAAAACCTTGTCAAAAT**cattgaATCAtcg**GGTTG -658
GCN

-657 AGCGATGTTGGCAATACCCAGTAGGAACTCCGATCCTCGAAAA**agGATAagcca**TTCCGTTCAATTGAAATCCAGCGTTGTGGA**aaGATAaga** -565
GATA **GATA**

-564 **at**GGAAGCAAGAGGGGCCTCCAACAGATACATTAGCGGTATCCTTGA**ccAGGGgaa**GGGAAAGTCAGAAGGCCTGAGGAAGGTCAGATTTGAC -472
STRE

-471 TAAATTAACCACTGCTGTATGTAATTTGGCTAAGGCTTCAAGCTTGTGAACTGACAGCTCTACGAGCTTGTGCACGGAAAAGGACATATTCGG -379

-378 GTTGCAGATCCCGGTTCCGACAGACACATTTT**TGCGCCCG**ACTATCGCAAGAGCAAGCTCAAGCTTGCTCTGGGCTTGGCTCACTCTGCGGCA-286
MRE

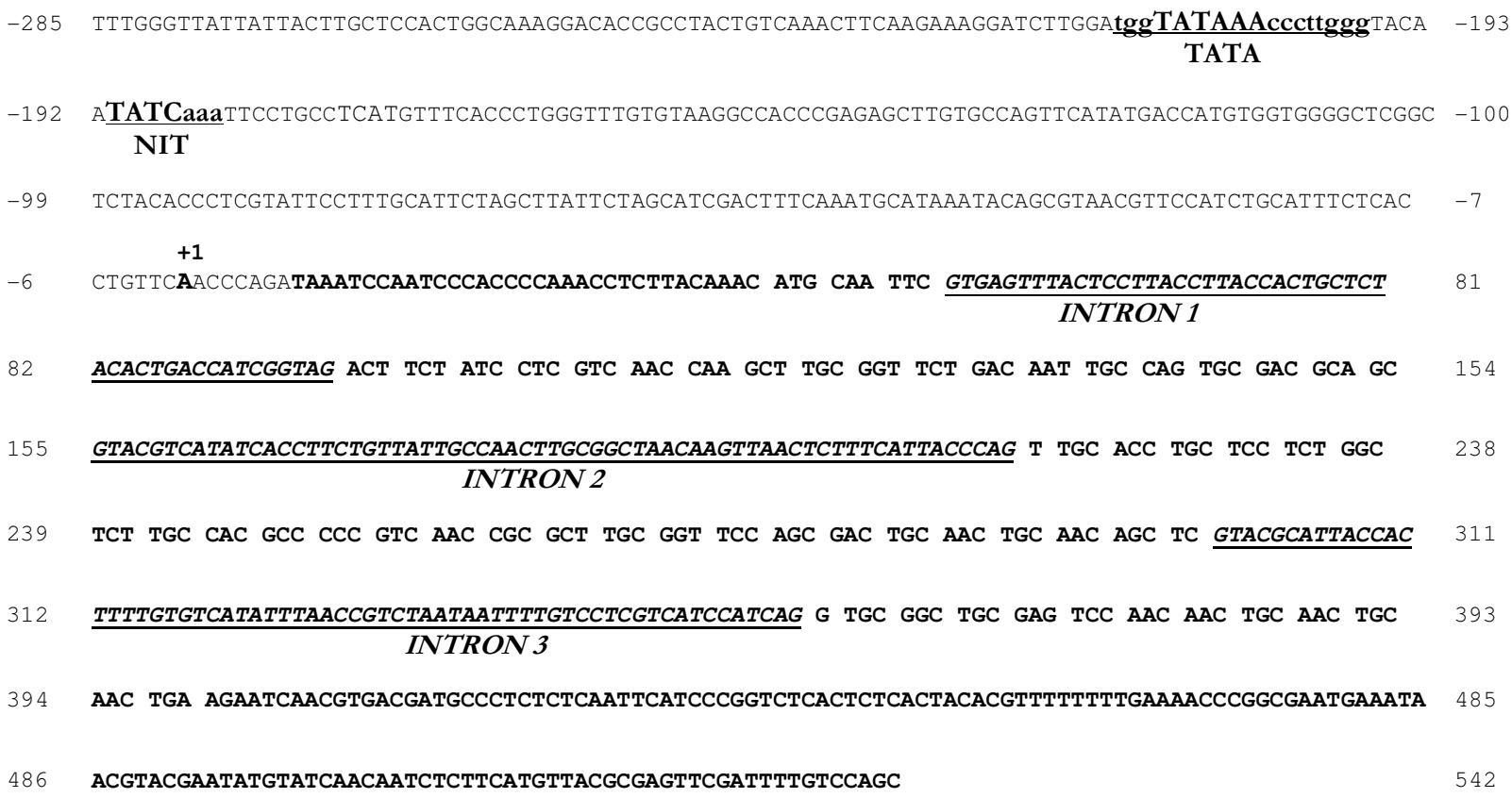
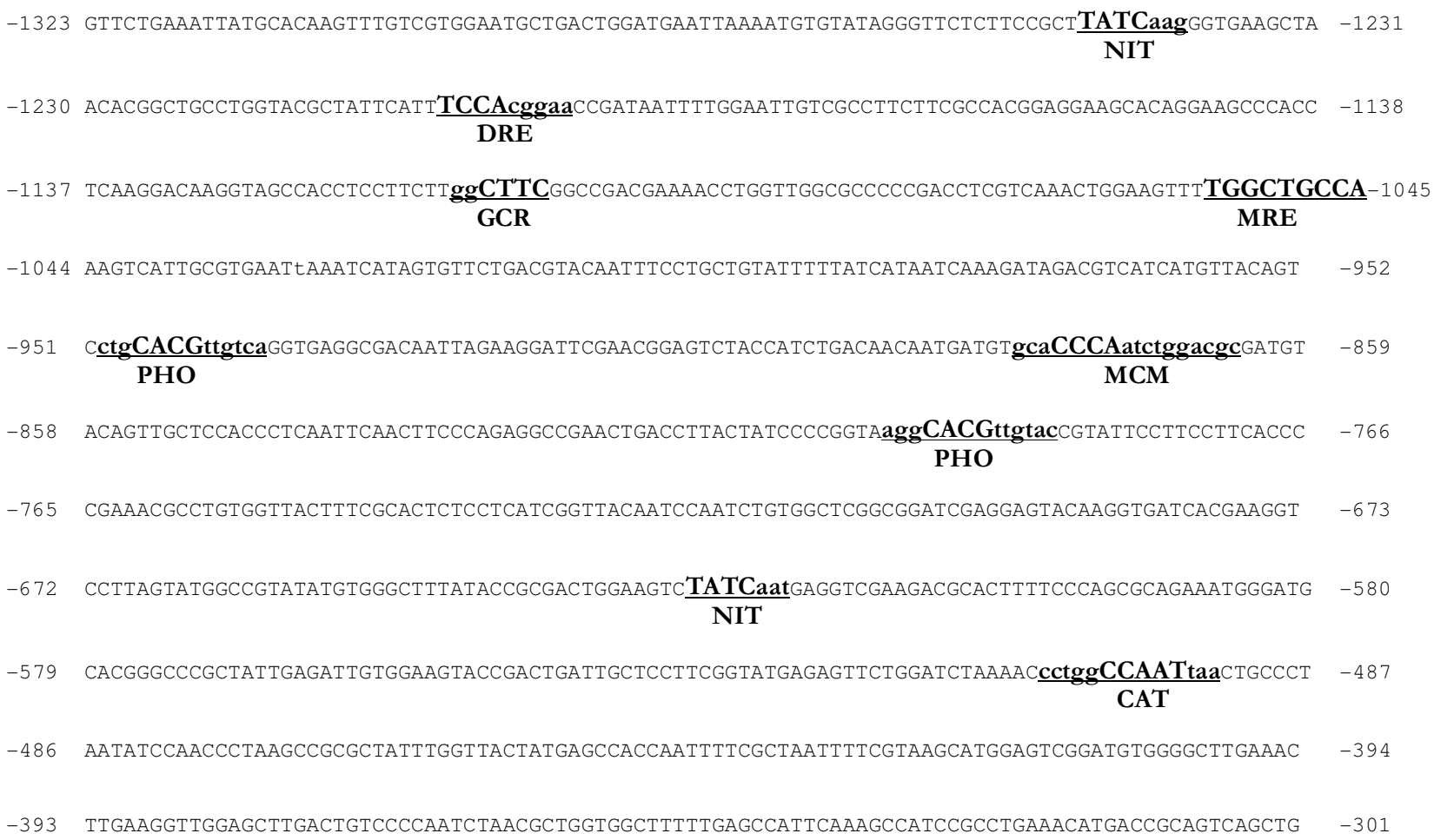


Figure 39: Nucleotide sequence of *HcMT1* genomic DNA. Responsive elements were shown in bold and underlined; introns sequences are shown in italic with underlined


-1323 GTTCTGAAATTATGCACAAGTTTGTCGTGGAATGCTGACTGGATGAATTAAAATGTGTATAGGGTTCTCTTCCGCTTATCaagGGTGAAGCTA -1231
NIT
-1230 ACACGGCTGCCTGGTACGCTATTCATTTCCAcggaaCCGATAATTTTGGAATTGTCGCCTTCTTCGCCACGGAGGAAGCACAGGAAGCCCACC -1138
DRE
-1137 TCAAGGACAAGGTAGCCACCTCCTTCTTggCTTCGGCCGACGAAAACCTGGTTGGCGCCCCCGACCTCGTCAAACTGGAAGTTTTGGCTGCCA-1045
GCR
MRE
-1044 AAGTCATTGCGTGAATtAAATCATAGTGTTCTGACGTACAATTTCCTGCTGTATTTTTATCATAATCAAAGATAGACGTCATCATGTTACAGT -952
-951 CctgCACGttgtcaGGTGAGGCGACAATTAGAAGGATTCGAACGGAGTCTACCATCTGACAACAATGATGTgcaCCCAatctggacgcGATGT -859
PHO
MCM
-858 ACAGTTGCTCCACCCTCAATTCAACTTCCCAGAGGCCGAACTGACCTTACTATCCCCGGTAaggCACGttgtacCGTATTCCTTCCTTCACCC -766
PHO
-765 CGAAACGCCTGTGGTTACTTTCGCACTCTCCTCATCGGTTACAATCCAATCTGTGGCTCGGCGGATCGAGGAGTACAAGGTGATCACGAAGGT -673
-672 CCTTAGTATGGCCGTATATGTGGGCTTTATACCGCGACTGGAAGTCTATCaatGAGGTCGAAGACGCACTTTTCCCAGCGCAGAAATGGGATG -580
NIT
-579 CACGGGCCCGCTATTGAGATTGTGGAAGTACCGACTGATTGCTCCTTCGGTATGAGAGTTCTGGATCTAAAACcctggCCAATtaaCTGCCCT -487
CAT
-486 AATATCCAACCCTAAGCCGCGCTATTTGGTTACTATGAGCCACCAATTTTCGCTAATTTTCGTAAGCATGGAGTCGGATGTGGGGCTTGAAAC -394
-393 TTGAAGGTTGGAGCTTGACTGTCCCCAATCTAACGCTGGTGGCTTTTTGAGCCATTCAAAGCCATCCGCCTGAAACATGACCGCAGTCAGCTG -301

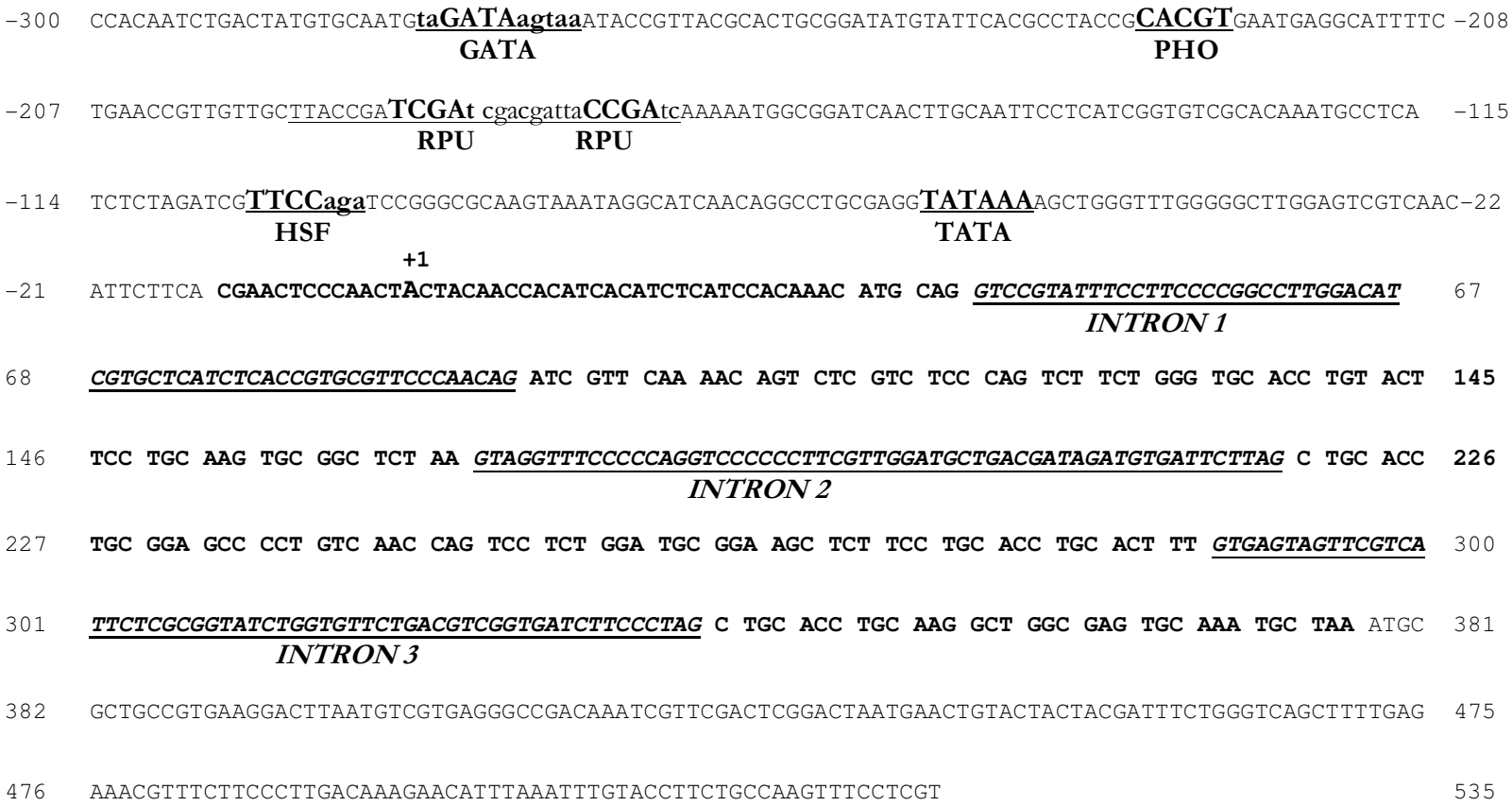


Figure 40: Nucleotide sequence of *HcMT2* genomic DNA. Responsive elements were shown in bold and underlined; introns sequences are shown in italic with underline.

4.6 Organization of *HcMT1* and *HcMT2* in fungal genome

To investigate the copy number of *HcMT1* and *HcMT2* in *H. cylindrosporum* genome, Southern blot analysis was performed using genomic DNA. Thirty micrograms of genomic DNA was digested with four different restriction enzymes: *Eco*R V, *Bam*H I. *Xho* I and *Pst* I, which do not cut *HcMT1* or *HcMT2* (Fig. 41). The digested DNA was capillary transferred to a nitrocellulose membrane and fixed with U.V cross link. Then the membranes were hybridized with radioactive labeled *HcMT1* and *HcMT2* cDNAs as probes. Probes were prepared using PCR by single labeling with radiolabelled α ^{32}P dCTP. The membranes were exposed to a phosphorimage screen and quantification of hybridization signals were done by using Bio Rad phosphorimage system (Bio Rad, Foster City, CA, USA). Single hybridization band was observed with genomic DNA digested with *Eco*R V, *Bam*H I, *Xho* I and *Pst* I (Fig. 42). These results showed that both the genes exist as single copy in the genome of *H. cylindrosporum.*

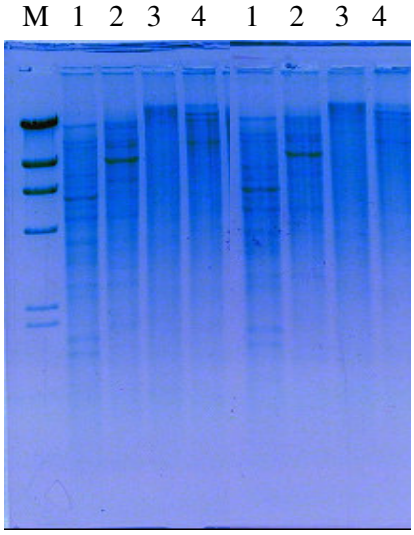


Figure 41: *H. cylindrosporum* genome DNA was digested with four different restriction enzymes *Eco*R V, *Bam*H I. *Xho* I and *Pst* I. The λ DNA digested with Hind III was used as a molecular marker (lane M**).** Lane 1: *Eco*R V; lane 2, *Bam*H I; lane 3, *Xho* I; lane 4: *Pst* I.

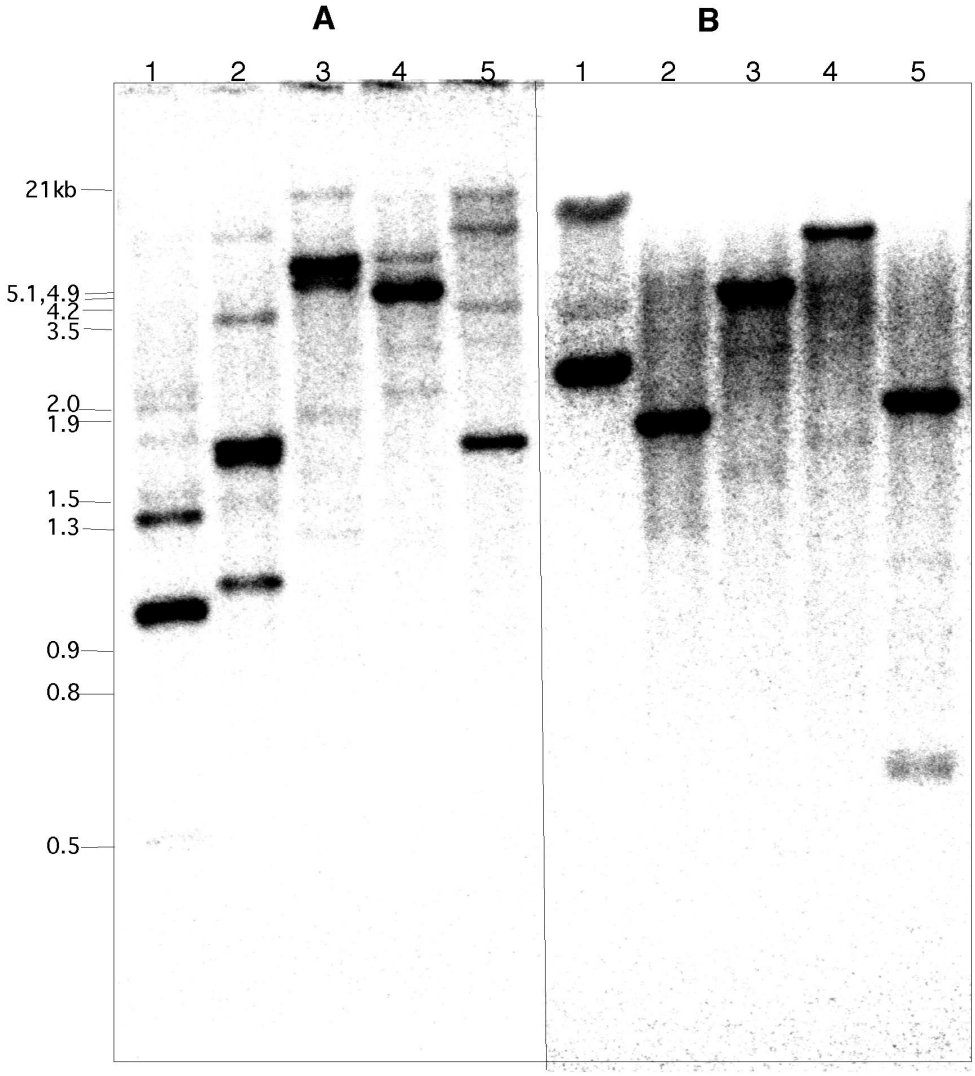


Figure 42: Southern blot analysis of gene copy number for A) *HcMT1* and B) *HcMT2*. Genomic DNA was digested with the indicated restriction enzymes and DNA fragments were separated on a 0.8% agarose gel, transferred to nylon membranes and hybridized with a radiolabelled *HcMT1* and *HcMT2* probes. The λ DNA digested with *Hind* III was used as a molecular marker (lane 1). Lane 2: *Eco*R V; lane 3, *Bam*H I; lane 4, *Xho* I; lane 5, *Pst* I.

4.7 Expression of metallothionein genes in presence of heavy metals

Competitive RT-PCR (cPCR) was used for the quantification of *HcMT1* and *HcMT2* transcripts. Competitive RT-PCR is based upon the co-amplification with the same primer pairs of the target sequence with a known amount of a DNA fragment (competitor), which differs from the target, by its size. Genomic DNA of *H. cylindrosporum* was used as competitor for cPCR. The genomic DNA was amplified using *HcMT1* and *HcMT2* primer pairs and amplified products were gel purified and cloned into pGEM-T Easy vector. The plasmid containing competitors were used to quantify *HcMT1* and *HcMT2* expression levels, a 490 bp long cloned *HcMT1* genomic DNA amplified PCR product for *HcMT1* and 295 bp long genomic DNA amplified PCR product in case of *HcMT2*. Before studying the expression levels of *HcMT* genes, various parameters such as $MgCl_2$, dNTPs concentration, primers concentration and number of PCR cycles were optimized for cPCR. Various concentration of $MgCl_2$, dNTPs and primers were used to obtain optimal amplification. For cPCR studies, 1.5 mM $MgCl_2$, 0.2 µm primers, and 100 µm dNTPs were optimized (Fig. 43a,b,c). To ensure that the competitor and the target DNA amplify with the same efficacy in the PCR mixture, equal volume of competitor was taken and amplified in different number of cycles (20-35). To reduce the effects of competitor for limiting factors such as primers and nucleotides, it was important that the amplification cycle number did not exceed the exponential phase. Following comparison of amplification products over the 20-35 cycles, no increase in the amplification was observed after 25 cycles (Fig. 43d). The cycle number was therefore deemed exponentially at 25 cycles and used through out the expression studies.

Before studying the expression of *HcMT* genes, the kinetics of the competitor when it is co-amplified with the corresponding target gene in plasmid DNA was studied. For this, various amounts of competitor was co-amplified with constant amount of target gene and depending on the band intensities of the competitor and target gene, constant amount of competitor was setup for expression of metallothionein gene in response to different heavy metals. For example, the optimal level of competitor was identified by co-amplifying with different concentration of competitor (2, 3, 4, 5 and 6 pg) and constant amount of *HcMT1* gene. Depending on the band intensities of the competitor and *HcMT1* gene, 4 pg of competitor was setup for expression of *HcMT1* gene in response to copper (Fig. 44).

Standard graphs were constructed based on the amplifying different known quantities of plasmid containing target DNA in the presence of constant amount of plasmid containing competitor. For example, different concentrations of plasmid containing target DNA (2, 5, 10, 15, 20 and 25 pg) was amplified with constant amount of plasmid containing competitor (4 pg) to obtain standard curve for copper studies (Fig. 45). The ratio of target versus competitor DNA amplification yields were plotted as a function of the initial target DNA concentration. All values were transformed in to logarithmic values to enable the construction of a linear graph. The correlation formula of the curve was used in the transformation of target/competitor DNA ratio to picograms of mRNA transcripts.

RNA was extracted from three independent batches of each treatment. Two reverse transcription reactions were performed from each RNA sample and three competitive PCR reactions were carried out with each cDNA sample. The results obtained in

replicates were expressed by reference to a control and transcript level was expressed using arbitrary units where 1 corresponds to expression level of control.

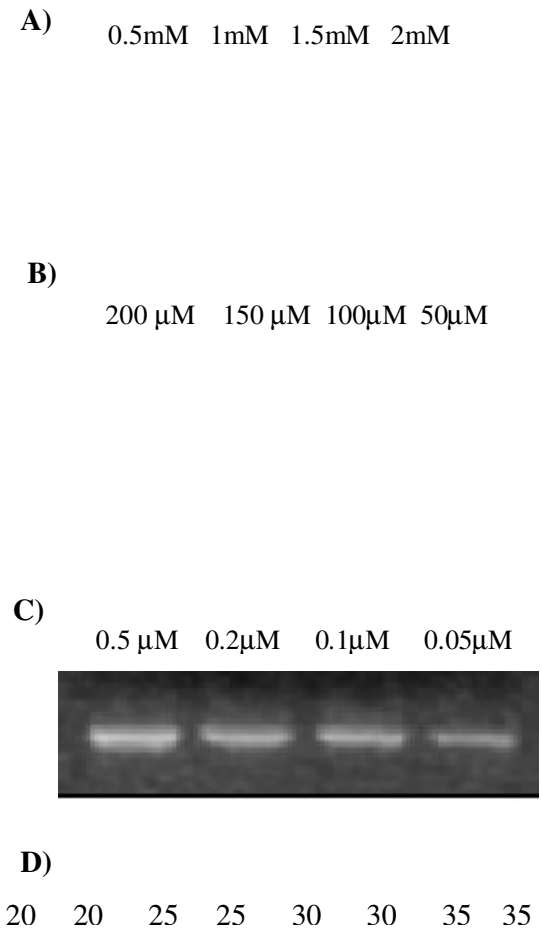


Figure 43: Optimization of PCR reaction for expression studies. Different concentrations of PCR components were tested for optimal amplification. A) 1.5 mM of $MgCl_2$, B) 100 µM of dNTPs, C) 0.2 µM of Primers were optimized. D) Equal volume of competitor was taken and amplified in different number of cycles and comparison of amplification products over the 20-35 cycles, no increase in the amplification was observed after 25 cycles.

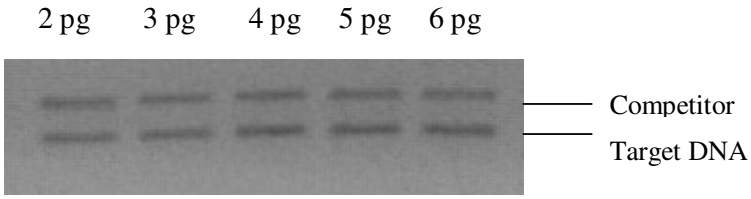


Figure 44: Optimization of competitor. Different concentrations (2, 3, 4, 5 and 6 pg) of competitor were co amplified with target DNA and depending on the band intensities of the competitor and target gene, constant amount of competitor was setup for expression studies.

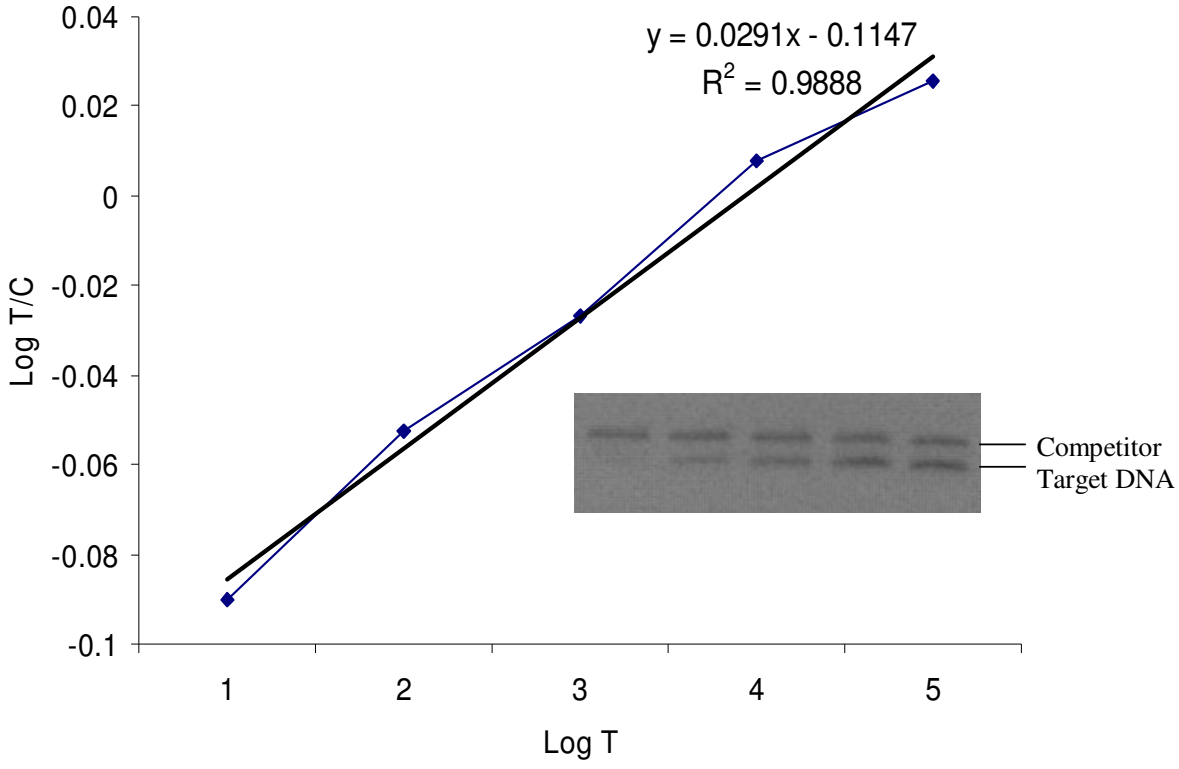


Figure 45: Standard graph to quantify *HcMT* genes expression by competitive RT-PCR. A range of different concentration of target DNA (2, 5, 10, 15, 20 and 25 pg) was amplified in the presence of constant amount of competitor. The ratio of target versus competitor DNA amplifications was calculated (log T/C).

4.7.1 Expression of metallothionein genes by different heavy metals

4.7.1.1 Kinetics of metallothionein genes by copper

To determine the induction of *HcMT1* and *HcMT2* genes in response to copper, the transcript accumulation kinetics was studied. For this, 15 days old *H. cylindrosporum* culture was treated with 320 µM of Cu for 0, 12, 24, 36, 48, 60 and 72 hours time intervals. Total RNA was isolated and cDNA prepared by RT-PCR.

The copper-induced accumulation of *HcMT1* mRNA was observed after 12 hours of treatment, afterwards mRNA accumulation increased sharply and a maximum 350 fold induction was observed at 24 hours as compared with untreated mycelium. Then there was decline in the expression of *HcMT1* after 24 hours treatment of copper and mRNA levels were decreased at 60 hours (Table. 10 and Fig. 46). In case of *HcMT2,* transcript accumulation levels were significantly increased to almost a maximum of 100 fold until 24 hours when compared with control. Transcript accumulation increased sharply at 12 and 24 hours of copper treatment, then decreased over the rest of the time course (Table 10 and Fig. 46). Over the 72 hour time course, maximum transcript accumulation of both genes was observed at 24 hours and decreased thereafter. The highest mRNA accumulation was recorded with *HcMT1,* whereas *HcMT2* was slightly less sensitive to Cu induction. Over the 24 hours time course, the most rapid response was observed with *HcMT1,* whereas the *HcMT2* response was less and the accumulation was much smaller in amount. After 24 hours, the *HcMT1* accumulation was decreased with increasing incubation time, but *HcMT2* transcript accumulation dropped very sharply and expression levels were almost undetected. In response to copper, *HcMT1* might be dominant over *HcMT2*.

Table 10: Induction kinetics of *HcMT1* and *HcMT2* genes in response to copper

Time (hours)	*HcMT1*	*HcMT2*
0	1.0±0.07e	1.0±0.25c
12	15.9±0.35d	59.1±3.50b
24	337.1±27.58a	113.7± 28.45a
36	288.9±18.65b	3.9±0.19c
48	187.7±12.50c	3.48±0.78c
60	8.4±0.98e	2.1±0.27c
72	1.6±0.65e	2.0±0.10c

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

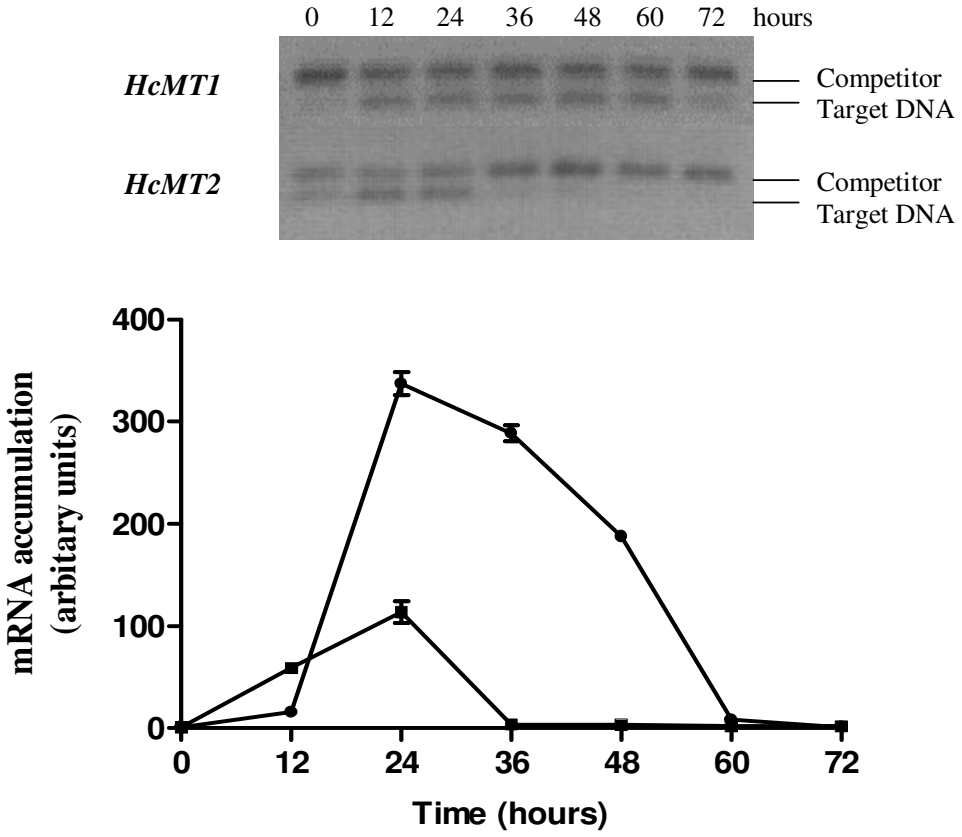


Figure 46: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after incubation in medium supplemented with 320 µM of Cu. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

4.7.1.2 Kinetics of metallothionein genes by cadmium

The induction kinetics of *HcMT1* and *HcMT2* mRNA transcript accumulation in response to cadmium was studied by transferring the 15 days old *H. cylindrosporum* mycelium to a medium supplemented with 21 µM of Cd for 0, 12, 24, 36, 48, 60 and 72 hours time intervals. Total RNA was isolated and cDNA prepared by RT-PCR.

Enhanced expression of *HcMT2* was observed after 12 hours and continued until 48 hours of cadmium treatment. A 40 fold higher induction level of *HcMT2* mRNA was observed with cadmium exposure for 48 hours. After 48 hours, the *HcMT2* transcript accumulation was decreased and this reduction continued until 72 hours (Fig. 47).The *HcMT1* mRNA was not induced by cadmium (Fig. 47). Over the 72 hour time course, *HcMT2* mRNA accumulation was markedly enhanced until 36 hours under cadmium stress, conversely *HcMT1* was not induced by Cd treatment with all the time periods tested.

The difference in expression level of *HcMT1* and *HcMT2* to cadmium might be a divergent pattern of gene regulation. The present results confers that *HcMT2* might be involved in intracellular cadmium detoxification.

Table 11: Induction kinetics of *HcMT1* and *HcMT2* genes in response to cadmium

Time (hours)	*HcMT1*	*HcMT2*
0	1.0±0.01d	1.0±0.2d
12	1.1±0.00ab	14.7±0.9c
24	1.1±0.00a	23.1±2.6b
36	1.1±0.00a	33.1±3.4a
48	1.0±0.01b	37.1±7.4a
60	1.0±0.01b	5.6±0.5d
72	0.9±0.01c	4.8±0.4d

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

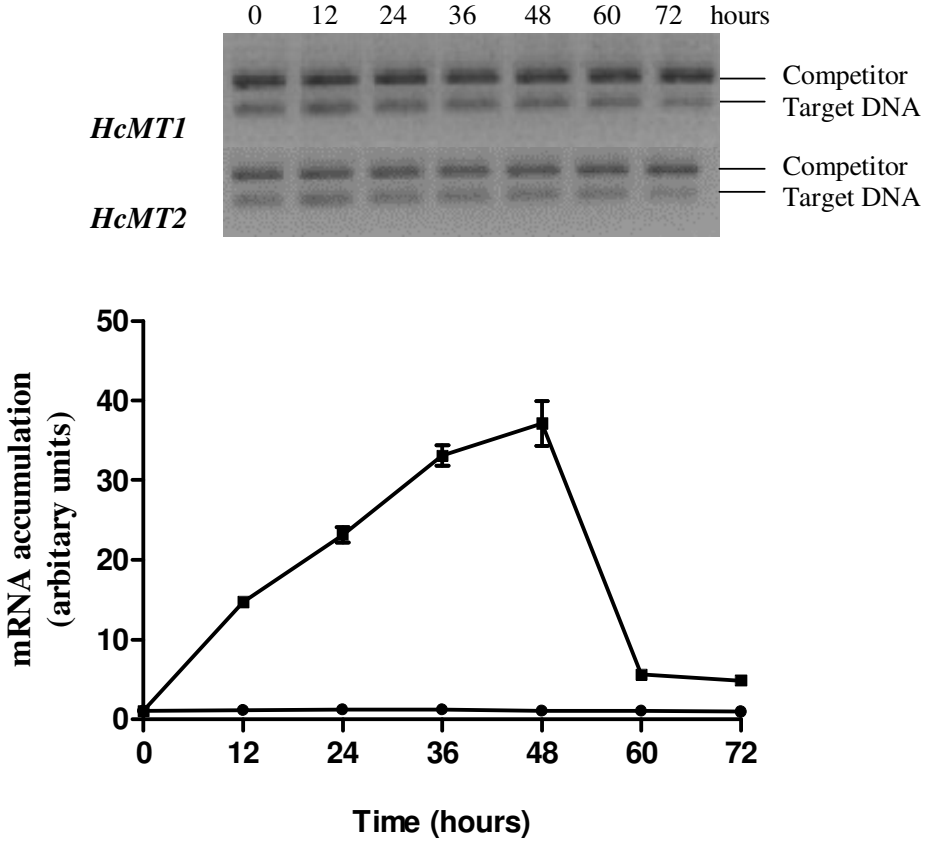


Figure 47: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after incubation in medium supplemented with 21 µM of Cd. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

4.7.2 Dose response of metallothionein genes by different heavy metals

4.7.2.1 Dose response of copper on metallothionein genes

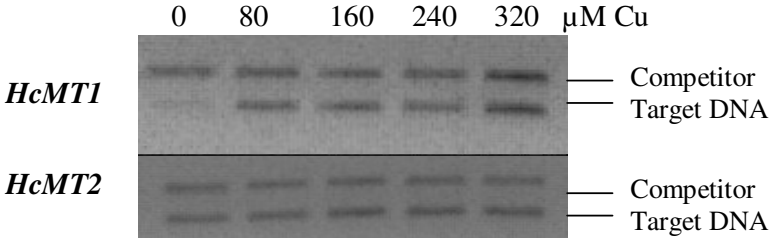
To study the metallothionein induction by different concentrations of copper, the fungus was first grown in MMN liquid medium for two weeks. Then the mycelium was transferred to fresh medium containing different concentrations of copper (0, 80, 160, 240 and 320 µM) and incubated for 24 hours. Total RNA was isolated and cDNA was synthesized.

Transcription of *HcMT1* was significantly increased with increasing concentrations of copper and a maximum of 344 fold higher induction was observed at 320 µM of copper treatment, when compared to control. The accumulation of mRNA was begun at 160 µM of copper treatment and thereafter a sudden increase in mRNA accumulation was observed at 240 µM and 320 µg/ml of copper treatment (Fig. 48). The mRNA accumulation of *HcMT2* was increased as the concentration of Cu increased and the maximum accumulation was recorded at 320 µM where it was almost 100 times higher than the control. Transcript accumulation of *HcMT2* was detected at 80 µM of copper treatment, thereafter significantly and progressively increased to 320 µM of copper (Fig. 48). The transcription of *HcMT1* and *HcMT2* were clearly up regulated under copper stress. But irrespective of Cu concentrations; *HcMT2* was much less sensitive to Cu induction than *HcMT1*.

Table 12: Dose responsive studies of *HcMT1* and *HcMT2* genes in response to copper

Cu conc.	*HcMT1*	*HcMT2*
0	1.0±0.09d	1.0±0.1c
80	2.4±0.37d	4.4±0.5c
160	11.8±1.07cd	28.4±2.7b
240	297.6±26.29b	84.1±15.1a
320	344.2±27.10a	96.6±14.2a

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)



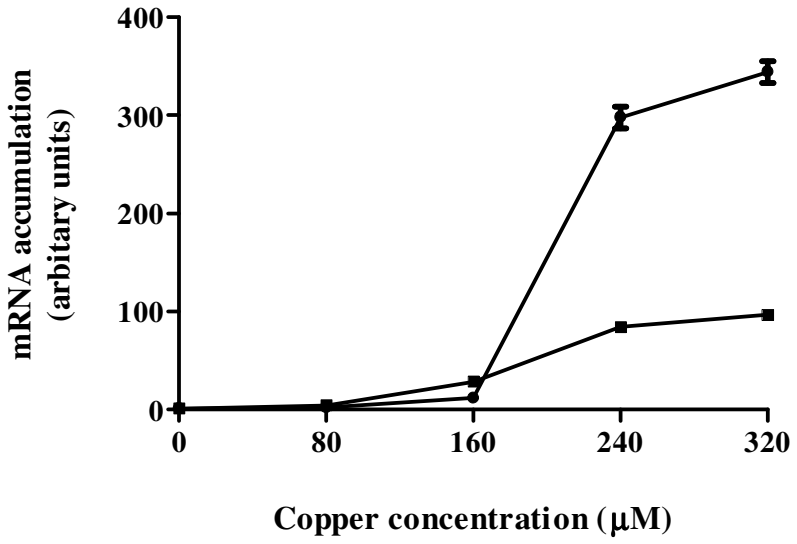


Figure 48: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after 24 hours incubation in medium supplemented with different concentrations of Cu. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

4.7.2.2 Dose response of cadmium on metallothionein genes

Fifteen days old *H. cylindrosporum* culture was treated with different concentrations of cadmium (0, 7, 14, 21 and 28 µM). The metal treated mycelium was harvested after 24 hours and the expression of *HcMT1* and *HcMT2* was studied. Results revealed that *HcMT1* expression was not induced by any of the concentrations of Cd used (Fig. 49). Cadmium-induced transcription of *HcMT2* increased as a function of metal concentration increased up to a maximum of 21 µM, thereafter decreased. Significant gradual increase of *HcMT2* transcript accumulation level was found till 21 µM of cadmium exposure and a maximum increase of 37 fold observed upon 21 µM of cadmium exposure compared with control. The expression of transcript accumulation levels dropped significantly at 28 µM of Cd concentration (Fig. 49).The study revealed that only *HcMT2* gene was induced by cadmium but not *HcMT1*.

Table 13: Dose responsive studies of *HcMT1* and *HcMT2* genes in response to cadmium

Cd conc.	***HcMT1***	***HcMT2***
0	1.0±0.01c	1.0±0.057d
7	1.2±0.02b	13.0±1.25c
14	1.2±0.03b	19.5±2.01b
21	1.4±0.03a	37.8±3.75a
28	0.9±0.02c	2.3±0.13d

Values sharing a common letter within the column are not significant at $P<0.05$ (Mean±SEM)

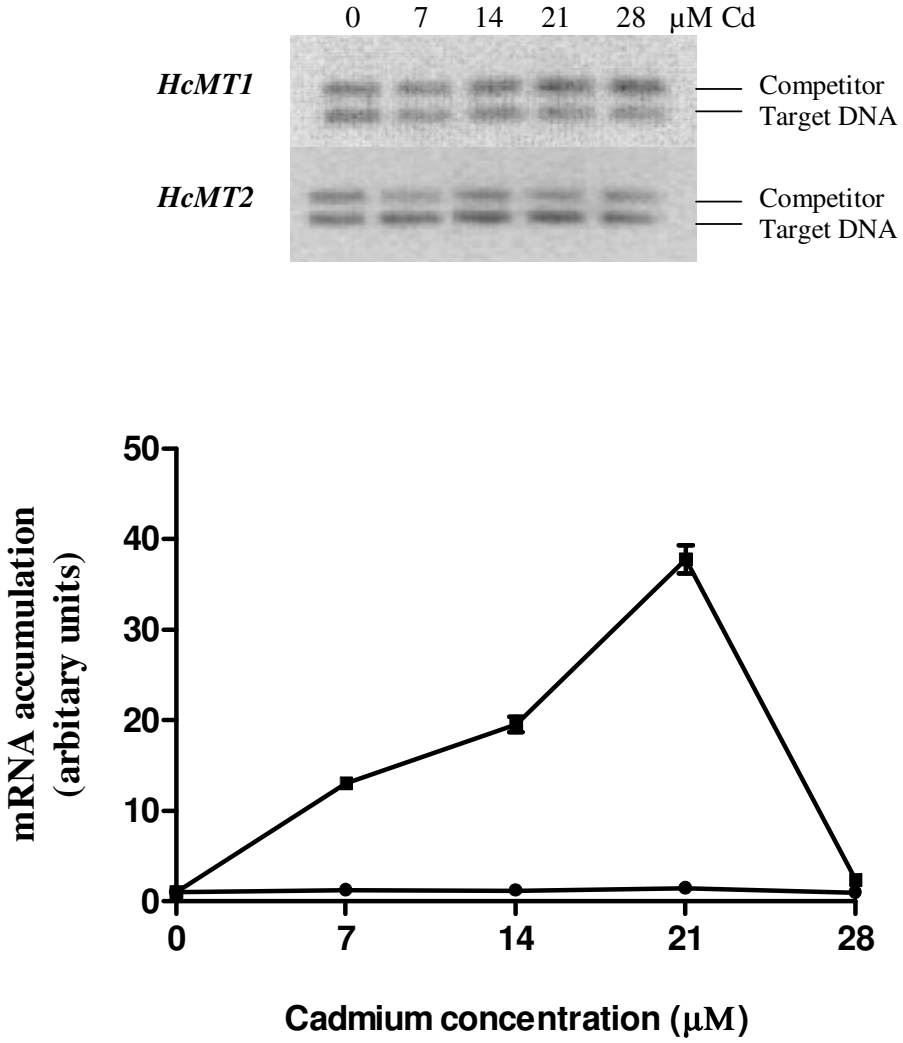


Figure 49: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after 24 hours incubation in medium supplemented with different concentrations of Cd. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

4.7.3 Expression of metallothionein genes in response to zinc, nickel and lead

To study, whether *HcMT1* and *HcMT2* genes was induced by Zn metal, which is a potent inducer of MTs in higher eukaryotes, 15 days old grown *H. cylindrosporum* culture was treated with different concentration of Zn (0, 0.5, 1 and 1.5 mM) for 24 hours and the expression of *HcMT1* and *HcMT2* was studied. Expression results indicated that both the genes were not affected with Zn stress and there was no significant increase in transcripts of both the genes. The results conclude that *HcMT1* and *HcMT2* were not induced by Zn (Table 14 and Fig. 50).

In an attempt to further document the regulation of *HcMT1* and *HcMT2*, the effect of various Ni and Pb concentrations were tested on the expression of *HcMT1* and *HcMT2*. For this, 15 days old *H. cylindrosporum* was treated with various concentration of Ni (0, 85, 170, 250 and 350 µM) for 24 hours. Expression results revealed that both the genes were not induced by Ni stress (Table 15 and Fig. 51). For Pb stress, 15 days old grown *H. cylindrosporum* was treated with different concentration of Pb (0, 25, 50, 75 and 100 µM) for 24 hours. Results revealed that Pb metal did not lead a significant up regulation of *HcMT1* and *HcMT2* transcription (Table 16 and Fig. 52).

Table 14: Dose responsive studies of *HcMT1* and *HcMT2* genes in response to zinc

Zn conc.	*HcMT1*	*HcMT2*
0.0	1.0±0.14a	1.0±0.09a
0.5	0.9±0.07b	0.8±0.13b
1.0	1.0±0.15a	1.0±0.08a
1.5	1.0±0.18a	0.9±0.09a

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

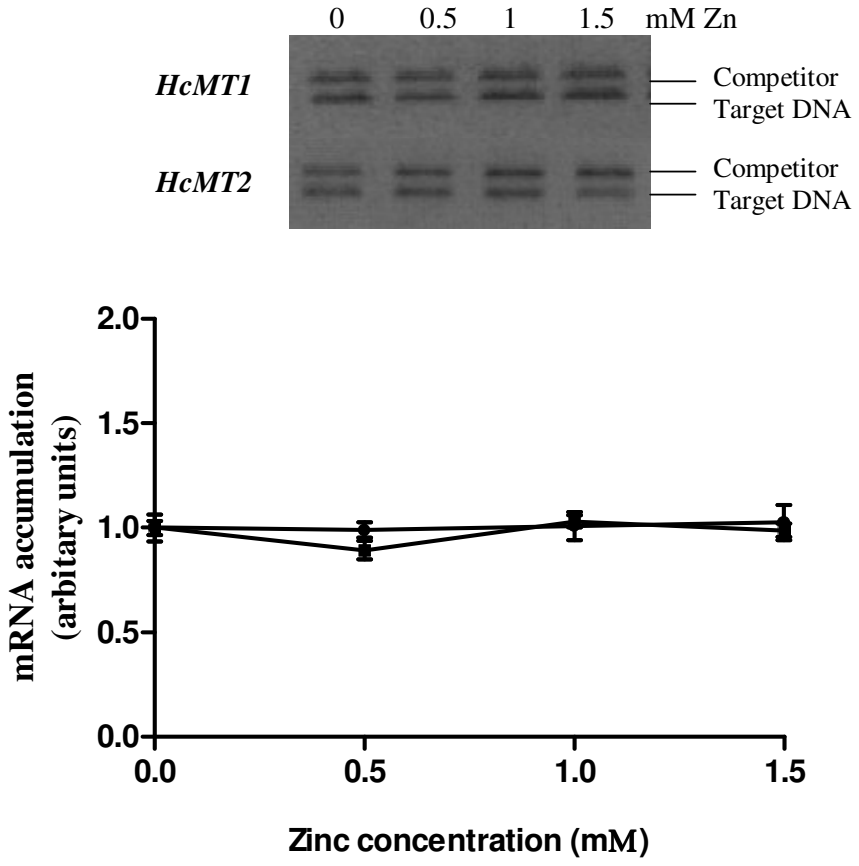


Figure 50: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after 24 hours incubation in medium supplemented with different concentrations of Zn. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

Table 15: Dose responsive studies of *HcMT1* and *HcMT2* genes in response to nickel

Ni conc.	*HcMT1*	*HcMT2*
0	1.0±0.06b	1.0±0.02a
85	1.1±0.02a	0.9±0.02b
170	1.1±0.04a	0.9±0.03b
250	1.1±0.08a	1.0±0.02a
350	1.1±0.02a	1.0±0.03a

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

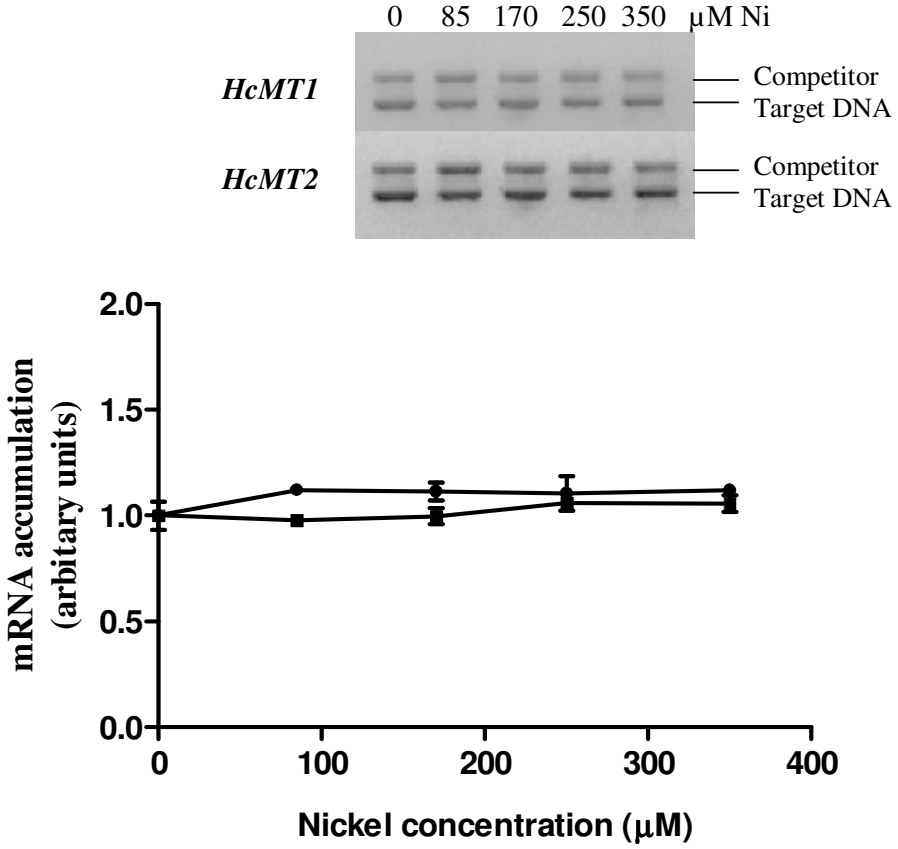


Figure 51: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after 24 hours incubation in medium supplemented with different concentrations of Ni. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

Table 16: Dose responsive studies of *HcMT1* and *HcMT2* genes in response to nickel

Pb conc.	*HcMT1*	*HcMT2*
0	1.0±0.02b	1.0±0.02a
25	1.1±0.01a	0.9±0.02b
50	1.0±0.03a	1.0±0.02a
75	1.1±0.00a	1.0±0.01a
100	0.9±0.03b	1.0±0.02a

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

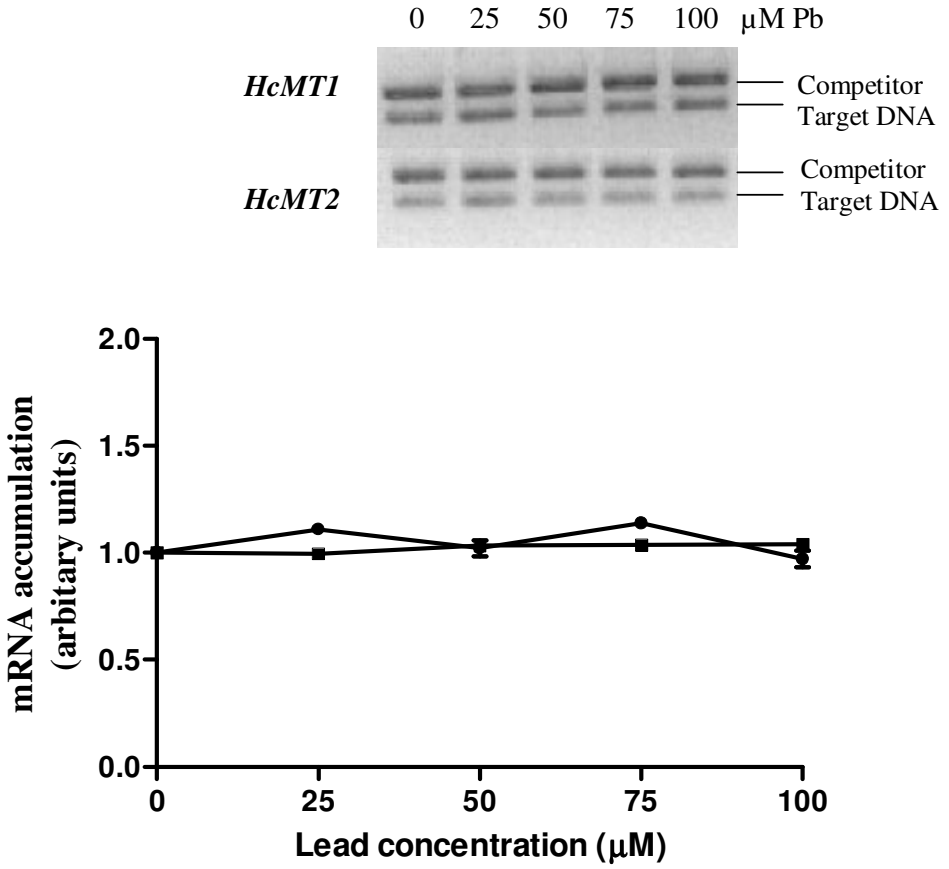


Figure 52: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after 24 hours incubation in medium supplemented with different concentrations of Pb. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

4.7.4 Expression of metallothionein genes in response to oxidative stress

Metallothioneins are mainly involved in metal detoxification. However, when metal in excess, it generates reactive oxygen species via the Fenton reaction. Several studies indicated that metallothioneins play a role in protecting against the effect of reactive oxygen species. To gain more information about the putative role of *HcMT1* and *HcMT2* genes in oxidative stress, the effect of H_2O_2 on both the genes were analyzed. Fifteen days old *H. cylindrosporum* culture was treated with 25 mM of H_2O_2 for different time intervals 0, 12, 24, 36, 48, 60 and 72 hrs. Total RNA was isolated and cDNAs were synthesized by RT-PCR method.

Expression levels of both *HcMT1* and *HcMT2* were increased in presence of oxidative stress. The transcript accumulation of *HcMT1* significantly increased till 48 hours and thereafter decreased. Initially 5 fold transcript accumulation was detected at 12 hours and continued to increase to 48 hours, where a maximum of 35 fold was detected. Then expression levels decreased over the remaining (Fig. 53). In the *HcMT2*, the transcript accumulation was significantly increased until 36 hours and maximum 6 fold induction were observed (Fig. 53). In comparison, the *HcMT2* gene was very less sensitive than *HcMT1*. The induction levels were not high compared to the expression levels under metal stress, suggesting the expression of *HcMT1* and *HcMT2* is more specific in response to metals than oxidative stress. However, these results suggests a role of MTs in maintaining the local redox balance either by sequestering copper and preventing potentially deleterious Fenton chemistry reactions.

Table 17: Induction kinetics of *HcMT1* and *HcMT2* genes in response to oxidative stress

Time (hours)	*HcMT1*	*HcMT2*
0	1.0±0.05g	1.0±0.10f
12	3.7±0.03f	2.4±0.05d
24	10.9±0.04c	4.6±0.06b
36	17.2±0.06d	7.8±0.07a
48	33.7±0.13a	2.5±0.04c
60	8.7±0.05d	1.3±0.05e
72	3.9±0.09e	1.0±0.08f

Values sharing a common letter within the column are not significant at P<0.05 (Mean±SEM)

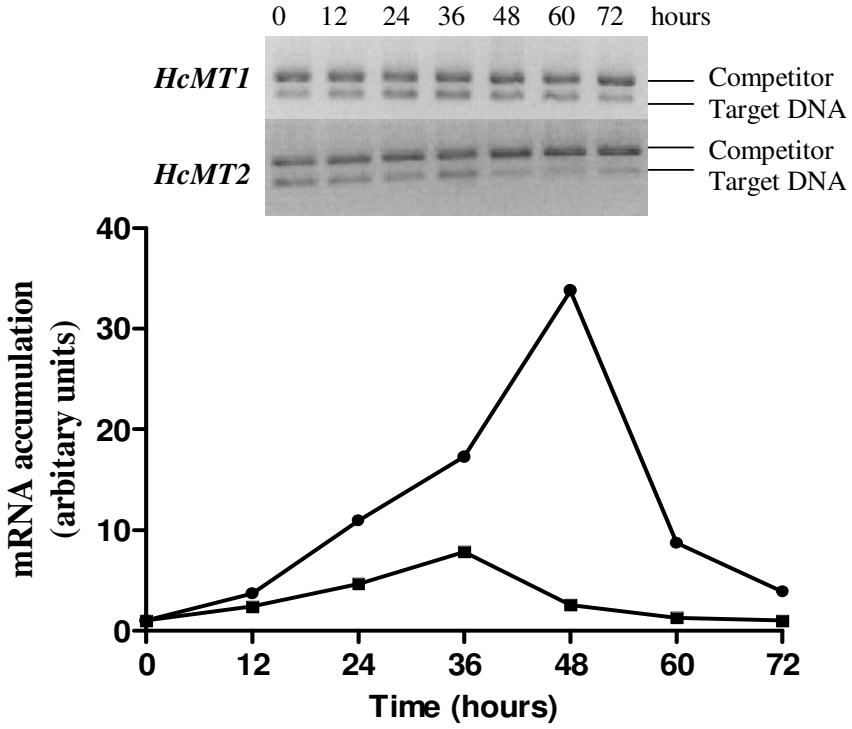


Figure 53: Expression of *HcMT1* (circles) and *HcMT2* (squares) in *H. cylindrosporum* after incubation in medium supplemented with 25 mM of H_2O_2. Transcript accumulation was quantified by competitive RT-PCR and expressed as arbitrary units where 1 is transcript level at zero time. Bars represent ± standard error (P≤0.05)

4.7.5 Yeast complementation studies

To characterize *HcMT* genes further, both genes were expressed in *Saccharomyces cerevisiae* mutant strains, which are unable to grow on high concentration of metals. Two copper-sensitive strains DTY3 (*MATα, leu2-3, 112his3$^{\Delta}$1, trp1-1, ura3-50, gal1 CUP1^{s}*) and DTY4 (*MATα, leu2-3, 112his3$^{\Delta}$1, trp1-1, ura3-50, gal1*, *cup1::URA3*) referred as *cup1^{s}* and *cup1$^{\Delta}$* respectively, used for the complementation studies for copper. The *CUP1* gene of *S. cerevisiae* encodes metallothionein protein, which is induced by only copper (Butt and Ecker, 1987). The wild type yeast DTY3 contains single copy of *CUP1* gene into genome and it confers resistant to copper up to 150 µM, which if deleted confers the yeast to copper sensitivity. The DTY4 (*cup1$^{\Delta}$)* yeast strain was developed from DTY3 wild type strain by deleting the *CUP1* gene. It is highly sensitive to Cu because of the complete disruption of *CUP1* gene, which regulates *cup1* expression under copper stress and unable to grow on copper containing media (Hamer *et al.,* 1985).

Cadmium sensitive *yap1* mutant of *S. cerevisiae* was used for cadmium complementation studies. *yap1* is a basic leucine zipper (bZIP) transcription factor required for oxidative stress tolerance; mediates pleiotropic drug and metal resistance. Nuclear localization and trafficking between the nucleus and cytoplasm is essential for Yap1p regulation. The *yap1* gene encodes a transcription factor related to the mammalian AP-1 complex that positively controls various genes involved in metal tolerance and, more generally, oxidative stress tolerance in yeast (Kuge and Jones, 1994). Its deletion renders the yeast mutant highly sensitive to Cd (Wu *et al*., 2003).

The complementation studies were carried out in yeast stains using p424 expression vector. Plasmid p242 is a high copy number yeast/*E. coli* shuttle vector. Plasmid p424

contains ampicillin for selection in *E. coli* and *TRP1* for yeast. The p424 expression vector is comprised of cassette, composed of the strong GDP promoter (glyceraldehyde- 3-phosphate dehydrogenase), flanked by a multiple cloning site (MCS) and the CYC1 terminator. The multiple cloning site based on pBIISK (Stratagene) provides nine unique restriction sites, which facilitates the cloning of genes. This cassette was placed into both the centromeric and 2μ plasmids of the pRS series containing *TRP1* marker (Mumberg *et al.,* 1995) (Fig. 55).

The ORF of *HcMT1* and *HcMT2* were obtained from cDNA using MT1F and MT1R and MT2F and MT2R primers introducing *Bam*H I and E*co*R I sites. Approximately 200 bp of ORF fragment was obtained for both genes (Fig. 54). The PCR products were digested with *Bam*H I and *Eco*R I and ligated into the yeast expression vector p424. The yeast vector p424-HcMT1 or p424-HcMT2 was constructed under the transcriptional control of the yeast GPD (glyceraldehyde- 3-phosphate dehydrogenase) promoter. Vector p424 and the construct p424-HcMT1 and p424-HcMT2 were introduced into *cup1*$^{\Delta}$ and *yap1* cells using lithium acetate procedure and transformed cells were selected by their capacity to grow in complete synthetic medium (SD), without Trp (p424 vector selection marker) and Ura (*cup1*$^{\Delta}$ strain selection marker) (SD-Trp-Ura medium) and SD medium without Trp for *yap1* mutant. For the functional complementation experiments, cultures of *cup1*$^{\Delta}$ and *yap1* yeast cells carrying either p424 or p424-HcMT1 and p424-HcMT2 were grown in respective medium at 30^{o}C and 220 rpm. Yeast cultures were adjusted to OD_{600} =1.0 and 5 μl of 10 fold serial dilutions were spotted on SD plates and on SD supplemented with 150 μM $CuSO_4$ and 40 μM $CdSO_4$ plates. Plates were incubated for 3 days at 30^{0}C and photographed. In parallel experiments, Falcon jars containing

20 ml of fresh SD-Trp-Ura and SD-Ura media were inoculated with mid-log precultures of *cup1*$^{\Delta}$ and *yap1* cells containing p424, p424-HcMT1 and p424-HcMT2 to attain a starting optical density of 0.02 at 600 nm. Cells were grown at 30^0C and 220 rpm and $CuSO_4$ (150 µM) and $CdSO_4$ (40 µM) were added 5 hours after inoculation. The optical densities of the cultures were measured at 3 hours interval for 42 hours.

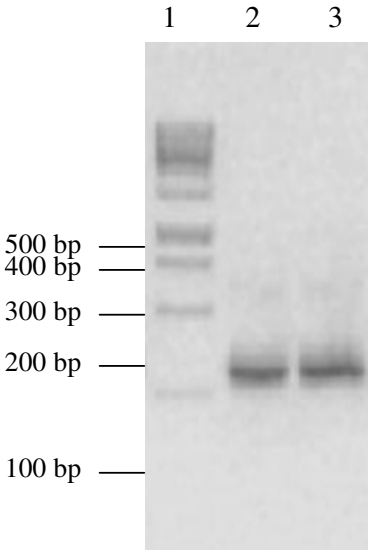


Figure 54: The ORF amplification of *HcMT1* and *HcMT2* using MT1F and MT1R and MT2F and MT2R primers (Primers contain *Bam*H I and E*co*R I sites) Lane 1: 100 bp ladder, Lane 2: ORF of *HcMT1* gene, Lane 3: ORF of *HcMT2* gene

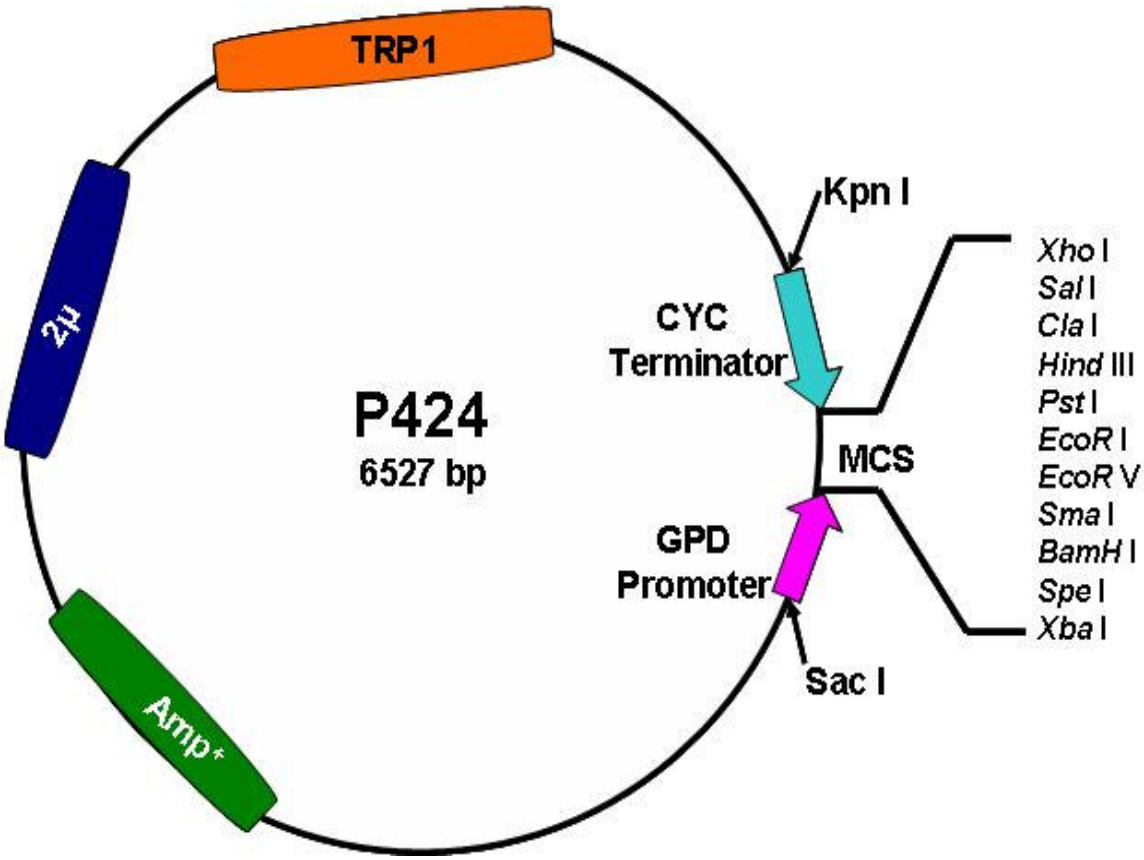


Figure 55: Structure and restriction map of p424 yeast expression vector (Adapted form Mumberg *et al.*, 1995). The p424 vector contain GDP promoter (glyceraldehyde- 3-phosphate dehydrogenase), flanked by a multiple cloning site (MCS) and the CYC1 terminator. The multiple cloning site composed of nine unique restriction sites for cloning of insert, it is based on pBIISK (Stratagene) provides. It contains 2μ for self replication, *TRP1* as marker for selection in yeast and Amp^+ as marker for selection in bacteria.

4.7.5.1 Copper studies

Complementation studies indicated that *cup1*^{s} cells were able to grow on 150 µM copper amended media as it contains single *CUP1* gene responsible for copper detoxification, whereas empty p424 transformed *cup1*$^{\Delta}$ cells were unable to grow at 150 µM copper supplemented media and p424-HcMT1 and p424-HcMT2 harboring *cup1*$^{\Delta}$ cells were able to grow on 150 µM copper supplemented media (Fig. 56). The *cup1*$^{\Delta}$ containing empty p424, p424-HcMT1 and p424-HcMT2 cells were tested for resistance to 150 µM of copper in a liquid medium growth assay for 42 hours. The results showed that the *cup1*$^{\Delta}$ containing empty p424 cells were unable to grow on 150 µM copper containing media and their growth was completely inhibited, whereas p424-HcMT1 and p424-HcMT2 transformed *cup1*$^{\Delta}$ cells growth was increased significantly with increasing time of incubation (Table. 18 and 19; Fig. 57). The present results indicated that both genes were responsible for copper and cadmium tolerance

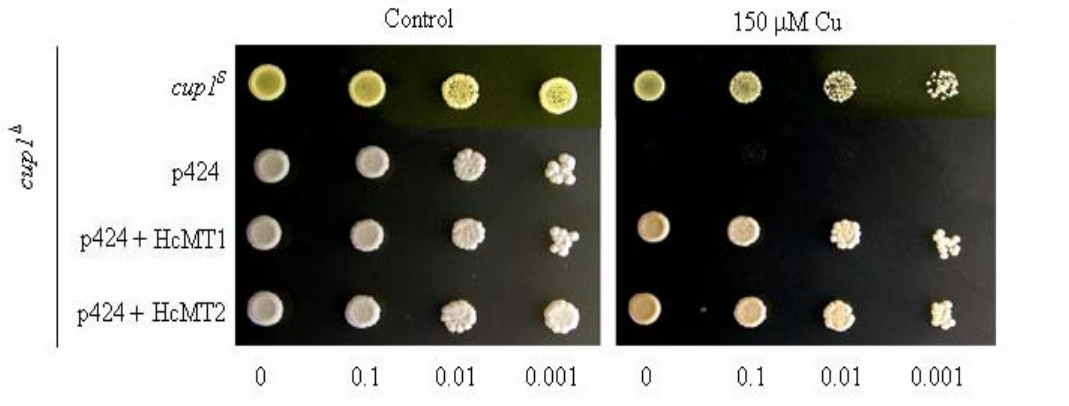


Figure 56: Functional complementation of the yeast mutants on SD medium supplemented (or not) with 150µM Cu. CUP1-null cells harbouring the control vector p424 (*cup1*$^{\Delta}$) or the construction p424-*HcMT1* and p424-*HcMT2* and a parental cells with a single CUP1 gene copy (*cup1*^{s}).

Table 18: Growth curve of *cup1*$^{\Delta}$ cells containing empty p424, p24-HcMT1 and p424-HcMT2 in 150 µM Cu amended media

Time (hours)	P424+control	P424+Cu	P424+HcMT1+ Cu	P424+HcMT2+ Cu
0	0.02±0.010	0.02±0.010	0.02±0.010	0.02±0.010
8	0.07±0.010	0.11±0.001	0.11±0.005	0.11±0.001
11	0.14±0.001	0.12±0.010	0.15±0.004	0.14±0.002
14	0.26±0.010	0.14±0.001	0.19±0.007	0.18±0.002
18	0.78±0.051	0.16±0.002	0.29±0.010	0.30±0.012
21	1.07±0.080	0.17±0.010	0.39±0.002	0.44±0.005
24	1.27±0.057	0.178±000	0.49±0.101	0.58±0.100
27	1.32±0.050	0.18±0.010	0.60±0.095	0.73±0.100
30	1.36±0.010	0.18±0.001	0.75±0.135	0.88±0.100
42	1.33±0.050	0.19±0.011	1.14±0.063	1.22±0.010

Mean±SEM

Table. 19: Two way ANOVA of yeast growth studies in copper amended medium

Source of Variation	Df	Sum-of-squares	Mean square	F
O.D	03	05.7	1.912	843.5***
Time	09	11.6	1.296	571.5***
O.D×Time	27	04.8	0.177	078.4***
Residual	80	00.2	0.003	

Values sharing a common letter within the fungus are not significant at P<0.0001

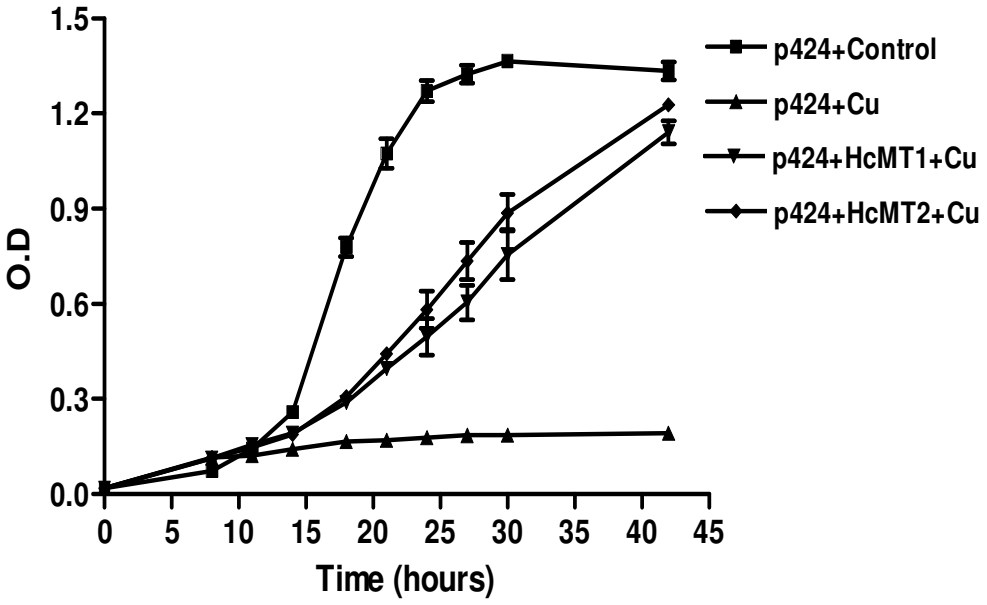


Figure 57: Growth curve of *cup1*$^{\Delta}$ cells in SD-trp medium transformed with empty vector p424 or with p424-*HcMT1* and p424-HcMT2 and *cup1*^{s} strain harbors a single copy of this MT gene and its growth is inhibited at 300µM $CuSO_4$. The Cu-tolerance threshold is reduced to 75µM CuSO4 in *cup1*$^{\Delta}$ supplemented (or not) with 150µM Cu

4.7.5.2 Cadmium studies

The *yap1* mutant cells were transformed with empty vector p424 or with p424-HcMT1 and p424-HcMT2 were spotted on SD medium supplemented (or not) with 40µM Cd. The complementation studies of *yap1* on Cd contain medium showed that *yap1* transformed with empty p424 vector was unable to grow at 40 µM cadmium, whereas the Cd-sensitive phenotype of the *yap* mutant was fully complemented by *HcMT1* and *HcMT2* (Fig. 54). Further, the restoration of Cd tolerance by expression of *HcMT1* and *HcMT2* were confirmed in liquid culture assays. Over 42 hours time course, the growth studies showed that the p424-HcMT1 and HcMT2 containing *yap1* cells growth was significantly increased in medium containing 40 µM Cd with respect to empty p424 containing cells (Table. 20 and 21; Fig. 55). The empty p424 harboring cells growth was very less. These results highlighted the key role of *HcMT* genes in Cd detoxification.

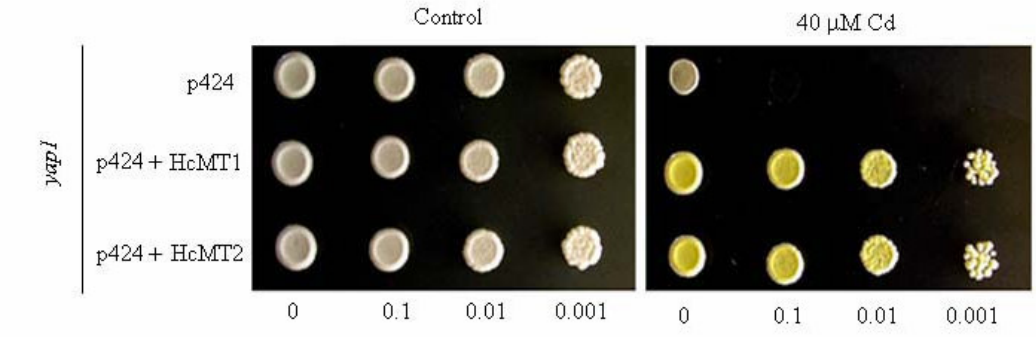


Figure 58: Functional complementation *yap1* yeast mutants on Cd containing medium. *yap1* mutant cells with empty vector p424 were unable to grow on Cd containing media, where as *yap1* mutant cells with p424-*HcMT1* and p424-*HcMT2* were grown well on Cd medium.

Table 12: Growth curve of *yap1* cells containing empty p424, p24-HcMT1 and p424-HcMT2 in 40 µM Cd amended media

Time (hours)	P424+control	P424+Cd	P424+HcMT1+Cd	P424+HcMT2+Cd
0	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
8	0.45±0.10	0.21±0.10	0.25±0.10	0.24±0.00
11	0.97±0.07	0.28±0.10	0.44±0.10	0.39±0.10
14	1.31±0.10	0.33±0.06	0.63±0.03	0.59±0.10
18	1.69±0.01	0.47±0.09	0.92±0.07	0.87±0.11
21	1.68±0.00	0.54±0.09	1.02±0.04	1.03±0.02
24	1.67±0.04	0.63±0.06	1.19±0.05	1.11±0.10
27	1.77±0.10	0.65±0.07	1.26±0.01	1.28±0.10
30	1.66±0.10	0.66±0.05	1.23±0.15	1.24±0.10
42	1.66±0.05	0.86±0.10	1.35±0.10	1.43±0.10

Mean±SEM

Table 13: Two way ANOVA of yeast growth studies in cadmium amended medium

Source of Variation	Df	Sum-of-squares	Mean square	F
O.D	03	10.2	3.42	178.6***
Time	09	21.5	2.39	124.8***
O.D×Time	27	02.6	0.02	005.7***
Residual	80	01.5	0.02	

Values sharing a common letter within the fungus are not significant at P<0.0001

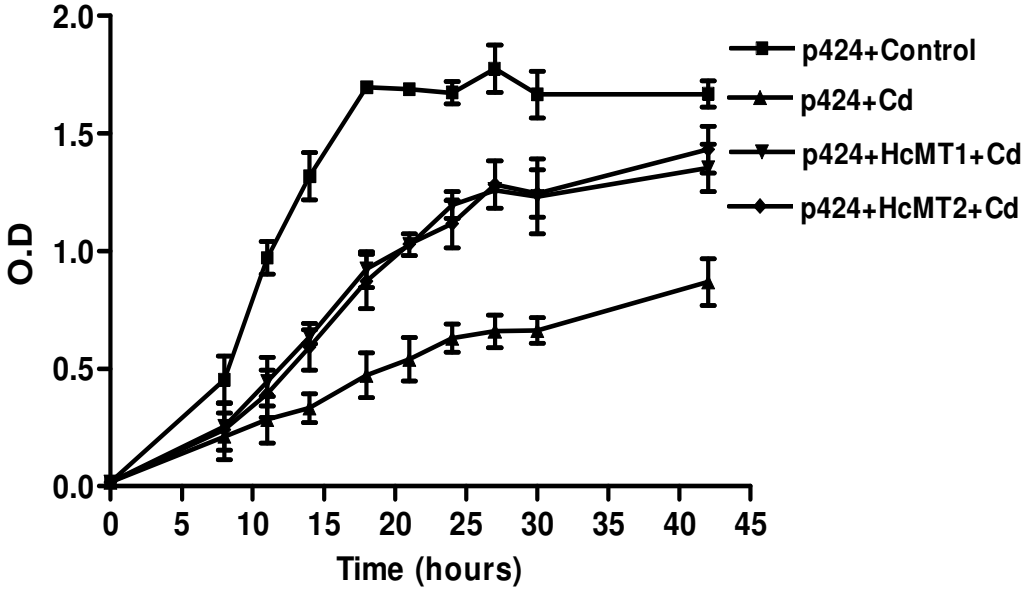


Figure 59: Growth curve of *yap1* cells in SD-trp medium transformed with empty vector p424 or with p424-*HcMT1* and p424-HcMT2 supplemented (or not) 40 µM Cd.

Discussion

5.1 Screening of *H. cylindrosporum* for their tolerance to different heavy metals

Mycorrhizal fungi are involved with a wide variety of activities that benefit plant establishment and growth. Ectomycorrhizal fungi (ECM) are found on most absorbing short roots of tree species in boreal and temperate forests. ECM fungi play dominant role in the nutrition of tress by mobilizing and transporting N and P to roots and provide plants with improved micronutrient, water acquisition and pathogen resistant (Smith and Read, 1997). In addition, ectomycorrhizal fungi are able to protect plants against toxic elements, including trace metals (Leyval *et al.,* 1997). This particular feature is important for enhancing seedling establishment on metal-polluted sites. The mechanism and extent of protection varies with type of host-fungus system as well as the metal pollutants present in the soil. Metals exhibit toxicity above certain concentration and these leads to expression of a detoxification mechanism for survival of organisms. Fungi have evolved a variety of mechanisms to respond or control of heavy metals. Mechanisms include reduced uptake of metals into the cytosol by extracellular chelation through extruded ligands, excretion of organic acids and binding onto cell-wall components. Intracellular chelation of metals in the cytosol by a range of ligands (glutathione, metallothioneins), or

decreased influx and increased efflux from the cytosol out of the cell or into sequestering compartments.

Many ectomycorrhizal fungi are tolerant of higher metal concentrations than their host plant species. Studies have indicated that colonization of tree roots by ectomycorrhizal fungi can increase the tolerance of their hosts in the presence of toxic concentrations of metals (Hartley *et al.,* 1997; Leyval *et al.,* 1997). Axenic screening of metal effects on ectomycorrhizal fungi in pure culture can give basic information regarding fungal metal tolerance. Studies using agar media have been conducted to determine sensitivity of a number of ECM fungal species to a range of potentially toxic metals (Jones and Hutchinson, 1988). However, there are various problems associated with using agar medium in studies of metal sensitivity (Gadd, 1983). Fungal biomass production determined in liquid media provides a more accurate assessment of metal sensitivity, as it is independent of growth form. Furthermore, liquid media allows accurate regulation of the concentration of metal to which the fungus is exposed (Hartley *et al.,* 1997).

The mechanisms involved in conferring the increase tolerance have proved difficult to resolve; they may be quite diverse and show considerable species and metal specificity, since large differences in response to metals have been observed, between fungal species and to different metals within a species (Hartley *et al.,* 1997; Hüttermann *et al.,* 1999). Variation in fungal response to trace metals is due to intrinsic physiological factors. Copper is an essential elements for fungal growth, even slight elevation in Cu concentration has a toxic effects on ectomycorrhizal fungi (Gruhn, 1989). Studies with

axenic culture of ectomycorrhizal species have shown that excessive amounts of Cu can cause a decrease in mycelial dry weight and diameter of mycelial growth on agar media (Gruhn and Miller, 1991; Jones and Hutchinson, 1986; Jones and Muehhlchen, 1994; Tam, 1995; Blaudez *et al.,* 2000a). In one study, *Laccaria laccata* was inhibited by Cu and Al concentration at 10 µg/ml, but not sensitive to the same concentration of Zn, whereas another ectomycorrhizal fungus *T. terrestris* was tolerant of Cu up to 500 µg/ml, Al up to 100 µg/ml and Zn 1000 µg/ml (Jones and Muehhlchen, 1994). A similar response was observed for Cu in *P. involutus* and *Laccaria laccata* grew at 2.5 mM Cu, whereas the *Scleroderma citrinum* growth was restricted at 2.5 mM Cu (Howe *et al.,* 1997). Similarly, *Suillus bovinus* and *T. terrestris* both protected *P. sylvestris* against Cu toxicity, the amount of Cu retained by the two fungi varied considerably (Van Tichelen *et al.,* 2001). Blaudez et al. (2000a) screened different ECM isolates of *P. involutus, P. tinctorius, S. bovinus, S. luteus* and *S. variegatus* in response to Cd, Cu, Ni and Zn and observed strong intraspecific variation in metal tolerance among the isolates. *S. luteus, S. variegates* and *P. tinctorius* were more tolerance to Cu, Cd and Zn when compared to *P.involutus*. Tam (1995) showed considerable variation between the ability of five ectomycorrhizal fungi to grow in a culture with a range of nine different heavy metals and found that *P. tinctorius* isolates showed varied degree of tolerance to Al, Fe, Cu, Zn, Ni, Cd, Cr, Pb and Hg. Several possible mechanisms of tolerance have been investigated for explaining the variation in response to copper among species. Some ectomycorrhizal fungi have been shown to sequester Cu in different locations in the hyphal cell wall, binding on the cell wall through the formation of polyphosphate granules (Gruhn, 1989) and copper precipitate on extranal hyphae (Meharg, 2003). Jones and Hutchinson (1988)

correlated the high mycelial phosphorus content in mycelium and its ability to take up high levels of metals. Ahonen-Jonnarth et al. (2000) found enhanced oxalic acid production in both mycorrhizal and non mycorrhizal tress in response to copper. Howe et al. (1997) and Bellion et al. (2007) suggest that binding of Cu by production of small cysteine rich proteins called metallothioneins in ectomycorrhizal fungi.

Zn is also micronutrient and considered less toxic to ectomycorrhizal fungi. The reasons for higher fungal tolerance of Zn than other trace elements have not been definitively elucidated. Studies showed considerable variation in Zn tolerance among ectomycorrhizal fungi. Symptoms of Zn toxicity in fungi are primarily described as a reduction in mycelial growth (Blaudez *et al.,* 2000a; Denny and Ridge, 1995; Hartley *et al.,* 1997) or as a reduction in ability to colonize a host plant (Hartley *et al.,* 1999; Hartley-Whitaker *et al.,* 2000a; Hartley-Whitaker *et al.,* 2000b). Isolates of *P. involutus* were less tolerant to Zn than any of those of *Amanita muscaria* (Brown and Wilkins 1985a). In another study, *A.muscaria* was found to be the most tolerant to Zn than all fungi tested (Colpaert and Van Assche 1992a). Colpaert and Van Assche (1992b) showed that *P. involutus* showed higher tolerance to Zn, whereas another species *Thelephora terrestris* showed less tolerance to Zn. Similarly, *Paxillus involutus* was sensitive to very low levels (25 ppm) of Zn in the media, whereas other species were not inhibited at concentrations up to 225 ppm Zn (Blaudez *et al.,* 2000a). Ectomycorrhizal fungi have been shown to improve seedling performance in Zn-amended environments (Adriaensen *et al.,* 2003; Brown and Wilkins, 1985a; Bucking and Heyser, 1994; Colpaert and VanAssche, 1992a; Denny and Wilkins, 1987a; Hartley-Whitaker *et al.,* 2000a). However, the way in which these fungi

ameliorate Zn toxicity is unclear. Most proposed mechanisms for Zn were binding in the extrametrical mycelium (Colpaert and VanAssche, 1992; Denny and Wilkins, 1987b). X-ray microanalysis suggested that Zn is bound to electronegative sites on the cell wall and never enters the fungal cytoplasm, thus minimizing toxic effects to the fungus (Denny and Wilkins, 1987b; Tam 1995). Fry et al. (2000) investigated Zn localization in *H. crustuliniforme- Picea abies* root tips and found that Zn accumulated mainly in cell walls of mentle hyphae, Hartig net hyphae and cortical cells. Denny and Ridge (1995) found production of a polysaccharide "slime" in fungi exposed to toxic levels of Zn and correlated to its ability to take up Zn from its surroundings.

In response to Ni, again high variation was observed in ectomycorrhizal fungi (McCreight and Schroeder, 1982). Jones and Hutchinson (1986) showed that *Scleroderma flavidum* was the only species of four tested which was able to increase tolerance of *Betula papyriferae* to nickel. In another study, *Scleroderma flavidum* was shown to be less sensitive to Cu and nickel than *Lactarius rufus* (Jones and Hutchinson, 1988). Tam (1995) found large variation among all ectomycorrhizal fungi tested in response to Ni. All isolates were less tolerant to low concentration of Ni (10 ppm). Similarly Blaudez et al. (2000a) found that *Suillus luteus, Suillus bovinus, Suillus variegatus,* and *P. tinctorius* were less tolerant of nickel, but *P. involutus* showed high degree of nickel tolerance. Brunner and Frey (2000) found accumulation of nickel in the Hartig net and cortex of *H. crustuliniforme- Picea abies.*

Cadmium is not an essential element for fungal growth and it is quite toxic to fungi as well as plants. Cadmium arrests growth of some ectomycorrhizal fungi at extremely low levels (McCreight and Schroeder, 1982). Fungal sensitivity to cadmium vary greatly among species, with *Suillus variegatus* having an effective concentration (EC_{50}) of just 0.04 mmol/m3 cadmium and *Amanita muscaria* having an EC_{50} of up to 1334-1379 mmol/m3 cadmium (Hartley *et al.,* 1997; McCreight and Schroeder, 1982). Another factor in toxicity levels was the presence or absence of other cations. The EC_{50} for cadmium of *Suillus variegatus* was 0.04 mmol/m3 in the absence of Zn ions, but when in the presence of 500 mmol/m3 Zn, the EC_{50} rose to 5.5 mmol/m3 (Hartley *et al.,* 1997). Various mechanisms were implicated for Cd tolerance in ECM fungi such as accumulation of Cd in the walls of extramatrical hyphae, excreation of organic acid, production of enzymatically synthesized proteins SOD, glutathionein and metallothionein, and vacuolar compartmentation. Binding of Cd to cell walls was shown to represent a substantial fraction of the metal accumulated by *P. involutus* and may be part of the mechanisms by which mycorrhizal fungi tolerate high amounts of Cd (Blaudez *et al.,* 2000a; Frey *et al.,* 2000). Using X-ray microanalysis, Brunner and Frey (2000) detected Cd in the Hartig net. In another study, oxalic acid reduced Cd uptake by more than 85% in *P. involutus* (Bellion *et al.,* 2006). Therefore, an increased oxalate exudation inducing a decreased Cd availability would be an efficient mechanism to avoid Cd entry into living cells of ectomycorrhizal fungi. Blaudez et al. (2000b) demonstrated the ability of the ectomycorrhizal fungus *P. involutus* to take up and further accumulate Cd in different compartments. Binding of Cd onto cell walls and accumulation of Cd in the vacuolar compartment may be regarded as two essential metal-detoxification

mechanisms. Jacob et al. (2004) found an induction of laccase activity and gene expression and production of polyphenolic compounds under Cd exposure, which may be an important determinant of the cellular response to excess metals in *P. involutus*. Glutathione and γ-glutamylcysteine was found to be increased under Cd exposure in *P. involutus* (Ott *et al.,* 2002; Courbot *et al.,* 2004).

Like cadmium, lead is not an essential element for biological function, and is found in the soil mainly as a result of pollution in industrialized areas. Accumulation of lead in the soil can inhibit ectomycorrhizal formation with its host plant (Chappelka *et al.,* 1991; Dixon, 1988; Dixon and Buschena, 1988; McCreight and Schroeder, 1982). Sensitivity of fungi to lead varies with species. Lead concentrations as low as 5 ppm inhibited ectomycorrhizal formation by *Suillus luteus*, whereas other ectomycorrhizal species were tolerant up to 200 ppm (Dixon and Buschena, 1988; McCreight and Schroeder, 1982). The morphological symptoms of lead toxicity in fungi are swollen and deformed mycorrhizal tips and secretion of an extracellular "slime" (Tam, 1995). An apparent tolerance of lead pollution by certain ectomycorrhizal species *in vitro* has been observed and found tolerance varies among species (Hartley *et al.,* 1997; Tam, 1995; Vodnik *et al.,* 1998; Blaudez *et al.,* 2000a). The extracellular slime may be a site of metal accumulation, preventing the poisonous element from entering the fungal cell (Tam, 1995). Inoculation of seedlings with ectomycorrhizal fungi has been shown to improve growth in lead-amended soil (Dixon, 1988; Dixon and Buschena, 1988; Jentschke *et al.,* 1998; Marschner *et al.,* 1996). A study by Hartley et al. (1997) showed that *P. involutus* was quite tolerant to lead contamination. Subsequent studies showed that mycorrhizal

infection of Norway spruce by *P. involutus* was shown to decrease lead content in the roots when compared to non-mycorrhizal controls (Jentschke *et al.,* 1998; Marschner *et al.,* 1996). Vodnik et al. (1998) found binding of lead in extramatrical hyphae in different ectomycorrhizal fungi. These findings support the suggestion that binding of the lead to the hyphal cell walls of the extrametrical mycelium and mantle and is indeed one mechanism of lead amelioration. Conversely, Jentschke et al. (1991) found lead accumulation in the cortical cells of mycorrhizal *Picea abies* but not in the Hartig net, hyphal mantle or in fungal cell wall. The mechanism of amelioration of lead toxicity, therefore, is not yet known.

Our findings are also in line with these findings. In the present study, the tolerance of *H. cylindrosporum* to various concentrations of Cu, Cd, Ni, Pb and Zn metals was determined in liquid MMN media. Among all metals tested, the *H. cylindrosporum* showed higher tolerance to Zn, Cu and Ni. Increasing concentration of Cu and Ni significantly decreased the growth of *H. cylindrosporum*. The growth was inhibited 50% at 160µM of Cu and 200 µM of Ni respectively. The *H. cylindrosporum* was very sensitive to Cd and Pb as their growth was inhibited 50% at 14 µM of Cd and 50 µM Pb respectively. *H. cylindrosporum* showed 30% growth reduction at 2 mM Zn. The increased tolerance of *H. cylindrosporum* to Zn, Cu and Ni might be due to the features of the trace metals. This was never observed for Cd and Pb, and is certainly due to considered as a non-essential elements and are highly toxic at low concentrations. The fungus was showed large variation in metal tolerance among metals tested. These results indicated that *H. cylindrosporum* isolate showed large variation in metal tolerance to all

metals tested. This supports the fact that ectomycorrhizal fungi have different degree of tolerance towards heavy metals. This is definitely because each ectomycorrhizal species has its own physiology and therefore its own specificity with respect to the metal tolerance levels. From the present data, we confer that the fungus tolerant to a particular metal did not imply tolerance to all other metals and might use different mechanisms to confer resistant to various metals.

5.2 Identification and molecular characterization of metallothionein genes of *H. cylindrosporum*

Metallothioneins comprise a group of low molecular weight, cysteine rich polypeptides that bind and sequester heavy metal ions. These proteins are founding in all living organisms and are mainly believed to function in heavy metal detoxification and in normal metabolism of essential trace metals such as zinc and copper (Kagi, 1993). The aim of this work was to characterize different MTs of the ectomycorrhizal fungus *H. cylindrosporum* and to study the specificity of their induction in an attempt to determine their possible functional role in heavy metal detoxification. Metallothioneins constitute a multigene family in many species including fungi. All animal and plant species have two or more MT gene, which are differentially regulated by different heavy metals (Hamer, 1986; Cobette, 2000). The yeast *S. cerevisiae* contains multigene MT (*CUP1* and *CRS5*) family, which play dominant role in copper detoxification (Ecker *et al.,* 1986; Culotta *et al.,* 1994). In *C. glabrata,* metallothioneins constitute a multigene family consisting of two distinct classes of genes with multiple isoforms of one class (Mehra *et al.,* 1989). We identified two different MTs from EST library of *H. cylindrosporum,* which show

similarity to fungal MTs (Lambiliotte *et al.,* 2004). There are no reports available on multigene family of metallothionein in ectomycorrhizal fungi. To best of our knowledge, this is the first report of the presence of a multigene family encoding MTs in an ectomycorrhizal fungus. As MTs are generally not identified by genome sequencing project, present results show that EST methods can be a suitable alternative to identify MTs. Out of ca. 4500 ESTs (corresponding to ca. 3500 unigenes) isolated by Lambiliotte et al. (2004) from *H. cylindrosporum* grown in the absence of heavy metal stress, we identified 6 MT ESTs corresponding to the two identified genes. If we consider that 77% of the ESTs in the library exist as less than 3 copies, this indicates that *HcMT1* and *HcMT2* are expressed at a high basal level. By contrast, transcripts of *PiMT1* were almost undetectable in *P. involutus* grown in the absence of heavy metal (Bellion *et al.,* 2007).

The *HcMT1* cDNA codes for 59 amino acids, contains a 177bp ORF. *HcMT2* cDNA encodes a 57 amino acids protein with an ORF of 174 bp. The coding sequences of *HcMT1* and *HcMT2* show 31% identical to each other at the amino acid level and 40% similarity at nucleotide level, indicating that the sequences of the two types of metallothionein genes are diverse and are encode two different MT proteins. MTs are made a single polypeptide chain and are comprised of two structural domains, which are capable of binding metals independently and are separated by short liker region (Hamar, 1986). The cysteine residues are the part of the characteristic of metallothionein (Kagi, 1993). The unique arrangement of C-x-C sequences, where X stands for a residue different from cysteine, is a typical feature of all fungal MTs studied so far. It was proposed to be essential for metal complexation and indicates that the cysteine residues

are specific metal ligands. The *HcMT1* and *HcMT2* contain two cysteine rich domains and are separated by 17 and 14 amino acids region respectively. The *HcMT1* contains 5 C-x-C motifs and two are at N-terminal part, other three at C-terminal part. The *HcMT2* contains 6 C-x-C motifs and are distributed equally at both terminal ends. Similarly in AM fungi, the *GmarMT1* and *GntMT1* contain six C-x-C motifs and distributed equally at both ends. The two cysteine domains of *GmarMT1* and *GntMT1* are separated by 28 and 24 amino acid spacer (Lanfranco *et al.,* 2002; Gonzalez-Guerrero *et al.,* 2007). Where as the *P. involutus PiMT1* gene codes for a relatively short (34 amino acids) MT and contains just one domain bearing the classical three C–X–C motifs (Bellion *et al.,* 2007). The alignment of *HcMT1* and *HcMT2* with other known fungal MTs showed that fungal MTs bear the C-x-C-x(2,3)-C signature at the N-terminal part together with a conserved Cys residue at the C-terminal end. This consensus sequence is far more restricted than the C-G-C-S-x(4)-C-x-C-x(3,4)-C-S-x-C consensus proposed as a signature of fungal MTs by Binz and Kägi (1999). The *HcMT1* and *HcMT2* gene showed high similarity with MTs of *A. bisporus, N. crassa, P. anserine, P. involutus* and *G. margarita*.

In *G. margarita, GmarMT1* gene is composed of two exons separated by a single 81 bp intron (Lanfranco *et al.,* 2002). *P. anserine* of *PaMT1* gene contains two exons and one intron (Averbeck *et al.,* 2001). Similarly *N. crassa* MT gene two exons were interrupted by a single intron (Munger *et al.,* 1987). In *C. glabrata*, no introns were found in two metallothionein genes (Mehra *et al.,* 1989). Interestingly in *H. cylindrosporum*, the *HcMT1* and *HcMT2* gene contain four coding exons and three non coding introns. These

organization patterns were found mainly in vertebrates and plants (Harlow *et al.,* 1989). The organization of the three coding exons has been classically described for metallothioneins in vertebrates (Nemer *et al.,* 1991).

5.3 Promoter analysis of *HcMT1* and *HcMT2*

The 5' upstream region of *HcMT1* and *HcMT2* were isolated using Universal genome walker Kit (Clonetech, Germany). The 5' upstream promoter region of *HcMT1* and *HcMT2* were analyzed for identification of transcription factors using Genomatix and Transfac softwares. The *HcMT1* and *HcMT2* promoters were shown to contain a putative metal responsive element (MRE) which suggests the possibility of metal regulated transcription. MREs of consensus TGCRCNC (where R stands for A or G and N for any of the four bases) are *cis*-regulatory DNA sequences that specifically bind metal transcription factors (MTF) and are essential for transcriptional induction upon heavy metal load (Stuart, 1985; Westin, 1988). Major target genes of MTFs are the genes encoding metallothioneins that have the ability to bind and thereby sequester heavy metals (Kägi, 1991; Palmiter *et al.,* 1992). These *cis*-acting MREs present in multiple copies in all MT promoters, are composed of a series of 13-15 bp imperfect repeats and are found in both orientations within MT promoters (Karin and Richardsri, 1984). The MRE sequences of *HcMT1* (TGCGCCCG) and *HcMT2* (TGGCTGCCA) are different and located at different position in promoter region. The divergence between MRE sequences of *HcMT* genes suggests considerable differences in their expression pattern. A total four different *cis*- acting MREs have been reported in yeast to control the expression of *CUP1*, the yeast metallothionein gene. Tandemly duplicated metal responsive

elements are responsible for metal stress response (Thiele and Hamer, 1986). The activation of *CUP1* occurs via the modulation of Cu ion-dependent Ace1p binding to the *CUP1* promoter MREs (Marjorette *et al.,* 1998). The promoter of *GmarMT1* of *G. margarita* contains three metal responsive elements which show high similarity with *CUP1* promoter MREs and they found enhanced activity of *GmarMT1* under limited carbon supply (Bergero *et al.,* 2007). In *N. crassa*, the CuMT gene contains one ARE (antioxidant responsive element), two MRE, four copper response elements, six stress regulatory elements (STRE) and five copper metallo-regulatory transcription factor binding sites (Kumar *et al.,* 2005). In *P. involutus*, *Pimt1* promoter sequence contains one putative metal regulatory sequence (MRE), similarly to yeast MTs. In the present study *HcMT1* MRE showed similarity with *Pleurotus ostreatus* MREs (Faraco *et al.,* 2003) also found that *P. ostreatus* MREs were activated under copper stress. The *HcMT2* MRE showed similarity with yeast.

GATA factors constitute a family of structurally related transcription factors that interact with the (A/T)GATA(A/G) consensus sequence present in fungi, metazoans and plants.. GATA factors are expressed in distinct developmental and tissue-specific patterns, and their involvement in the regulation of cell-specific gene transcription is well established (Simon, 1995) In addition, the results suggest that GATA elements are involved in the regulation of *C. elegans* MT gene transcription (Moilanen *et al.,* 1999). GATA elements are also present in several invertebrate MT genes that show highly restricted patterns of expression, including the *Drosophila Mto* gene (Bonneton *et al.,* 1996, Durliat *et al.,* 1995) and sea urchin *spMTA* and *spMTB* genes (Nemer *et al.,* 1991 and 1995). In fungi

GATA factors act as transcription factors or repressors in a number of different processes, ranging from nitrogen source utilization to mating-type switching (Scazzocchio, 2000). In fungi there are no such repots reports available on GATA factors role in relation to MT induction. The presence of GATA factors in *HcMT1* and *HcMT2* promoter might be involved in variety of process including metal stress.

S. cerevisiae metallothionein gene *CUP1*, was transcriptionally activated in response to heat shock and glucose starvation through the action of heat shock transcription factor (HSF) and a heat shock element located within the *CUP1* promoter upstream regulatory region (Tamai *et al.,* 1994). Bergero et al. (2007) found HSF in *GmarMT1* promoter of *G. margarita* and observed the enhanced activity of *GmarMT1* under glucose starvation. Liu and Thiele (1997) demonstrate that transcription of the *S. cerevisiae* MT gene *CUP1* was strongly activated by oxidative stress and *CUP1* transcriptional activation was dependent on a functional *CUP1* promoter heat shock element (HSF). Considering together, the HSF in HcMT1 and HcMT2 promoter might be involves in transcription of MT genes under variety of stimuli such as heat/oxidative stress and glucose starvation.

Apart from MRE, GATA and HSF, *HcMT1* and *HcMT2* promoters contained other standard stress response elements implicated in metal response such as response to phosphate starvation (PHO) (Ogawa and Oshima, 1990) and response to nitrogen utilization (NIT) (Fu and Marzluf, 1990). However, the number and variety of potential regulatory elements in *HcMT1* and *HcMT2* promoter regions was different. *HcMT1* contained response elements that were not present in *HcMT2* promoter region such as

STRE, which are known to be responsible for multiple stress response or enhance the response of other elements (Treger *et al.,* 1998) and GCN, which responds to amino acid and nitrogen starvation related stress (Arndt and Fink, 1986; Tate and Cooper, 2007). On the other hand, *HcMT2* promoter contained several additional regulatory elements such as GCR that are involved in regulating glycolytic enzymes presumably in response to heavy metal stress in the production of organic acids (Uemura *et al.,* 1997), DRE (Katzmann *et al.,* 1994), involved in drug or heavy metal efflux mechanisms, MCM (Fassler *et al.,* 1997) which may be involved in osmosensing as well as heavy metal tolerance induced pathways, RPU (Suarez *et al.,* 1995) involved in regulation of purine utilization pathways in response to heavy metal or other stress responses and CAT a generic or basal level regulator of transcription found in a variety of fungal promoters. However, it has been shown that that CAT elements when found in proximity of other elements can amplify the response (Kato, 2005). Based on sequence analysis, following the criteria suggested for promoter evolution (Miller *et al.,* 1996; Raijman *et al.,* 2008; Zhang, 2003), it appears that *HcMT2* is likely originated by duplication of *HcMT1* followed by changes in its sequence to acquire new elements such as STRE elements and DRE elements as well as duplication of existing elements such as PHO. It has been shown that cells respond to multiple stresses in a synergistic manner, and exposure to one form of stress leads to transient stress-hardening or cross-tolerance to other forms of stress (Kültz, 2003). Thus duplication and acquisition of new stress response elements facilitate additional adaptations that are stressor-specific and aimed at reestablishing cellular homeostasis. The additional stress response elements in addition to MREs in both *HcMT1* and *HcMT2* suggest differential regulation and function of *HcMT1* and *HcMT2*. For example, the

presence of DRE is responsible for more differential response of *HcMT2* than *HcMT1*. Thus, *HcMT2* exhibits a more pleiotropic role similar to *PiMT1* of *P. involutus.* As shown in results section, the differences in the elements occurring in promoters of *HcMT1* and *HcMT2* suggest diversification of stress response function and level of response between *HcMT1* and *HcMT2* as seen from the expression analyses.

5.4 Differential regulation of *HcMT1* and *HcMT2*

The mRNA transcript accumulation pattern of *HcMT1* and *HcMT2* were quantified by competitive RT- PCR method in *H. cylindrosporum*. Higher eukaryote MTs are generally induced by a wide array of metals and stress conditions (Cobbett and Goldsbrough, 2002). To study the metallothionein induction, *H. cylindrosporum* was first grown in Melin's liquid medium containing different concentrations of heavy metals such as Cu, Cd, Zn, Pb and Ni. To study the optimum time for maximum induction of MT, the fungus was grown in Melin's medium with 320 µM of Cu and 21 µM of Cd concentration for different time intervals 0, 12, 24, 36, 48, 60 and 72 hrs. Oxidative stress treatment was imposed by incubating the culture supplemented with 25 mM H_2O_2.

In fungi, the metallothionein gene expression is induced by mainly metal exposure and oxidative stress. By contrast, each of the fungal MTs studied so far is induced by a limited number of heavy metals, most of them being induced by Cu and metallothioneins bind Cu with high affinity (Lerch, 1980; Münger and Lerch, 1985; Culotta *et al.,* 1994; Cobine *et al.,* 2004; Kumar *et al.,* 2005). It was also the case of both MTs characterized in this work. However, *HcMT1* was specifically regulated by Cu, not by Cd, Zn, Pb and

Ni whereas *HcMT2* showed a dual regulation since it was induced by Cu and Cd, not by Zn, Pb and Ni. The expression levels of *HcMT2* were significantly higher in the presence of Cu than Cd. In general fungal MTs were inducible by Cu. For example, *PaMT1* from *P. anaserina* was up-regulated by only Cu ions and expression was increased in a concentration depended manner. The addition of Cd, Zn and Mn ions not induced a significant upregulation of *PaMT1* (Averbeck *et al.,* 2001). In *C. glabrata,* northeren analysis showed that both the genes coding MTs were induced by Cu and Ag in a concentration depended fashion, where as Cd failed to induce both MT genes (Mehra *et al.,* 1989). Similarly, in the present investigation also *HcMT1* and *HcMT2* was significantly increased due to Cu and not affected by Cd. The *HcMT1* and *HcMT2* transcript accumulation was increased in a concentration depended fashion as induction increased with increasing concentration of copper treatment ranging from 80 to 320 µM. In comparison, *HcMT1* was more responsive to copper than *HcMT2*. The difference in *HcMT1* and *HcMT2* gene expression in response to copper might be due the different pattern of regulation of the genes. In the yeast *S. cerevisiae,* resistance to the toxic effects of copper is conferred by a two metallothionein genes, which is encoded by the *CUP1* gene and *CRS5* gene. The transcription of *CUP1* and *CRS5* metallothionein genes are induced only by copper and not by other heavy metals. The *CUP1* is more responsive to copper than *CRS5* (Butt *et al.,* 1984; Culotta *et al.,* 1994). In AM fungi, Lanfranco et al. (2002) also reported *GmarMT1* from *G. margarita* responded to Cu but not to Cd exposure. Similarly, Gonzalez-Guerrero et al. (2007) reported *GntMT1* from *G. intraradices* induced by Cu, not to Cd. In *N. crassa,* MT characterized by Cobine et al. (2004) and Münger et al. (1987) was inducible by Cu, not by Cd, Zn, Co or Ni. Münger

et al. (1987) detected rapidly increasing amounts of metallothionein mRNA in *N. crassa* in response to copper shock, metallothionein mRNA was detected for a time period of more than 30 hours. In the present investigation also *HcMT1* transcript accumulation was sharply increased first 24 hours and continued till 48 hours due to Cu, where as none of the tested Cd concentration did not induce the *HcMT1*. In many plants including *A. thaliana* MT RNAs are strongly induced by Cu. The metal-regulated expression of *Arabidopsis* MT genes is more similar to that of fungal MT genes, with greatest induction by copper (Murphy *et al.,* 1997). Altogether, the *HcMT1* of *H. cylindrosporum* can be classified in this group and *HcMT1* might be involved mainly in intracellular copper detoxification. This is not the case of *HcMT2* which is also induced by Cd.

Cadmium inducible MTs have been identified in different fungi (Jacob *et al.,* 2004; Courbot *et al.,* 2004; Jaeckel *et al.,* 2005; Bellion *et al.,* 2007). In *Colletotrichum gloeosporioides*, Hwang and Kolattukudy (1995) reported the induction of metallothionein genes by Cu and Cd. Kameo et al. (2002) also reported the induction of metallothionein by Cu and Cd in *Beauveria bassiana*. Similarly Jaeckel et al. (2005) also reported the larger amounts of MT transcripts in presence of Cd and Cu in an aquatic fungus *Heliscus lugdunensis*. Contrary to the above, in the filamentous cynobacterium *Oscillatoria bravis*, a MT gene was markedly increased under Cd and Zn (Liu *et al.,* 2003). Bellion et al. (2007) showed that the Cd-inducible MTs of *P. involutus* are also induced by Cu. They also showed that *PiMT1* response to Cu was faster and more intense than response of Cd. They also suggested that the isolated MT might have higher binding capacity for Cu than Cd. This is also the case of *HcMT2,* where it was induced more in

presence of Cu than Cd. The regulation of *HcMT2* appears to be similar to that of *PiMT1* from *P. involutus* (Bellion et al. 2007), both being induced by Cu and Cd, not by Zn. As mentioned by these authors in case of *PiMT1*, the encoded proteins could be involved in the response of mycorrhizal fungi to Cu and Cd stress. This hypothesis can be updated on the basis of present results. Indeed, *HcMT1* is much more responsive to Cu induction than *HcMT2* and not induced by Cd. This suggests that it could be specifically involved in Cu detoxification whereas *HcMT2*, as *PiMT1*, which shows a dual regulation, could have a more pleiotropic role. In the present study, *HcMT1* is the dominant MT gene in conferring copper tolerance to *H. cylindrosporum*. The contribution of *HcMT2* to copper tolerance is minor in relation to *HcMT1*.

Apart from MTs, there are other enzymatically synthesized cysteine rich peptides, phytochelatins, were involved in intracellular metal ions detoxification. PCs are mainly induced by cadmium and their respective roles were extensively studied in plants (Cobbett, 2002). Limited studies are available on phytochelatins in fungi, *C. glabrata* (Zhou and Goldsbrough, 1995), *S. pombe* (Clemens and Simm, 2003) and *Heliscus lugdunensis* (Jaeckel *et al.,* 2005) produced phytochelatins in response to Cd. The role of glutathione as a metal chelator in fungi is clearly established (Pocsi *et al.,* 2004). Intracellular glutathione hinders the progression of heavy metal-initiated cell injuries by chelating and sequestering the metal ions themselves. Under Cd exposure, an increase in nonprotein thiols glutathione and its precursor γ-glutamylcysteine were observed in *L. laccata* (Dameron *et al.,* 1989; Galli *et al.,* 1993) and *P. involutus* (Courbot *et al.,* 2004). Conversely, these studies have highlighted the complete lack of phytochelatins (PCs) in

P. involutus (Courbot *et al.,* 2004) and *Suillus bovinus* (J. V. Colpaert et al., personal communication). However, putative gene sequences encoding enzymes involved in the synthesis of glutathione and γ-glutamylcysteine have been identified in expression sequence tag (EST) databases obtained from the ectomycorrhizal fungi *H. cylindrosporum* and *P. involutus.* The presence of glutathione and γ-glutamylcysteine genes in *H. cylindrosporum* prompts us to reconsider the role of phytochelatins in response to Cd.

Although some MTs of higher eukaryotes have been shown to bind Zn (Cobbett and Goldsbrough, 2002), there are only few reports of Zn-inducible MTs in fungi. *CUP1* and *CRS5* from *S. cerevisiae* have extensively related to Cu handling, but recently Pagani et al. (2007) defined the dual role of *CRS5*, the involvement of the *CRS5* Cu-metallothionein of *S. cerevisiae* in Zn detoxification. Tucker et al. (2004) described a MT like protein in *Magnaporthe grisea* which showed a very high affinity to Zn. Considering these reports, although none of the MTs identified to date in ectomycorrhizal fungi is induced by Zn, the presence of Zn-specific MT(s) in these fungi (including *H. cylindrosporum*) cannot be ruled out. The influences of Ni and Pb on expression of metallothionein genes were reported mainly in vertebrates, but there is no direct evidence for induction of metallothionein in fungi by Ni and Pb (Cobbett, 2000). In the present study, both *HcMT* genes were not induced by Ni and Pb.

Metals and oxygen are chemically linked in biological systems. Metals and oxygen play important roles in enzymatic reactions, metabolism, and signal transduction; however,

metals and oxygen react to form highly toxic oxygen-derived free radical species. It is generally accepted that metallothioneins are devoted to the regulation of the metabolism of essential trace metals and to chelation of toxic metals. Nowadays, there is increasing evidence that MTs also act as free radical scavengers (Andrews and Geiser, 1999). Metallothionein gene transcription is induced by a wide range of metal ions as well as chemicals that generate oxidative stress (Kagi, 1991). Recent studies have shown that metals, including iron, copper, chromium, and vanadium undergo redox cycling, while cadmium, mercury, and nickel, as well as lead, deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of reactive oxygen species as superoxide ion, hydrogen peroxide, and hydroxyl radical. Agents capable of mediating formation of free radicals are known to induce the MT mRNAs (Bauman *et al.,* 1991). In *S. cerevisiae,* Gralla et al. (1991) demonstrated that ACE1 is the transcription factor for Cu induction of SOD1 gene expression and metallothionein. Both metallothionein and CuZnSOD eliminates free radicals, which are formed by Fenton and Haber-Wise reaction. Gonzalez-Guerrero et al. (2007) reported that *GntMT1* from *G. intraradices* expression was enhanced by paraquat, which is potent oxidative inducer. For example, a plant type 2 metallothionein was reported to respond to oxidative stress by Mir et al. (2004). It was also the case of *HcMT1* and, at a lower extent, *HcMT2*. Expression levels of both *HcMT1* and *HcMT2* were increased in presence of oxidative stress. The *HcMT1* is more responsive than *HcMT2*. This suggests a role of *H. cylindrosporum* MTs in maintaining the local redox balance either by sequestering copper and preventing potentially deleterious Fenton chemistry reactions (Zhang *et al.,* 1999).

The ability of *HcMT1* and *HcMT2* to function as MT was assessed in *S. cerevisiae* mutant strains which are unable to grow on high concentrations of various metals, and growth was then monitored on both control and metal supplemented media. In *S. cerevisiae*, *CUP1* encodes a Cu-thionein that is induced by and binds to Cu. $cup1^{s}$ strain harbours a single copy of this MT gene and its growth is inhibited at 300µM $CuSO_4$. The Cu-tolerance threshold is reduced to 75µM CuSO4 in $cup1^{\Delta}$ strain (*cup1::URA3*), which has no functional copies of *CUP1* (Hamer *et al.,* 1985). The empty vector carrying $cup1^{\Delta}$ cells growth was inhibited at 150µM $CuSO_4$, whereas the same cells carrying p424-*HcMT1* and p424-*HcMT2* were able to grow at a similar rate to parental $cup1^{s}$ cells. *yap1* strain transformed with the empty vector was unable to grow at 40µM Cd, whereas the Cd-sensitive phenotype of the *yap1* mutants was complemented by *HcMT1* and *HcMT2*. Further, the restoration of Cd and Cu tolerance in liquid culture assays confirmed that p424-*HcMT1* and *HcMT2* $cup1^{\Delta}$ and *yap1* cells grew well in medium containing 150 µM $CuSO_4$ and 40 µM $CdSO_4$ whereas the growth of p424 $cup1^{\Delta}$ cells was inhibited. Surprisingly, the results showed that *HcMT1*, which is not induced under Cd stress *in vivo* in *H. cylindrosporum*, tolerant for Cu and Cd. This might be because of expression of genes in heterologus system. Similar observation was found by Lanfranco et al. (2002), *GmarMT1* encodes metallothionein capable of conferring tolerance against Cu only. When *yap1* and $cup1^{\Delta}$ strain were transformed with *GmarMT1*, they became resistant to Cu as well as Cd. In *S. cerevisiae,* metallothioneins bind a variety of transition metal ions *in vitro*, its synthesis is only induced *in vivo* by copper (Thiele, 1992). In *Festuca rubra*, *mcMT1* enhanced by Cu and Cd, but functional complementation studies confirmed the *mcMT1* confer resistant to Cu, Cd, Zn and Pb (Ma *et al.,* 2003).

5.5 Conclusions

The impact of different heavy metals such as Cu, Cd, Ni, Pb and Zn on *H. cylindrosporum* was assessed. The fungus showed considerable variation in metal tolerance among metals tested and it concludes that fungus might employ different mechanisms to tolerate different metals. The present study shows experimental proof that ectomycorrhizal fungus *H. cylindrosporum* encodes different MTs, with differences in pattern of regulation. *HcMT1* is highly responsive to Cu induction and is likely involved in the detoxification of this metal. *HcMT2* is responsive to Cu and Cd induction. The Cd might be detoxified by *HcMT2* or other intracellular mechanism as it is know that a variety of metal protection strategies co-exist in a single organism. The lack of induction of both *HcMT1* and *HcMT2* genes in response to Zn, Ni and Pb indicates that *H. cylindrosporum* might be employed by different other proposed mechanisms like extramatrical cell wall binding, chelation through organic acids, increased or decreased efflux and vacuolar compartmentation to detoxification of Zn, Ni and Pb. The presence of different putative regulatory elements and their position in *HcMT1* and *HcMT2* promoter might be a region for different regulation pattern for Cu and Cd. Further studies are required to understand the metal regulated transcription in *H. cylindrosporum*. These results suggest that MTs might have specialized in terms of metal binding ability and functions within each life form to adapt different environmental conditions or specific endogenous metabolic requirements. In conclusion, these results show that the identification and functional verification of different MTs in ectomycorrhizal fungi and the study of their regulation is a prerequisite for a better understanding of heavy metal

tolerance of these fungi and subsequently for the determination of their ability to detoxify and improve heavy metal tolerance of their host plant.

SUMMARY

Metallothioneins are a class of low molecular weight cysteine rich proteins that bind to large amounts of metal ions such as Zn^{2+}, Cu^{2+}, Cd^{2+} or Hg^{2+} (Hamer, 1986). MTs were identified from different filamentous fungi (Lanfranco *et al.,* 2002). The occurrence of MTs and PCs or both, in ectomycorrhizal fungi still a matter of debate and the metal tolerance mechanisms are not well understood in mycorrhizal fungi. The molecular regulation of MTs and PCs has been investigated, to a limited extent in ectomycorrhizal fungi (Bellion *et al.,* 2007). In the present investigation metallothionein genes of ectomycorrhizal fungus *Hebeloma cylindrosporum* were cloned and characterized. The expression of these genes was studied in the presence of different metals and their functions were assayed by yeast complementation.

Axenic screening provides a simple and rapid process for determining fungal response to increasing metal doses, and identifying possible mechanisms of tolerance. In the present study, the tolerance levels of *H. cylindrosporum* to various concentrations of copper, cadmium, zinc, lead and nickel was assessed by growing pure mycelial cultures in liquid MMN media *in vitro*. The growth of *H. cylindrosporum* was adversely affected with increasing concentrations of metals. Among all metals tested, the *H. cylindrosporum* showed higher tolerance to Zn, Cu and Ni. The growth was inhibited 50% at 160µM of Cu and 170 µM of Ni respectively. There was no significant growth reduction observed in all concentrations of Zn tested. The *H. cylindrosporum* was very sensitive to Cd and Pb as their growth was completely inhibited at 14 µM of Cd and 50 µM Pb respectively.

These results indicated that *H. cylindrosporum* showed large variation in metal tolerance to all metals tested and confers that the fungus is more tolerant to one or more types of metals not to other metals (Meharg, 2003).

Two partial cDNAs encoding metallothionein (MT) like polypeptides designated *HcMT1* and *HcMT2* were identified from an EST library of the ectomycorrhizal fungus *H. cylindrosporum* (Lambiliotte *et al.,* 2004) and primers designed (HcMT1F and HcMT1R and HcMT2F and HcMT2R) for the amplification of metallothionein gene. Genomic DNA was isolated from *H. cylindrosporum.* Total RNA was isolated and cDNA prepared by RT-PCR method. Both cDNA and genomic DNA were used for PCR to amplify metallothionein genes. The results showed that HcMT1F and HcMT1R primers amplified the 490 bp long fragment of genomic DNA and 295 bp of cDNA. In the case of HcMT2F and HcMT2R primers, 295 bp long fragment of genomic DNA and 210 bp of cDNA were obtained. Corresponding full length of *HcMT1* and *HcMT2* were obtained by RACE PCR according to manufactures instruction (5'/3' RACE kit, Roche, USA). The PCR products amplified from genomic DNA and cDNA were cloned and transformed into DH5α *E. coli* cells by the heat shock method. The positive clones were selected and the plasmid DNA was isolated. Plasmid containing inserts were confirmed by PCR with insert specific primers and sequenced. Sequence analysis was performed with BLAST program (Altschul *et al.,* 1997) using the nucleic acid and predicted amino acid sequences deposited in multiple data bases.

The *HcMT1* and *HcMT2* contained 4 exons and 3 introns and all the 3 introns contained conserved intron junctions GT-AG. The ORF of *HcMT1* and *HcMT2* contained 177 bp

(59 amino acids) and 174 bp (57 amino acids) respectively. *HcMT1* contains 13 Cys residues and two C-x-C motifs at N-terminal part and three at their C-terminal portion, which is characterization of MTs. The *HcMT2* sequence contains 14 cystein and six C-x-C motifs are equally distributed at both ends. *HcMT1* and *HcMT2* were 31% identical to each other and the similarity was 40%. The alignment of *HcMT1* and *HcMT2* with other known fungal MTs showed that fungal MTs bear the C-x-C-x(2,3)-C signature at the N-terminal part together with a conserved Cys residue at the C-terminal end. This consensus sequence is far more restricted than the C-G-C-S-x(4)-C-x-C-x(3,4)-C-S-x-C consensus proposed as a signature of fungal MTs by Binz and Kägi (1999) (http://www.expasy.ch/cgi-bin/lists?metallo.txt). The alignment of sequence shows that this latter consensus sequence resembles the signature of basidiomycete MTs, which is C-x(3, 4)-C-x-C-x(3)-C-x-C at the N-terminal end together with C-x-C at the C-terminal end.

The genomic clones of *HcMT1* and *HcMT2* and the adjacent DNA sequence, 1200 kb upstream from *HcMT1* and *HcMT2* start codon of *H. cylindrosporum* was cloned using the Universal Genome Walker kit (Clonetech Laboratories, Inc., Germany). In order to explain whether the differential expression of *HcMT1* and *HcMT2* is due to differential regulation, we performed computational analysis of their respective upstream regions of the promoters. Both *HcMT1* and *HcMT2* promoters contained the standard stress response elements implicated in metal response such as metal response element (MRE), general stress response (GATA), response to phosphate starvation (PHO), response to nitrogen utilization (NIT), and heat shock (HSF). *HcMT1* contained STRE (multiple stress response element), and GCN, which were not present in *HcMT2* promoter region.

The *HcMT2* promoter contained several additional regulatory elements such as GCR, DRE, MCM, RPU and CAT. The difference and location of potential regulatory elements in *HcMT1* and *HcMT2* promoter regions might be the reason for different regulation pattern.

To study the metallothionein induction by different heavy metals, the fungus was first grown in Melin's liquid medium for two weeks. Then the mycelium was transformed to fresh medium containing different concentrations of heavy metals. For dose responsive studies, the fungus was grown in different concentrations of Cu (0, 80, 160, 240 and 320 µM), Cd (0, 7, 14, 21 and 28 µM), Zn (0, 0.5, 1 and 1.5 mM), Ni (0, 85, 170, 250 and 350 µM) and Pb (0, 25, 50, 75 and 100 µM) for 24 hours. For induction kinetics studies, the fungus was grown in Melin's medium with 320 µM of Cu and 21 µM of Cd concentration for different time intervals 0, 12, 24, 36, 48, 60 and 72 hours.

Competitive RT- PCR was used to quantify the mRNA accumulation of *HcMT1* and *HcMT2*. The competitor sequence was plasmid cloned genomic DNA fragment. In a PCR reaction containing a RT-cDNA sample and a known amount of plasmid cloned competitor, primers HcMT1F and HcMT1R amplify simultaneously the 295bp long cDNA fragment and the 490 bp long competitor fragment. In the case of *HcMT2*, 210 bp long cDNA fragment and the 295 bp long competitor fragment was obtained with HcMT2F and HcMT2R primers. Standard curves were constructed by co-amplifying different known amounts of target DNA with a constant amount of competitor. A standard curve was obtained by plotting the log values of the amplification ratios of target

DNA/competitor against the log values of the target DNA (pg of target DNA) added to PCR mix before amplification.

The kinetics of *HcMT1* and *HcMT2* transcript accumulation results showed that transcript accumulation was observed maximum at 24 hours and decreased thereafter. The highest mRNA accumulation was recorded with *HcMT1* which was induced by ca. 350 fold at 24 hour treatment. *HcMT2* was slightly less sensitive to Cu induction; at 24 hours, transcript level was ca. 100 times higher than control. *HcMT2* was also induced by Cd. Transcript accumulation was maximum at 48 hour treatment. It was ca. 40 times as high as control (zero time). By contrast, *HcMT1* was not induced by Cd.

Dose responsive studies showed that the induction of *HcMT1* and *HcMT2* increased as a function of Cu concentration increased. Maximum 300 folds of *HcMT1* accumulation was observed at 320 µM. In case of *HcMT2*, the maximum accumulation was recorded at 320 µM where it showed almost 100 times higher than the control. In response to Cd, transcription of *HcMT2* increased up to a maximum of 21 µM and decreased thereafter. *HcMT1* expression was not induced by any of the concentrations of Cd used. Zn, Pb and Ni metals were not induced expression of *HcMT1* or *HcMT2* genes. The present results show that both MTs are having a particular pattern of regulation. *HcMT1* is very sensitive to Cu induction and is probably involved in the detoxification of this metal. *HcMT2* has a more pleiotropic role as it involved in Cu and Cd detoxification.

To gain more information about putative role of *HcMT1* and *HcMT2* genes in oxidative stress, the effect of H_2O_2 on both the genes were analyzed. Fifteen days old *H. cylindrosporum* was treated with 25 mM of H_2O_2 for different time intervals 0, 12, 24, 36, 48, 60 and 72 hours. Expression levels of both *HcMT1* and *HcMT2* were increased in presence of oxidative stress. The maximum induction was observed 48 hours and 36 hours for *HcMT1* and *HcMT2* respectively. The induction levels were not high compared to the expression levels under metal stress, suggesting the expression of *HcMT1* and *HcMT2* is more specific in response to metals than oxidative stress.

To characterize *HcMT* genes further, both genes were expressed in two *Saccharomyces cerevisiae* mutant strains, which are unable to grow on high concentration of metals. Two copper-sensitive strains $cup1^s$ and $cup1^{\Delta}$ and one cadmium sensitive yap1 mutant were used. The yeast vector p424-HcMT1 or p424-HcMT2 was constructed under the transcriptional control of the yeast GPD (glyceraldehyde- 3-phosphate dehydrogenase) promoter. Vector p424 and the construct p424-HcMT1 and p424-HcMT2 were introduced into $cup1^{\Delta}$ and *yap1* cells using lithium acetate procedure and transformed cells were selected on appropriate complete synthetic medium (SD). For the functional complementation experiments, cultures of $cup1^{\Delta}$ and *yap1* yeast cells carrying either p424 or p424-HcMT1 and p424-HcMT2 were spotted on SD supplemented with 150 µM $CuSO_4$ and 40 µM $CdSO_4$ plates. Plates were incubated for 3 days at 30°C and photographed. In parallel experiments, Falcon jars containing 20 ml of fresh SD-Trp-Ura and SD-Ura media were inoculated with mid-log precultures of $cup1^{\Delta}$ and *yap1* cells containing p424, p424-HcMT1 and p424-HcMT2 to attain a starting optical density of 0.02 at 600nm. Cells were grown at 30°C and 220 rpm and $CuSO_4$ (150 µM) and $CdSO_4$

(40 µM) were added 5 hours after inoculation. The optical densities of the cultures were measured at 3 hour interval for 42 hours.

Complementation studies indicated that p424-HcMT1 and p424-HcMT2 harbouring *cup1*$^{\Delta}$ cells were able to grow on 150 µM copper supplemented media, where as p424 transformed DTY4 cells growth were restricted. *yap1* transformed with p424 vector was unable to grow at 40 µM cadmium, whereas the Cd-sensitive phenotype of the *yap* mutant was fully complemented by *HcMT1* and *HcMT2*. Further, the restoration of Cd and Cu tolerance by expression of *HcMT1* and *HcMT2* were confirmed in liquid culture assays. The p424-*HcMT1* and *HcMT2* *cup1*$^{\Delta}$ and *yap1* cells grew well in medium containing 150 µM $CuSO_4$ and 40 µM. These results highlighted the key role of *HcMT* genes in Cu and Cd detoxification.

The present study results show that *H. cylindrosporum* encodes two different metallothionein genes and each of them have a particular pattern of regulation which are specifically induced by Cu and Cd respectively. Altogether, these results show that the identification and functional verification of different MTs in ectomycorrhizal fungi and the study of their regulation is a prerequisite for a better understanding of heavy metal tolerance of these fungi and subsequently for the determination of their ability to improve heavy metal tolerance of their host plant.

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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

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

Autoclave 20 minutes at 121^0C for 15 minutes.

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 and autoclave 20 minutes at 121°C.

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. Pour plates and store at 4°C.

IPTG stock solution (0.1M)

1.2 g IPTG

Add water to 50 ml final volume. Filter sterilize and store at 4°C.

X-Gal (2ml)

100 mg 5-bromo-4-chloro-3-indolyl-D-galactoside dissolve in 2ml N,N´-dimethylformamide. Cover with aluminum foil and store at 20°C.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

YPD medium

Peptone	20 g/L
Yeast extract	10 g/L
Agar (for plates only)	20 g/L

Add H_2O to 950 ml and autoclave. Allow medium to cool to55^0C and then add dextrose (glucose) to 2% (50 ml of a sterile 40% stock solution).

SD medium

Yeast nitrogen base (without amino acids)	6.7 g/L
Agar (for plates only)	20 g
H_2O	850 ml
10X Dropout Solution	100 ml

10X Dropout Solution

Nutrient	10X Concentration
L-Adenine hemisulfate salt	200 mg/L
L-Arginine HCl	200 mg/L
L-Histidine HCl	200 mg/L
L-Isoleucine	300 mg/L
L-Leucine	1000 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tryptophan	200 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L
L-Valine	1500 mg/L

To make one liter of 10X –Ura/–Trp Droupout Solution, combine all amino acids except Uracil and Traptophan.

Appendix II

Genomic DNA Extraction buffer

Sodium acetate	100 mM
Na_2EDTA	50 mM
NaCl	500 mM
SDS	1%

PEG/LiAc solution (polyethylene glycol/lithium acetate)

Prepare fresh just prior to use.

PEG 4000	40%
TE buffer	1X
LiAc	1X

TBE Buffer (10x)

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

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)

Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

MOPS (5X)

3-morpholinopropane sulphonic acid	20 mM
Sodium acetate	5 mM
Na_2EDTA	5 mM (pH 7.0)

SSC 20X

NaCl	3M
Sodium citrate	0.3 M (pH 7)

Wash buffer I

2X SSC
0.1% SDS

Wash buffer II

1X SSC
0.1% SDS

TE buffer 10X

Tris-HCl	0.1 M (pH 8)
Na_2EDTA	10 M (pH 8)