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Review article

Advances in metallotionein studies in forest trees

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Abstract

Metallothioneins (MTs), which widely distributed in eukaryotic and prokaryotic organisms, are a class of cysteine-rich heavy metal-binding proteins with low molecular weights. Their biological functions differ among organisms and isoforms. Increasing environmental pollution due to industrialization have led to the accumulation of heavy metals in soils with consequent health risks to human. Forest tree MTs may be involved in plant metal tolerance and accumulation and as metal chelators scavenge reactive oxygen species (ROS). They are playing increasingly important roles in environmental cleanup. This paper review the classification, function, expression, and characteristics of forest tree MTs and the proposed directions for future research. Further studies will clarify the roles of forest tree MT genes in metal homeostasis and scavenging of ROS and assess the applicability of forest tree MTs to phytoremediation of contaminated soils.

Keywords: Metal homeostasis; metallothionein; phytoremediation; *Populus*; ROS.

Abbreviations: ABA- abscisic acid; AmMT- *Avicennia marina* metallothionein; AtMT- *Arabidopsis thaliana* metallothionein; BjMT- *Brassica juncea* metallothionein; CAT- catalase; ClMT- *Citrullus lanatus* metallothionein; Cys- cysteine; FeMT- *Fagopyrum esculentum* metallothionein; GhMT- *Gossypium hirsutum* metallothionein; GPX- glutathione peroxidase; MTs- metallothioneins; OsMT- *Oryza satiral* metallothionein; PcMT- *Porteresia coarctata* metallothionein; PtMT- *Populus trichocarpa* × *deltoides* metallothionein; ROS- reactive oxygen species; SOD- superoxide dismutase; ThMT- *Tamarix hispida* metallothionein.

Introduction

With industrialization, heavy metal pollution becomes increasingly serious, which leads to significant deleterious not only for the environment but also for human health. Evolutionarily, plants have developed a number of regulatory mechanisms, including heavy metal absorption, transportation, chelation and regional isolation, and detoxification to adapt to the increasing concentrations of metal ions. Phytoremediation has been recently extensively reviewed, and several species of hyperaccumulators have been extensively investigated. Forest trees play important roles in human lives (Yang et al., 2011). Especially, trees are good candidates for phytoremediation because of their rapid growth, extensive root systems, and ease of propagation and transformation. Metallothioneins (MTs), are low molecular weight, cysteine-rich, heat-stable, metal-binding proteins that are common in plants. The cysteine residues (Cys), which conserved across MTs sequences of different species probably play an important role in metal chelation. Recently, increasing numbers of reports have indicated that plant MTs may play important roles as they exist in animals and fungi (Cobbett and Goldsbrough, 2002; An et al., 2006). Many studies suggest that MTs might be involved in biological processes as diverse as apoptosis, growth, embryonic development, microspore development, senescence, fruit development, maturation and stress responses (Murphy et al., 1997; Giritch et al., 1998; Yu et al., 1998; Cobbett and Goldsbrough, 2002; Liu et al., 2002; Bhalerao et al., 2003; Akashi et al., 2004; Lee et al., 2004). Plant MTs function in metal metabolism and detoxification due to their metal-binding activity and inducibility by heavy-metal ions (Cobbett and Goldsbrough, 2002; Bhalerao et al., 2003). In rice, several type 1 and type 2 *MTs* genes have been identified from the rice MT family (Zhou et al., 2005). Their exhibited induction patterns vary by the metals to which they are exposed, sucrose starvation, salt stress and other stimuli (Zhou et al., 2005). *MTs* genes involved in heavy metal metabolism, seed development, and antioxidation (Yu et al., 1998; Wong et al., 2004; Zhou et al., 2005). Expression and characterization analysis of type 2 metallothionein from grey mangrove species (*Avicennia marina*) in response to metal stress, the result support the hypothesis that AmMT2 may be involved in processes of metal homeostasis or tolerance *in A. marina* (Huang and Wang, 2010). In other plant species, such as *Arabidopsis thaliana* and *Gossypium hirsutum*, they have similar functions (Gou et al., 2003; Xue et al., 2009). Understanding the biological roles of plant MTs is important to speed the forest tree breeding process and enhance forest tree productivity. Biotechnological applications of MTs are an ultimate goal in MTs research. The recent advances in our knowledge regarding the classification of forest tree MTs, their functions and their use in phytoremediation are described in this review.

1. General classification and function of plant MTs

1.1 Classification of plant MTs

Metallothioneins (MTs) are a family of cysteine-rich (20%~30%), low-molecular-weight (2~7 kDa), cytoplasmic metal-binding proteins. MTs are present in various eukaryotic organisms including fungi, invertebrates, mammals, and plants, as well as some prokaryotes. The first plant MTs were discovered in roots of soybean by Casterline and Barnett (1982). Plant MTs are known to play a role in metal metabolism and detoxification due to their metal-binding activity and inducibility by heavy-metal ions. They are quite different from the animal MTs in amino acid sequence, charge, homocysteine number and distribution pattern (Mi et al., 2004). Plant MTs are divided into four types based on the arrangement of Cys residues (Cobbett and Goldsbrough, 2002).

1.2 Function of plant MTs

Multiple MTs types have been reported in plants like rice, hybrid poplar, oil palm and lichens, and many cDNAs encoding *MTs* genes have been isolated (Abdullah et al., 2002, Kohler et al., 2004, Zhou et al., 2006, Bačkor and Loppi, 2009). Most information about their putative function is derived from mRNA expression studies. In plants, *MTs* gene expression has been found to be influenced by metal ions, osmotic stress, drought, dark-induced senescence, cold/heat shock, UV irradiation, oxidative stress and pathogen attack, as well as by various plant hormones and endogenous factors (Foley and Singh, 1994; Mir et al., 2004; Berta et al., 2009). Generally, type 1 genes are predominantly expressed in roots, type 2 in leaves, type 3 in ripening fruits and type 4 in seeds (Cobbett and Goldsbrough, 2002), however, the expression characteristics of each type of plant *MTs* gene differs from species to species. Moreover, plants MTs are involve in many important developmental processes, such as fruit maturation, root growth and apoptosis (Mir et al., 2004; Yuan et al., 2008). In *A. thaliana*, MTs form a diverse family, with seven translated genes belonging to four types (Cobbett and Goldsbrough, 2002), and different types have unique and overlapping functions in homeostasis, heavy metal detoxification and cells antioxidative defenses (Murphy et al., 1997; Miller et al., 1999; Lee et al., 2004).

1.2.1 MTs function as metal chelators

The main functions of plant *MTs* genes are related to metal metabolism and detoxification. Expression of *MTs* genes can be induced not only by essential metals such as copper (Cu) and zinc (Zn), but also by nonessential toxic metals like cadmium (Cd). In plants, the first evidence for the role of MTs in Cu and Cd tolerance was provided by Zhou and Goldsbrough (1995). In their study, two *A. thaliana MTs* genes were expressed in MT-deficient yeast (*Saccharomyces cerevisiae*). Further evidence for the function of metal metabolism and detoxification for various MTs in plants has been confirmed by knock-down and overexpression lines, tissue-specific and metal-regulated expression, and characterization of MT-metal complexes (Guo et al., 2003; Freisinger, 2007; Yuan et al., 2008). In *A. thaliana*, the expression of *MTs* was related to Cu stress (Murphy and Taiz, 1995). When expressed in metal-sensitive yeast, plants MTs are able to increase tolerance of the yeast to Cu, Cd and Zn stress (Zhou and Goldsbrough, 1995; Roosens et al., 2004). PcMT3 from *Porteresia coarctata* showed enhanced expression level when exposure to heavy metals like Cd, Zn and Cu (Usha et al., 2011). MTs are known as major transition metal ion binding proteins in cells, mostly complexing with essential metals such as Cu and Zn, but having less binding ability to Cd, mercury and lead (Pb). Type 1, type 2, type 3 and type 4 plant MTs have been identified, and each may have higher affinity for same heavy metals than others (Foley et al., 1997).

1.2.2 MTs scavenge reactive oxygen species (ROS)

In addition to metals, other factors are known to increase the expression levels of MTs. Some researchers hypothesize that MTs can protect plants and their DNA against oxidative damage by removing free radicals. Akashi et al. (2004) suggested that ClMT2 induction contributes to the survival of wild watermelon (*Citrullus lanatus sp.*) under severe drought and high light stress conditions and they were first to demonstrate that a plant MT with antioxidant properties functioned as an ROS scavenger. Transgenic tobacco (*Nicotiana tabacum*) plants overexpressing *GhMT3a* showed increased tolerance to ROS stresses compared with wild-type plants. These results indicated that *GhMT3a* was an effective ROS scavenger and that its expression could be regulated by abiotic stresses through ROS signaling (Xue et al., 2009). Recently, the research on the antioxidant properties of plant MTs was increasing. However, the interplay between metal binding and ROS scavenging is currently not clear. During ROS scavenging process, metals would be released when ROS species are bound to the Cys residues of the MTs. Some authors have proposed that the released metals might be involved in a signaling cascade. Based on these results, a model mechanism of MTs action in plants has been proposed (Fig. 1).

1.2.3 Other possible roles for MTs

The expression of *MTs* genes is affected not only by metal and oxidative stress but also by responses to abiotic stress (Brkljacic et al., 2004; Jin et al., 2006). *GhMT3a* was up-regulated not only by heavy metal ions, reactive oxygen species (ROS), and Zn and Cu in cotton seedlings, but also by high salinity, drought, low temperature, abscisic acid (ABA) and ethylene (Xue et al., 2009). Guo et al. (2003) reported that *AtMTs* can be induced by senescence and suggested that *MTs* may be involved in the remobilization of metal ions from senescing leaves. Bhalerao et al. (2003) reported that *MTs* were abundant in a cDNA library derived from senescing (*Populus tremula* × *tremuloides*) leaves. The induction by senescence may also be related to ROS removal or signaling.

2. Classification of poplar *MT* genes

Thus far, there has been little research on *MTs* genes in forest trees. *MTs* genes have only been reported from hybrid poplar roots of *Populus trichocarpa* × *deltoides* (Kohler et al., 2004). The poplar genome contains at least six different *MT* genes divided into three subgroups, corresponding to the previously described type 1, type 2 and type 3 *MTs* described in other plants (Cobbett and Goldsbrough, 2002). Type 4 *MTs* sequences were neither found in our cDNA libraries nor in the NCBI poplar ESTs (Guo et al., 2003). Sequence alignment of the cDNAs with genomic sequences showed that the *MTs* genes contained one to three introns. The intron sizes ranged from 71 to 275 bp. In *PtdMT1a*, *PtdMT1b*, *PtdMT2a*, and *PtdMT2b*, the first intron (82–258 bp) was inserted upstream of the ATG start codon. All of the *MTs* genes contained putative TATA and CAAT boxes in a region between 8 to 635 bp upstream of the predicted start codon. A variety of other regulatory elements were also found with high homology among several *MTs* genes . For example, *PtdMT1a* and *PtdMT1b* both contained MYC and MYB-binding sites involved in drought- and abscisic acid-regulated gene expression. Both *PtdMT2a* and *PtdMT2b* contained ABRE-like and MYB-binding sequences required

Table 1. Functions of *MTs* genes have been identified in some plant species.

Specie	Gene	Function	Reference
Arabidopisis thanliana	*AtMT*	Tissue specific expression and induction during senescence and in response to Cu	Guo et al., 2003
	AtMT1	Enhances tolerance to Cd in yeast	Lee et al., 2004
	AtMT2	Enhances tolerance to Cd in sensitive yeast	Lee et al., 2004
	AtMT2a	Enhances resistance to Cd in *Vicia faba* guard cells	Lee et al., 2004
	AtMT3	Enhances resistance to Cd in *Vicia faba* guard cells	Lee et al., 2004
	AtMT4a	Enhances Cu and Zn tolerance in *A. thanliana*	Rodríguez et al.,2010
Brassica juncea	*BjMT2*	Enhances Cu and Cd tolerance in *E. coli* and *A. thaliana*	An et al., 2006
Citrullus lanatus	*ClMT2*	Drought stress induced	Akashi et al., 2004
Fagopyrum esculentum	*FeMT3*	Enhances drought and oxidative tolerance in buckwheat	Samardzic et al., 2010
Gossypium hirsutum	*GhMT3a*	Enhances biotic stress tolerance in transgenic tobacco and yeast	Xue et al., 2009
Oryza satiral	*OsMT1a*	Plays roles in Zn homeostasis and enhance drought tolerance in transgenic rice	Yang et al., 2009
	OsMT2b	Plays roles in root development and zygotic embryo germination of rice	Yuan et al., 2008
Porteresia coarctata	*PcMT3*	Cd, Cu and Zn induced	Usha et al., 2011
Thlaspi caerulescens	*MT2*	Contributes to the metal adapted phenotype in *T. caerulescens*	Hassinen et al., 2009
	MT3	Cu homeostasis function in *T. caerulescens*	Roosens et al., 2004
Avicennia marina	*AmMT2*	Enhances Zn, Cd, Cu, Pb tolerances in transgenic *A. marina*	Hunag and Wang, 2010
Populus alba L	*MT1*	Zn induced	Castiglione et al., 2007
	MT2	Cd, Cu, Zn induced	Macovei et al., 2010
	MT3	Water deficit induced and as ROS scavenger	Berta et al., 2010
Populus trichocarpas× deltoide	*PdtMT1a*	Zn induce expression in root	Kohler et al., 2004
	PdtMT1b	Zn induced expression in root	Kohler et al., 2004
	PdtMT2a	Enhances Cd tolerance in yeast and Zn induced expression in root, leaves and stem	Kohler et al., 2004
	PdtMT2b	Enhances Cd tolerance in yeast and Zn induce expression in root, leaves and stem	Kohler et al., 2004
	PdtMT3a	Enhances Cd tolerance in yeast and Zn induce expression in root, leaves and stem	Kohler et al., 2004
	PdtMT3b	Zn induce expression in leaves	Kohler et al., 2004
Tamarix hispida	*ThMT3*	Enhances Cd, Zn, Cu and NaCl tolerance in transgenic yeast	Yang et al., 2011

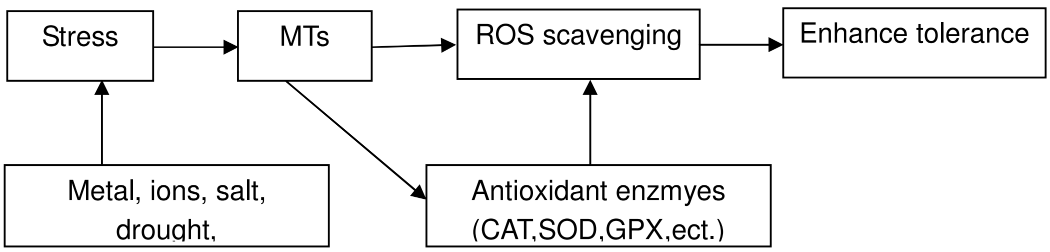

Fig 1. A model mechanism of MTs action in plants

for stress and senescence, gibberellin-responsive element and G-boxes.

3. Expression patterns of poplar MTs

MTs gene expression patterns in model plants were described above. *PtdMTs* play different roles in different developmental stages, such as root development and leaf senescence. *PtdMT1* was predominantly expressed in roots. *PtdMT2* was expressed in roots, leaves, twigs and stems, indicating broader tissue-specific expression. *MT3b* was preferentially expressed in leaves (Cobbett and Goldsbrough, 2002). The transcripts levels of poplar *MTs* genes increased with aging, metal stress, and viral infection (Bhalerao et al., 2003; Kohler et al., 2004). The involvement of MTs in response to plant water stress and recovery was assessed by analyzing gene expression in leaves and the cambial zone of white poplar. Expression of *Populus alba MT2a* and *MT3a* in leaves and roots was higher as water stress increased (Street et al., 2006; Bogeat et al., 2007). The occurrence of multiple MTs in poplar may reflect their diversity of function in heavy metal sequestration and in other mechanisms essential for plant growth. The transcript levels of poplar *MTs* increased during senescence, heavy metal treatment and virus infection (Kohler et al., 2004). Because no recent comprehensive reviews on this subject are available, we wish to briefly summarize the current knowledge regarding the MTs forms in forest trees.

4. MTs functions in metal tolerance and accumulation in forest trees

Kohler et al. (2004) reported that in hydroponically-grown poplars, treatment with Zn affected the expression of *PtdMTs* (*PtdMT1a*, *PtdMT1b*, *PtdMT2a* and *PtdMT2b*). The longer the trees were exposed, the expression levels were higher, suggesting that *Populus MTs* genes might function as enhancers of Zn tolerance. Kohler et al. (2004) also found that the overexpression of *PtdMTs* (*PtdMT1a*, *PtdMT1b*, *PtdMT2a*, *PtdMT2b*, *PtdMT3a* and *PtdMT3b*) increased the Cd tolerance of transgenic yeast. Macovei et al. (2010) discovered that white poplar (*Populus alba*) cells grown in cell suspensions with different doses of Cd (150 μM), Zn (2 mM) and Cu (100 μM) were all died, while 27%, 28% and 21% of cells transformed with the *VFMT2* gene survived, suggesting that *MT2* may contribute to enhancing heavy metal tolerance. In our lab, a *ThMT3* (GenBank No. EH057039) gene from *Tamarix hispida* was cloned. Expression analysis revealed that *ThMT3* was induced by Cd, Zn, Cu and NaCl (Yang et al., 2011). Transgenic yeast expressing *ThMT3* showed increased tolerance to Cd, Zn, Cu, NaCl stress, and ROS. Three antioxidant genes (SOD1, CAT1, and GPX1) were specifically expressed under Cd, Zn, Cu, and NaCl stress. Cu increased the expression levels of SOD1, Zn increased the expression levels of CAT1, and whereas NaCl induced the expression of SOD1 and GPX1, suggesting all three antioxidant enzymes participate in the process of scavenging superoxide radicals in MT transgenic yeast. The altered expression of heavy metal transportation system genes in the vacuole of transgenic yeast, however, is different from that found in most plant *MTs* gene studies (Hassinen et al., 2011). *ThMT3* are primarily involved in the homeostasis of cellular Cd and Zn rather than Cu (Yang et al., 2011). The results in Yang et al. (2011) study showed that ThMT3 not only scavenged ROS but also enhanced plant tolerance of heavy metals and the absorption of Zn and Cd. Although some preliminary studies on the *ThMT3* gene and its mechanism have been done, its mechanism of tolerance to heavy metal is still unclear. What upstream signaling pathway regulates *ThMT3* gene upstream expression? Which genes coordinate with *ThMT3* to improve resistance to heavy metal stress? The precise functions of forest tree *MTs* genes and the pathway of metal tolerance needs further investigation. However, many specifically or preferentially expressed *MTs* genes have been identified in plant (Table 1).

5. Forest tree MTs function as ROS scavenger

Drought, high salinity, and low temperatures are three important abiotic stresses that are commonly encountered by plants growing in natural environments. One of the most common and crucial consequences of these stresses is the generation of ROS in plants, which can damage cellular constituents and act as signals for ameliorating oxidative stress. Drought stresses enhance the generation of ROS in plants, which lead to a series of enzyme reaction adverse to plant (Apel and Hirt, 2004). *MTs* gene expression level of *Populus euphratica* growing in the Negev desert increased as amount of precipitation decreased, while ROS levels declined (Brosche et al,. 2005). These results indicate that MTs may be involved in scavenging ROS. Expression of *MTs* induced the production of a series of active oxygen defense enzymes, but these enzymes were transcribed at different levels from other defense enzymes involved in ROS. Thus, MTs play a role in ROS detoxification, but the specific pathways and mechanisms have not been determined. In a survey of the role of MTs involved in heavy metal detoxification, plant growth and development mechanisms under Cu stress and the expression levels of same genes were changed, in particular, oxidative stress regulating proteins decreased in abundance. When plants were attacked by pathogens that induced their defense systems, MTs were metal chelators with ROS scavenging activity and modulated oxidative signal. MTs associated with the down-regulation of oxidative signal patterns have also been described in poplar (Smith et al., 2004) and rice (Wong et al., 2004). Lower *MTs* transcript levels detected in the experiments could be associated with a transient ROS accumulation, which in turn would be necessary to trigger a general resistance response. However, the precise functions of plant MTs remain elusive.

6. *MTs* transgenic forest tree used for phytoremediation

Phytoremediation of heavy-metal contaminated land using trees has considerable potential, and the approach is gaining increasing interest (Pulford and Watson, 2002). Poplars and willows have already been tested in phytoremediation. At one polluted site, poplars were found to accumulate Cd and Zn at such high levels (800 mg kg/1), thus, that fallen leaves had to be removed and treated (Laureysens et al., 2004). Large areas of land that had been contaminated with Cd, Pb, and Zn are currently in agricultural production in the Campine region of Belgium. After willows were used in short-rotation systems, soil pollution was reduced to levels safe for crops (Witters et al., 2009). Felix (1997) reported that *Salix viminalis* had the highest metal-accumulating ability among several plants tested. Guerra et al. (2009) analyzed the differential gene expression in plants exposed to 30 μM and 60 μM Cu for 12 and 24 hours respectively and found that Cu increased the metal accumulation in their roots of *P. deltoides*, which suggested that *Populus* could be bred for the purpose of phytoremediation (Guerra et al., 2009). Poplar MTs are a genefamily of proteins. Many reports conclusively demonstrate that the expression of *MTs* genes are involved in several processes, including metal ion homeostasis and heavy metal detoxification, which suggested that *MTs* genes could be used in phytoremediation. The expression of *MTs* genes is up-regulated as metal ion concentrations increases, suggesting that ability of plants to amend polluted soil by absorbing heavy metal ions is correlated with MT genes. The expression of forest tree *MTs* genes in transformed model plants including *A. thaliana*, tobacco and yeast enhanced the ability of metal uptake, transport and sequestration (Yang et al., 2011; Kohler et al., 2004). Whether using transgenic forest trees for phytoremediation could be effective remains to be determined. Poplar species and their hybrids are becoming the model plants for forest tree biotechnology studies (Bradshaw et al., 2000), providing the molecular biology and genetic engineering foundation on which to investigate forest tree phytoremediation of land contaminated by heavy metals.

7. Future prospects and conclusions

Researches on MTs cover the fields of biochemistry, environmental hygiene, medicine, toxicology, pathology, and nutrition. Most *MTs* genes are cloned from herbaceous plants, although a few came from forest trees, which are quite different from herbaceous plants in biological processes such as growth, senescence, fruit development, maturation, and the basic mechanisms implicated in plant metal tolerance and homeostasis. Some forest trees in extreme environments can withstand severe environmental stress conditions, indicating that they may have efficient stress-related genes. Genes isolated and identified from forest trees growing in extreme environment have attracted much attention for research institutions worldwide. The study of *MTs* genes in forest trees is at the beginning stages, but the results could have broad applicability and huge market potential. Because of their deep root systems and high biomass yields, poplars are becoming important sources of wood and are also candidates for phytoremediation, they have huge economic and ecological value. Genetic engineering of plants for enhanced phytoremediation has obvious environmental benefits (Linacre et al., 2003). *MTs* genes involved in metal uptake, transport and sequestration have been studied in herbaceous model plants such as *A. thaliana*, tobacco and yeast. Overexpression of *MTs* in plants improves heavy metal tolerance. The transformation of *MTs* genes from different sources into forest trees and the breeding of new cultivars that can tolerate and accumulate heavy metals will benefit both the environment and human health.

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