REVIEW ARTICLE

Plant Metallothioneins as Regulators of Environmental Stress Responses

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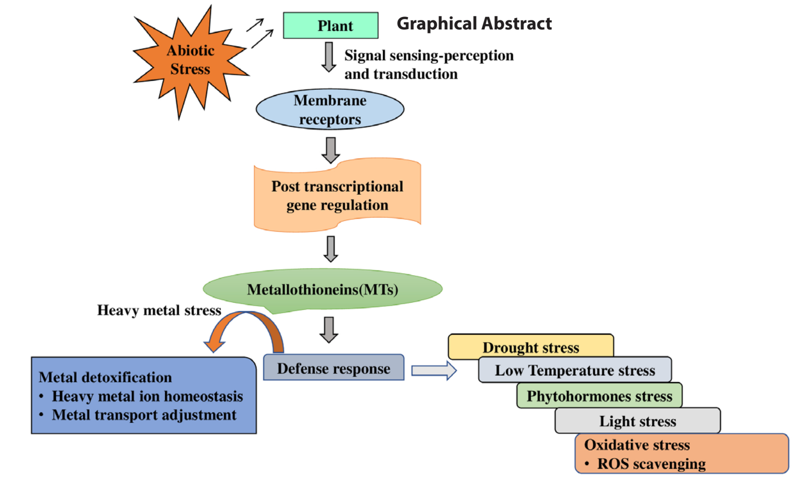
ABSTRACT

Scientific research has focused to delineate the core aspects of stress tolerance mechanisms and to apply the crucial components for enhancing the tolerance of plants and their growth under stressed environments. Metallothioneins (MTs) are known to play pivotal roles in stress regulation. They are known in bacteria, fungi, and all other eukaryotic organisms. MTs are low molecular weight, cysteine-rich proteins. They are important metal chelators and also possess antioxidant properties. MTs work upon reactive oxygen species (ROS), whose production is induced by different stresses and is known to damage various cellular biomolecules. MT proteins are classified into two classes, plant MTs belong to class II type, while animal MTs belong to class I type. MTs have been investigated in the vast number of plant species and their crucial roles in stress modulation has been uncovered. Hence, MTs are a promising tool for stress management, acting as essential stress biomarkers and ensuring food security in the coming future to achieve sustainable development goals worldwide. This review discusses the roles of MTs in plants under different abiotic stresses, including drought, salinity, low temperature, light, in oxidative stress regulation and heavy metals homeostasis and detoxification including toxic nanoparticle management in plants.



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INTRODUCTION

Plants are sessile organisms and need to survive in challenging environments induced by physical and chemical stresses. Plants are essential to meet the needs of the human diet. With the increasing demand for food due to the rising population, enhancing food production is a challenging and uphill task. A new paradigm is a need for present climate change mitigation, including improved agricultural practices with genetic modifications and improved plant breeding techniques (Fita *et al.*, 2015). Abiotic stresses include water availability (flooding and drought), temperature (cold and heat), heavy metals, salinity, chemicals, and light encompassing UV-B radiation. These are detrimental to plant growth and development and lead to huge crop losses every year worldwide. Drought and salinity predominantly affect most of the agricultural land worldwide (Soda *et al.*, 2016). Reactive oxygen species (ROS) are a major outcome of abiotic stresses and cause severe damage at cellular levels (Das *et al.*, 2014). Environmental contamination with metals is also a major problem threatening crop productivity and yield throughout the world. It is of foremost importance to combat such multiple stresses to save crops and to also sustain and improve their yields to meet the demands of the growing population.

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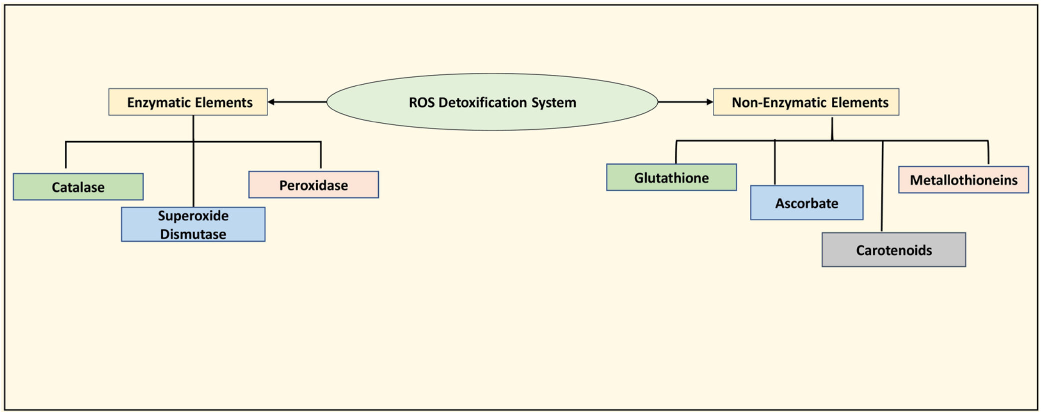

Fig. 1: A schematic flow diagram of ROS detoxification system in plants including metallothionein.

Plants have unique machinery, including molecular mechanisms for defense against such stresses. The defense system includes morphological modifications, ROS scavenging, compatible solutes, and molecular chaperones. Hence, plants have suitable resisting mechanisms to overcome such damage at both cellular and genomic levels. At the cellular level, modifications in cellular structure and cell cycle and cell division, changes with the synthesis of different hormones like ethylene, salicylic acid, abscisic acid, and jasmonic acid are reported (Kazan, 2015; Wani *et al*., 2016).

A complex molecular network upholding both upstream and downstream effects at the genomic level plays key role. Upstream effects involve signaling molecules like polyamines, ROS, phytochromes, and stress hormones, whereas downstream effects involve gene regulation, particularly transcription factors (TFs) (He *et al*., 2018; Khan *et al*., 2018). Through stress regulatory genes or transcription factors, post-transcriptional regulation is an essential tool of investigation for improved crop yield. A large number of TFs have been investigated to play roles in stress regulatory pathways. ROS detoxifying agents include certain enzymes and antioxidant molecules along with metallothioneins (Fig. 1). Metallothioneins (MTs) are constitutively expressed and activated by both endogenous and exogenous agents spatially and temporally. It is one of the cytosolic protein family implicating essential metal homeostasis and modulation of various stresses other than metal ions (Mekawy *et al*., 2020). Hence, it is a research topic of immense value for investigating the abiotic stress biology of plants and stress-responsive factors, including metallothioneins.

METALLOTHIONEINS: THE FIRST LINE OF CELLULAR DEFENSE

Metal toxicity with Ca, Cu, Zn, and other heavy metals impose severe physiological defects in plants, hampering plant growth and development (Kupper and Andresen, 2016). Metal chelators play important roles in the detoxification and homeostasis of metals. Metallothioneins are among the first line of cellular defense for heavy metal detection in polluted soils and aquatic areas and are used to screen and detect heavy metal pollutants (Gutiérrez *et al*., 2015). With their characteristic feature of metal sequestration, they are crucial members of ROS-induced cell injury inhibition or reduction. The Cys-residues having thiol group (-SH) are oxidized, leading to their direct role in ROS elimination, maintaining the cellular ROS levels, and shielding the cell and defending it from harmful ROS-induced injuries (Mekawy *et al*., 2020).

Major intracellular metal-binding agents include organic acids, amino acids, phytochelatins (PCs), and metallothioneins (MTs). Both PCs and MTs have characteristic cysteine-rich residues. MTs are known to occur in bacteria, fungi, plants, and animals except for Eubacteria. Initially, MT was isolated from the horse kidney and was revealed as a Cd binding protein (Thirumoorthy *et al*., 2011; Kepinska and Milnerowicz, 2015). An MT protein (Ec) was first isolated from wheat germ and the first plant MT protein to be characterized and purified. It binds to Zn and is regulated by abscisic acid rather than metals (Jin *et al*., 2014). MTs are among the highly abundant transcripts in plants. Numerous genes have been investigated in different plants, including tomato, wheat, rice, and soybean, as well as in the model plant *Arabidopsis*, which uncover the roles of MTs in basic physiological activities, including embryo development, along with the response to abiotic stress (Yang *et al*., 2015). Various external stimuli uphold the expression of MTs in plants but their regulatory mechanisms modulating their expression is still not resolved (Khalid *et al*., 2020). Due to the increasing interest in plant metallothioneins, the vast database of sequences is available for creating a considerable research base for researchers (Hassinen *et al*., 2011; Khalid *et al*., 2020). MTs are found in different eukaryotes, like liverworts (*Marchantia polymorpha*), mosses including (*Syntrichiaruralis*, *Physcomitrella patens*, *Grimmiapilifera*), algae (*Sargassum binderi*), and various higher angiosperms like *Arabidopsis*, *Cicer arietinum*, *Musa acuminata*, *Triticum* and many more.

Characterization of plant MTs using molecular techniques like overexpression, knockdown of *MT* gene, tissue-specific expression, characterization of protein-metal complex, and expression of *MT* gene based on metal regulation has been carried out as per the advancements in the study (Hassinen *et al*., 2009). *Mimulus guttatus* being the first cloned *MT* gene in plants (Zúñiga *et al*., 2020). MTs are generally classified into Class I or Class II based on the arrangement of the cysteine-containing thiol groups. These MTs are distinguished into four types as discussed by Zúñiga *et al*. (2020) and are organ-specific. pMTs belong to Class II type. pMTs have been categorized into four subfamilies, namely MT1, MT2, MT3 and MT4 identified in different plants (Table 1-4). MT1, MT1a of chickpea, and MT1c of *Arabidopsis* are class I MTs. MT2 of chickpea and MT2a, MT2b of *Arabidopsis* belong to class II MTs. In a similar context, MT3 of banana and *Arabidopsis* are placed in class III, whereas class IV includes MT4a and MT4b from *Arabidopsis* and *Triticum aestivum* (Benatti *et al*., 2014).

MT1 is found in roots and investigated in *Pisum sativum*, *Zea mays*, and *M. guttatus*. MT2 transcripts have been observed in the aerial parts like leaves and stems in *Arabidopsis*. MT3 has been identified from ripened fruits in *Vitis vinifera, Malus domestica, Actinidia deliciosa*, and *Elaeis guineensis*. MT4 is known to be specific for expression in germinating pollen and seeds. They are also reported in reproductive as well as vegetative tissues (Dabrowska *et al*., 2012). The anti-apoptotic defense is also an important regulatory mechanism modulated by MTs in plants (Golestannejad *et al*., 2016). Their expression depends on various stimulations, including heat, cold, drought, oxidative stress, and excess accumulation of heavy metals. Characteristic dumbbell conformation, including cysteine residues with sulfhydryl groups, enables the gene to bind to heavy metal ions. Because of their structural features, they participate in metal detoxification (Hrynkiewicz *et al*., 2012). Hence, MTs have roles in stress management through metal ion homeostasis and metal transport adjustment. MTs are known to be important antioxidant agents with metal-chelating properties (Hassinen *et al*., 2011). In sweet potato, MTs play important antioxidant roles against hydroxyl and peroxyl radicals (Huang *et al*., 2014). MTs form mercaptide bonds, and perform their action involving buffering metal concentration in the cytosol. MTs are investigated under *in vivo* conditions to release accumulated zinc on the increase of ROS and RNS (Reactive Nitrogen Species) (Drzeżdżon *et al*., 2018).

Role of MT in other stresses, including both abiotic and biotic, like drought, low temperature, oxidative stress, light, and phytohormones are also known (Sharma *et al*., 2016; Mekawy *et al*., 2018b). Expression levels of MTs are also affected by microorganisms present in the substrate (Dąbrowska *et al*., 2012; Hrynkiewicz *et al*., 2012). Along with oxidation and dehydration, MTs also play roles in plant development and other stress modulating roles, including root development, fruit ripening, senescence, hormonal modulations, regulation of various metalloenzymes, suberization, DNA damage repair, and in the regulation of cell growth and proliferation (Moyle *et al*., 2005; Cherian and Kang, 2006; Yuan *et al*., 2008) (Fig. 2).

STRUCTURAL ANALYSIS AND BIOSYNTHESIS

Metallothionein (MT) protein is an evolutionarily conserved cysteine-rich protein of a low molecular weight of <10 kDa, which covalently binds with heavy metal ions (Khalid *et al*., 2020). The first discovery of Zn-bound MT protein (Ec-1) from mature wheat embryo has curiously involved more research in the structural analysis of MT protein. 3-D structural data of any of the pMTs (1-3pMTs) have not yet been reported, with the only exception being Ec-1 of wheat (Khalid *et al*., 2020).

The variations reported in the primary structures among different MTs, reflect their distinct structural properties with molecular structures. The distinct arrangement of cysteine, structural variations like sequence length, amino acids composition, and cysteine arrangement and numbers differentiate the metallothioneins from one another (Leszczyszyn *et al*., 2013).

Being flexible in structural confirmation, plant metallothioneins possibly contain histidine and aromatic residues (Isani *et al*., 2014). Low kinetic ability and high stability of MT gene thermodynamically with metal complexes add positive characteristics to its metal binding capacity. The two terminals, C and N-terminal have α and β domains, respectively. The C-terminal contains a stable α domain, whereas N-terminal having β domain, which is reactive. The cysteine-rich domains were separated and linked by four to seven amino acids (Zúñiga *et al*., 2020). Plant MTs are of four types with type 1, 2, and 3 with 40 and 15 amino acids linker portions (Cys-free spacer) between cysteine-rich domains. The cysteine-rich

Table 1: Roles of metallothioneins (MT1) in plants under multiple abiotic stresses.

S. No.	*Plant*	*Roles for stress tolerance*	*References*
1.	*Arabidopsis thaliana*	Homeostasis of Cu, Cd, and Zn	(Zimeri *et al*., 2005), (Guo *et al*., 2008)
2.	*Paxillus involutus*	Tolerance to Cu	(Bellion *et al*., 2007)
3.	*Brassica rapa*	Increased Cd tolerance with ROS scavenging	(Kim *et al*., 2007)
4.	*Brassica campestris*	Cu, Cd and ROS tolerance	(Lv *et al*., 2013)
5.	*Festuca rubra*	Sequestration of metals	(Ma *et al*., 2003)
6.	*Oryza sativa*	Higher tolerance to Ni, Cd, Pb and Zn and drought tolerance via ROS scavenging	(Malekzadeh and Shahpiri 2017)
7.	*Oryza sativa*	Enhanced accumulation of Hg and As	(Shahpiri and Mohammadzadeh 2018a,b)
8.	*Cajanus cajan*	Increased tolerance to Cd and Cu	(Sekhar *et al*., 2011)
9.	*Ziziphus jujuba*	Lower the Cd toxicity	(Li *et al*., 2016)
10.	*Chloris virgata*	Tolerance to salinity, carbonate and drought stress	(Nishiuchi *et al*., (2007)
11.	*Elsholtzia haichowensis*	Cu accumulation and tolerance	(Xia *et al*., 2012)
12.	*Zea mays*	Zn, Cd and Pb tolerance	(Duan *et al*., 2019)
13.	*Nicotiana tabacum*	Cd accumulation and tolerance	(Sidik *et al*., 2019)

Table 2: Roles of metallothioneins (MT2) in plants under multiple abiotic stresses.

S.No.	*Plant*	*Roles for stress tolerance*	*References*
	Quercus suber	Resistance to Cd, Cu, and Zn	(Domenech *et al*., 2007)
2.	*Oryza sativa*	Suppress ROS production and Cu toxicity	(Liu *et al*., 2015)
3.	*Hevea brasiliensis*	Tolerance to Zn and Cu	(Zhu *et al*., 2010)
4.	*Saccharum sp.*	Increased tolerance to drought, salinity, Cu, and Cd	(Guo *et al*., 2013)
5.	*Iris lacteal*	Decreased production of H_2O_2	(Gu *et al*., 2015)
6.	*Silene vulgaris*	Tolerance to Cu	(Van Hoof *et al*., 2004)
7.	*Citrullus lanatus*	Resistance to drought and light stress	(Akashi *et al*., 2005)
8.	*Olea europaea*	Metal homeostasis	(Dundar *et al*., 2015)
9.	*Brassica juncea*	Tolerance to Cd and Cu	(Zhigang *et al*., 2006)
10.	*Brassica campestris*	Resistance to Cd, Cu, and ROS	(Lv *et al*., 2013)
11.	*Thlaspi caerulescens*	Increased Zn and Cd tolerance	(Hassinen *et al*., 2009)
12.	*Bruguiera gymnorrhiza*	Tolerance to Zn, Cu, Cd, and Pb	(Huang *et al*., 2011)
13.	*Pisum sativum*	Resistance to Cu	(Turchi *et al*., 2012)
14.	*Arabidopsis thaliana*	Excess metal ions removal from trichomes, oxidative stress	(Zhu *et al*., 2009)
15.	*Suaeda salsa*	ROS, oxidative and metal tolerance	(Jin *et al*., (2017)
16.	*Azolla filiculoides*	Metal homeostasis	(Fumbarov *et al*., 2005)
17.	*Cicer microphyllum*	Multiple abiotic stress responses	(Singh *et al*., 2011)
18.	*Allium sativum*	Cd tolerance	(Zhang *et al*., 2006)
19.	*Sedum alfredii*	Cu tolerance	(Zhang *et al*., 2014)
20.	*Helianthus annuus*	Cr stress tolerance	(Agar *et al*., 2019)
21.	*Avicennia marina*	Cu_2 +, Fe_2+, Zn_2 +, Ni_2+ and Cd_2+	(Babaei-Bondarti and Shahpiri 2020)

Table 3: Roles of metallothioneins (MT3) in plants under multiple abiotic stresses.

S. No.	*Plant*	*Roles for stress tolerance*	*References*
1.	*Fagopyrum esculentum*	ROS scavenging	(Brkljacic *et al*., 2004)
2.	*Porteresia coarctata*	Zn, Cd and Cu tolerance	(Usha *et al*., 2011)
3.	*Tamarix hispida*	Enhanced Cu, Zn, salinity and Cd tolerance	(Yang *et al*., 2015 a)
4.	*Thlaspi caerulescens*	Zn and Cd tolerance	(Hassinen *et al*., 2009)
5.	*Gossypium hirsutum*	Modulating H_2O_2 levels	(Xue *et al*., 2009)
6.	*Arabidopsis thaliana*	Removal of excess metal ions	(Guo *et al*., 2003)
7.	*Oryza sativa*	Tolerance to salinity and heavy metals	(Mekawy *et al*., 2018b) (Gautam *et al*., 2012).
8.	*Brassica napus*	Resistance to As3+ stress	(Pan *et al*., 2018)

Table 4: Roles of metallothioneins (MT4) in plants under multiple abiotic stresses.

S.No.	Plant	Roles in stress tolerance	References
1.	*Arabidopsis thaliana*	Cu and Zn tolerance	(Llorente *et al*., 2010)
2.	*Hordeum vulgare*	Aleurone layer binding Zn	(Hegelund *et al*., 2012)
3.	*Brassica napus*	Zn tolerance	(Mierek-Adamska *et al*., 2018)

domains have variable arrangements of cysteines. Type 4 has cys-free spacer of 15 amino acids between the cysteine-rich domains. The N-terminal domain has variable cys-residues, whereas the C-terminal domain has conserved six Cys-residues arranged in a consensus manner in type 1, 2, and 3 but has three cys-rich domains in type 4 with six, six, and five cys-residues in each (Mekawy *et al*., 2018b). MTs have a significant primary structure with CC, CXC, and CXXC motifs. These motifs help in toxic metal detoxification and essential metal homeostasis to bind mono- or bivalent metal ions, usually Cu^+, Zn^{2+}, and

Cd^{2+}. Comparative analysis reveals rapid replacement of ions, including Zn and Cd in β-domain, than α-domain. The thiol groups in MTs are myriad of powerful antioxidant properties with oxidative stress prevention activities. The metal ions bind to these metal-thiolate clusters provides high kinetic labiality with thermodynamic stability.

A strong resemblance in the structure of class III MTs with glutathione (GHS), concludes the possibility of synthesis of MTs with thiol group-rich tripeptides or their precursors (Sharma *et al*., 2016). Experiments indicating the inhibition of MTs and hampered Cd tolerance when exposed with buthionine sulfoxamine, an inhibitor of γ-glutamylcysteine, also reduce the GHS concentration. Enzymes lacking for GHS synthesis in mutants of fission yeast (*Schizosaccharomyces*) also represents class III MTs. Results of *Silene cucubalus* indicate the synthesis of pMTs from γ-glutamylcysteine dipeptidyl transpeptidase (Khalid *et al*., 2020).

With the advancement in structural determination and deeper insights of their functional analysis, such proteins are interesting tool for researchers, and hence their detection and metallation pathways have been identified and are to be uncovered. Analytical methods for determining MTs in organisms are well discussed by Ryvolova *et al*. (2011) and by Krystofova *et al*. (2012). Such methods involve both bioanalytical methods and immunochemical methods for the detection of metallothioneins in different organisms (Fig. 3). Recent data has revealed the pathway of metallation of MT2 in *Cicer arietinum* using mass spectroscopy. A single metal-thiolate cluster was formed when 14 cysteine thiolate groups were coordinated with up to five Zn^{2+}or Cd^{2+} ions. At the N-terminal, binding of Cd^{2+} was reported as up to three ions were coordinated with eight cysteine residues whereas two Cd^{2+} were coordinated with six cysteine residues at C-terminal part. The non-coordinated cysteine-thiolate groups were free for reacting with ROS scavenging. The metallation occurs in a stepwise manner, revealing the formation of sub-metallated species, thus preventing the accumulation of apo-MT species. This leads to an advantage for free binding sites for essential toxic metal ions (Salim *et al*., 2020).

PLANT METALLOTHIONEINS: ROLES IN DIFFERENT ABIOTIC STRESSES

The crop plants face substantial yield losses due to various stresses like drought, leading to osmotic stress and cellular dehydration due to the reduced cytosolic and vascular water content. The primary responses, including drought, salinity, and temperature stress induce several secondary responses like generation of reactive oxygen species (ROS) e.g. superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), which can cause

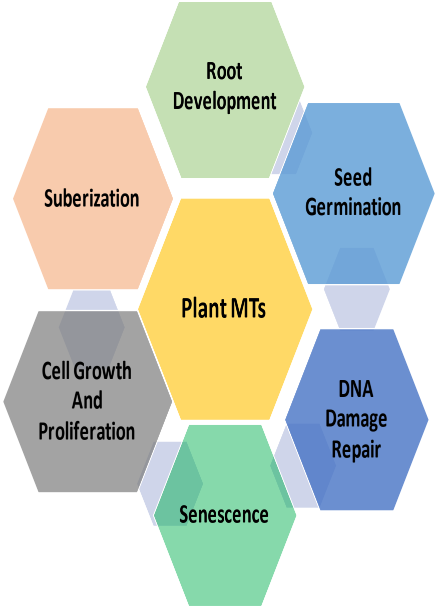

Fig. 2: Plant metallothioneins (pMTs) having different roles during growth and development.

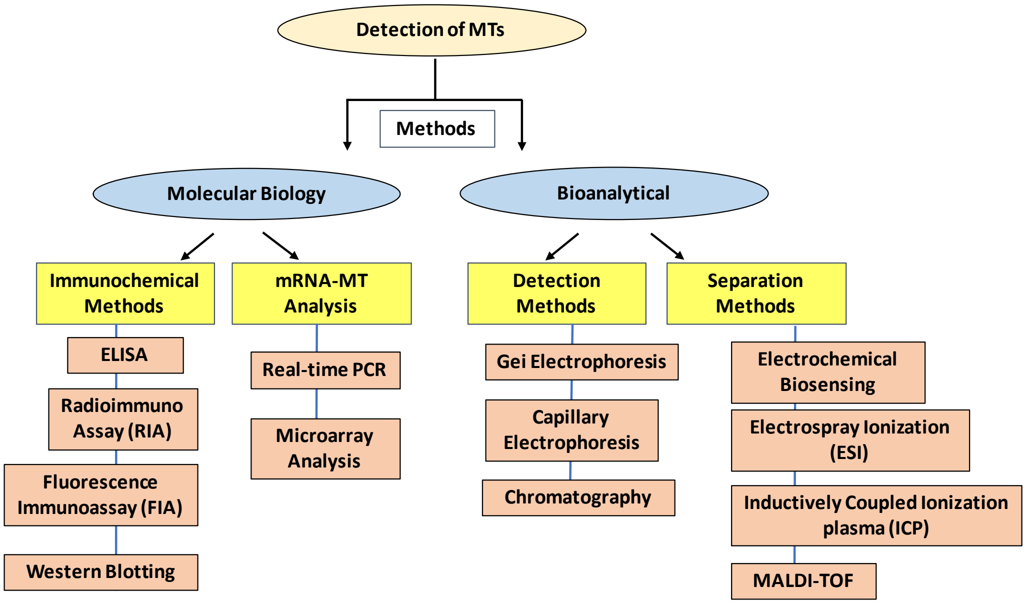

Fig. 3: A flowchart with analytical methods for detection of metallothioneins (MTs) in organisms.

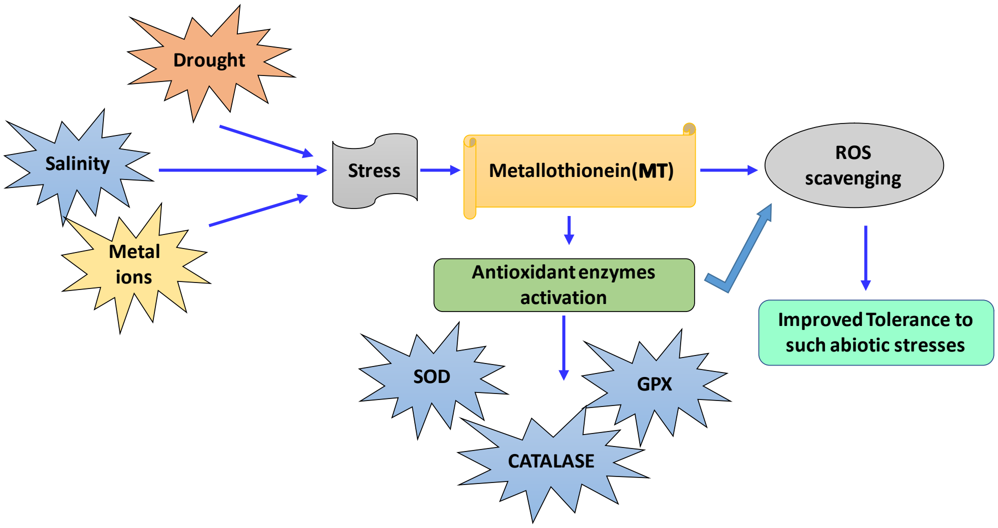

Fig. 4: The predicted mechanism of action of metallothioneins in plants with modulation of redox status.

damage in the DNA by modifying the protein expression level. Moreover, by destroying the phospholipids and glycolipids (Fig. 4), ROS causes significant damage to cellular components and metabolism and acts as important signaling molecules and byproducts of stress responses of toxic nature. Plants have developed an antioxidant system for scavenging ROS during stress conditions (Hassinen *et al.*, 2011; Kumar *et al.*, 2012). Stimulation-based activation of MTs was investigated, and MTs were found to play roles in different abiotic stresses including, drought, oxidative, and metal detoxification, all being correlated. The most important roles include chaperone roles of heavy metals and scavenging reactive oxygen species. Indirect regulation of stress tolerance mechanisms in plants via *MT* genes via their function as heavy metal reservoirs such as zinc reservoirs and this action is needed including the release of zinc molecules when there occurs an increase in levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bell and Vallee, 2009).

MTs are important in modulating ROS concentrations. ROS not only acts in negative aspects but are also important central signalling molecules. MTs are activated by ROS activity and maintain redox status (Mir *et al.*, 2004).Metallothionein gene from rice has been used to investigate *Arabidopsis* under various abiotic stresses, including salinity, drought, and metal stresses. *OsMT-3a* was constitutively expressed in *Arabidopsis* with upregulation under different abiotic stresses with heavy metal stress ($CdCl_2$). Increased tolerance to NaCl, PEG and $CdCl_2$ with enhanced vegetative growth was reported in transgenic plants. Higher levels of Cd^{2+} in shoots and roots with low Na+ and hydrogen peroxide levels were observed in transgenic *Arabidopsis* plants. Higher levels of ascorbate peroxidase and catalase (CAT) have also been reported (Mekawy *et al.*, 2020).

Drought and Oxidative Stress

The identification of the antioxidant activity of the MT gene was demonstrated in wild watermelon (Citrullus lanatus). In vitro analysis of CLMT2 showed suppression of the degradation of genomic DNA by hydroxy radical activity. Hence, under drought conditions and under high light, it affects the peroxy radicals, which were detoxified by MT gene (Akashi *et al.*, 2005). OsMT1a is predominantly expressed in the roots of Oryza sativa with L.cv. Lapar9 encoding type I metallothionein that was induce by drought. This dehydration effect was related to Zinc accumulation. The antioxidant machinery involving peroxidase (POD), catalase and ascorbate peroxidase (APX) were higher in expression than wild-type rice plants.

Drought tolerance related to zinc-induced transcription factors could be validated by their accumulation in the overexpressing lines, indicating ROS scavenging along with zinc homeostasis (Yang *et al.*, 2009). *FeMT3* in *Fagopyrum esculentum* was found in the whole embryo, the vasculature of roots, guard cells, and mesophyll cells. Enhanced accumulation of *FeMT3* could be seen in the leaves upon H_2O_2 and drought treatment up to 2.4 and 3.1 folds, respectively. Overexpression of *FeMT3* to prove the ROS mechanism in yeast resulted in positive roles through real-time PCR (Brkljacic *et al.*, 2004). *Arabidopsis* plants harboring chickpea *MT* gene showed strongly enhanced drought tolerance up to 30-folds. Improved physiological aspects, including higher biomass, longer roots, larger siliques, and better survival ratio, were documented. The biochemical analysis resulted in higher proline and cysteine amino acids and higher accumulation of antioxidant molecules(Dubey *et al.*, 2019). Heterologous expression analysis of chickpea *MT* gene in *Arabidopsis*has been carried out by Dubey *et al.* (2019) (Fig. 5). Interestingly the overexpression of *MT* gene is underway in chickpea, along with its homologous expression analysis (Data unpublished).

MT is known to protect cells from electrophiles and oxidants and react with sulfhydryl groups. *QsMT2* was reported in *Quercus suber* cork tissue responsive to oxidative stress. The mRNA of *QsMT2* was expressed in some specific areas of the meristem, cortex fibres, endodermis, phloem, and tracheary elements, and upon stress treatment, the gene expression was enhanced. Oxidative stress enhances the expression of the *MT* gene due to its role in senescence. Lignification, suberization, and senescence enhance oxidative stress and ROS production, leading to enhanced expression of *MT2*. The highest expression was reported in the cell dividing areas, including meristem initials and proliferating areas, because of the need for redox status balance (Mir *et al*., 2004).

Salinity Stress

Salinity is one of the significant environmental abiotic stresses suitable only for alkaline/salt-tolerant vegetation. Salt stress, along with drought stress, contributes to global crop losses. The intriguing research regarding the roles of stress-related traits with their mechanism of action led to the discoveryof MT in various salt-tolerant plants. MT gene from *Oryza sativa* (*OsMT1e-P*) was isolated from a salinity tolerant variety 'Pokkali', which resulted in better growth and survival effects under saline conditions when ectopically expressed in tobacco with better seed set and good viability features. The stress tolerance includes scavenging of ROS identified to be the primary salinity stress tolerance mechanism (Kumar *et al*., 2012). Recent research on the *Oryza sativa* cytoskeleton protein known as intermediate filament-like gene (*OsIFL*), is identified to play key roles in protecting tobacco plants from salinity stress with enhanced accumulation of chlorophyll and maintaining ion homeostasis along with high K^+/Na^+ ratio. This protein is investigated to have interaction with stress-responsive genes, including MT in the nucleus. Hence upon salinity stress, both of these genes interact and result in enhanced tolerance to salinity. This interaction was studied using Bi-molecular Fluorescence Complementation (BiFC) assay (Soda *et al*., 2016).

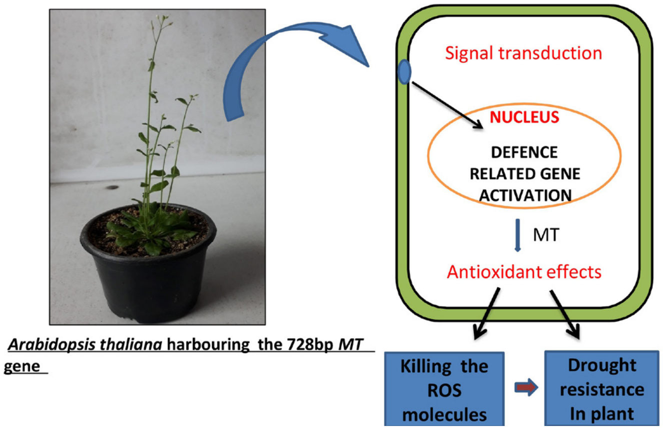

Fig. 5: Transgenic *Arabidopsis thaliana* harboring the 728 bp *MT* gene of chickpea was investigated upon drought stress, the cell membrane receptors communicate signals leading to activation of *MT* gene providing defense through antioxidant effects (Dubey *et al*., 2019).

Suaeda salsa a salt-tolerant plant harbors a type 2 *MT* gene responsible for salt tolerance, which has been validated in yeast and *Arabidopsis*, confirming tolerance to salinity, oxidative stress, and mental stress, including Cd^{2+}. The overexpressed and transgenic machinery showed a higher concentration of Cd^{2+} with lower Na^+ ions and higher survival ability. Excess Na^+ ions were prevented by three mechanisms that involve removal and storage of excess ions in the vacuole, activation of Na^+ ions influx transporter genes, and transportation *via* plasma membrane to apoplast through antiporters (Jin *et al*., 2017). Date palm is a salt-tolerant plant that faces extreme salinity conditions. *PdMT2A* is identified as a salt responsive type 2 MT gene in date palm. In yeast, this MT gene was found to confer salinity, drought, and oxidative stress tolerance upon overexpression. Heterogeneous expression in *Arabidopsis* revealed regulatory roles on HKT (high-affinity K+ transporters) with the maintenance of a high K+/Na+ ratio and less accumulation of Na^+ ions when compared to wild-type plants. The seedlings showed higher biomass, broader leaves, and longer roots (Patankar *et al*., 2019).

Light stress

The concentration of antioxidant enzymes increases upon high light stress conditions, especially in the leaves. Such exposure

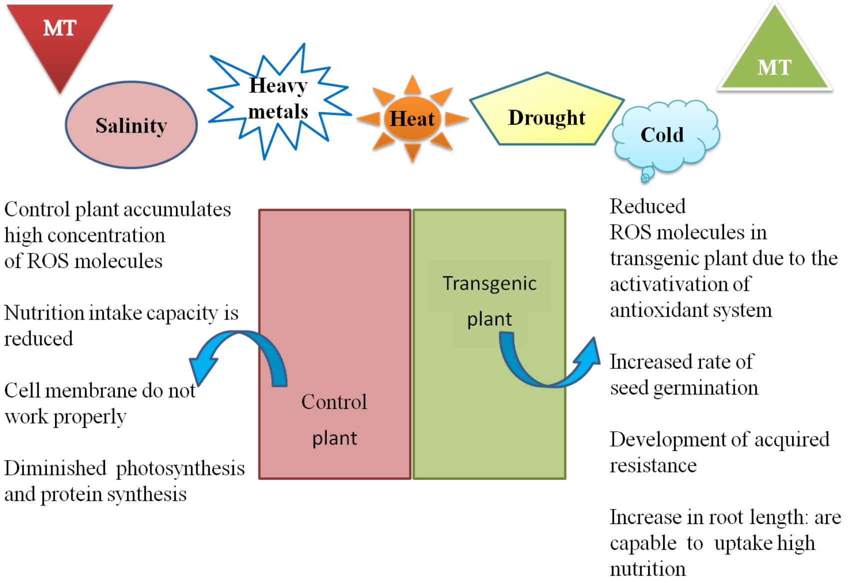

Fig. 6: Illustration of effects of different stresses on both transgenic and non-transgenic *Arabidopsis thaliana* with transgenic plant harboring *MT* gene and the non-transgenic plant is without *MT* gene.

leads to necrotic injuries in plants due to oxidative damage. *Citrullus lanatus* has been investigated to possess citrulline as a scavenger of hydroxyl radicals, but is expressed at later stages; an *MT* gene was identified to tolerate such stress. Upon high light stress, the leaves are affected, and this interrupts photosynthesis related to oxidative stress, leading to DNA damage. *MT* gene was highly expressed and accumulated under high light stress. Its expression leads to anti-oxidative defence (Akashi *et al*., 2005). Not much work has been carried out on the roles of MT in light stress on different plants. Hence, this is an exciting topic of research.

Low-temperature Stress

Low-temperature stress is one of the stresses which needs to be spotted with a keen interest for the roles of pMTs, as not many investigations have been done and no recent data has been published. Cold stress has been an initiator of ROS molecules, which damage plants' cellular metabolism, leading to survival issues. This freezing temperature creates specific changes in the gene expression patterns. The low-temperature stress, along with H_2O_2 causes oxidative stress. In *Arabidopsis thaliana, AtMT2a* has been investigated to have roles in the anti-oxidative defense system with cold stress regulation. This gene was upregulated upon cold stress leading to the balancing of ROS in the cytosol, with increased mRNA levels. However, the *Arabidopsis* mutants having *mt2a* were sensitive to cold stress, and higher levels of H_2O_2 were observed in the mutant plants. *CAT2* gene is a class of antioxidant genes that has been mutated, leading to *cat2* mutant forms. These mutant forms in *Arabidopsis* overexpressing *AtMT2A* have revealed improved catalase (CAT) function, with higher enzymatic functions, whereas mt2a harboring AtMT2A complemented CAT activity enhanced the enzymatic activity of the CAT gene (Zhu *et al*., 2009). Cold storage of fruits is a conventional process that induces specific proteins upon cold treatment. Apple fruit is also subjected to cold storage. It has been identified in two clones that pAMT1 and pAMT2 were upregulated upon the cold treatment and that their expression is very sensitive to temperature. Both these MT clones were having different expression patterns related to fruit development. With an increase in leafage, the expression levels of both genes were also increased (Reid and Ross, 1997).

Metal Stress Tolerance

Due to increased environmental pollution concerning heavy metals, the health risks are expanding, and the survival ability is at risk in the present world. One of the most crucial measures for heavy metal remediation includes phytoremediation. This includes precise regulatory mechanisms like heavy metal chelation, absorption, transportation, and isolation to specialized regions in the cell and detoxification. MTs are known to be involved in heavy metal stress responses, metabolism, and anti-oxidation (Zhou *et al*., 2006).

All four types of MTs are found to be involved in metal stress regulation. Both essential (Cu and Zn) and non-essential metals (Cd) are detoxified. Important crops including *Oryza*, *Brassica, Cicer*, *Cajanus*, *Ziziphus*, *Saccharum*, *Pisum*, and many more have shown tolerance to metals, with roles of MTs. MT3 and MT4 are identified in *Hordeum vulgare*, where MT3 is known for metal homeostasis of Cu and Zn with housekeeping roles. MT4 was found to be involved in the storage of Zn and local binding in an aleuronic layer of grain. MT4 is hence a vital topic in biofortification (Hegelund *et al*., 2012). *ScMT2-1-3*, a novel MT identified from sugarcane, has roles in tolerance to NaCl, PEG along with Cd^{2+} and Cu^{2+} metals. Copper detoxification, as well as storage, is the central role of this gene. However, real-time expression analysis identified the upregulation of *ScMT2-1-3* under $Cu+^{2}$ stresses, while down-regulation was observed in Cd^{2+} stress (Guo *et al*., 2013). *ThMT3* of *Tamarix hispida,* expressed in *Salix matsudana*, showed increased tolerance to Cu metal with increased adventitious roots and higher nitric oxide (NO) levels. NO induces adventitious roots and hence increases Cu tolerance (Yang *et al*., 2015a). *CsMT3* from cucumber was heterologously expressed in *E. coli*, showing higher tolerance to Cu^{2+} and Cd^{2+}. The highest expression of *CsMTL3* was observed in leaves, but the highest expression was found in roots (Xu *et al*., 2018). Rice is an important staple crop that is also affected by heavy metals. The studies indicate that *Oryza sativa* MT is responsible for the detoxification of heavy metals, including Cr(VI) or Cr(III), with treatments showing higher accumulation in the roots than shoots. Cr chelation was carried out mostly in roots by *OsMT1-b,* whereas *OsMT2-c* was found to have a higher expression for H_2O_2 accumulation in shoots (Yu *et al*., 2019).

MTs are expected to undergo some specific mechanism of action for metal tolerance in plants that could be metal and plant-specific. These include cytoplasmic metal efflux or cytosolic metal binding, leading to sequestration of metals in vacuoles (Küpper and Kochian, 2010). *Phytolacca americana* is known as hyperaccumulator of cadmium and used by Zhi *et al*. (2020) to study cadmium tolerance in this plant. *PaMT3-1* was over-expressed in tobacco plants and the results were observed. 100mM $CdCl_2$ was given to the plants, indicating similar Cd accumulation in both wild-type and transgenic roots, but the transgenic leaves had variation in results. The Cd content was reduced in leaves, indicating a low transportation coefficient in the transgenic plants. This pointed towards the phytoremediation aspect of this gene in plants. *Cucumis sativa* was investigated with MT4 induced with relative concentrations of Cd but was less affected with Zn when heterologously expressed in *E. coli*. Overexpression of MT4 in cucumber, *CsMT4* with heterologous expression in *E. coli*, led to increased uptake of Cd in those tissues, where phytochelatin-like peptides were highest. Homologous expression of *CsMT4* with less tolerance to Zn indicated binding capacity and preference for different metal ions, concerning structural composition with an arrangement and N-terminal Cys-residues (Duan *et al*., 2019).

Avicennia marina was recently investigated for heavy metal stress toxicity in mangrove plants. The Type 2 MT from *A. marina*was found to have roles in the accumulation of various heavy metals, including Cu^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+}, and Cd^{2+}. This *MT* gene binds to iron and activates catalase enzymes for balancing hydrogen peroxide in plants(Babaei-Bondarti and Shahpiri, 2020). Regulation in Cd and Cu tolerance by*MT* gene was recently studied in *Physcomitrella patens*. Heterologous expression of *PpMT2* in yeast cells and *Arabidopsis* plants was conducted by Liu *et al*. (2019). The results prove high tolerance to Cd and Cu tolerance in both systems. *OsMT2b* and *OsMT2c* from rice were expressed in the vascular bundle phloem of nodes and anthers.

The highest expression of both genes was in node I. The expression was not affected by Cu, Zn, Fe, and Mn deficiency, whereas excess of Cd and Zn did not change the expression levels. A slight decrease in expression levels could be seen with excess Cd levels. The knockout mutants of both *OsMT2b*and *OsMT2c* reduced the grain yield with decreased zinc distribution in panicle, but increased levels were reported in upper nodes with reduced pollen viability. The knockout mutant of *OsMT2b* resulted in Cu distribution in various organs. Sulfur (S) deposition in node I was an indicator of MT proteins. Less S was observed in the knockout mutants, whereas it was high in the wild-type plant, mostly in phloem cells. Expression of *OsMT2b* and *OsMT2c* in yeast resulted in increased Zn or Cu tolerance, hence detoxifying these heavy metals (Lei *et al*., 2020). *OsMT1e,* highly expressed in rice roots, were induced by Cd exposure in overexpressing lines, with improved tolerance to Cd stress, enhanced growth of roots and shoot elongation, chlorophyll, and dry weight content. The RNAi lines were opposite in effect to *OsMT1* over expressing plants, with sensitive and delayed poor growth (Rono *et al*., 2021).

Hence, MT is an important genetic engineering tool for molecular enhancement in crop plants facing metal stress. Enormous research regarding MTs in different crops has revealed the roles of plant MTs in multiple stresses. These investigations could reveal crucial roles of MTs in different abiotic stresses, enhancing the interest for researchers to uncover the hidden essential roles in stress regulation that might still be untouched and need much more in-depth research (Fig. 6).

NANOPARTICLES (NPS): EFFECTS ON METALLOTHIONEINS

Silver nanoparticles come into view in the present review due to their harmful contaminant behavior in the environment in which plants grow and perish. Wheat plants exposed to Ag ions and silver nanoparticles (AgNPs) in the sand matrix resulted in oxidative stress in wheat roots with the accumulation of oxidized glutathione. *MT* gene was also induced to express detoxification, a line of defence (Dimkpa *et al*., 2013). Cao *et al*. (2020) had recently presented the results of the interaction of arbuscular mycorrhizal (AM) fungi Glomus Caledonium with maize involving the effects on MT with the treatment of AgNPs. Normally this Ag increases the relative expression of *MT* genes in maize roots. The fungal inoculation led to alleviation of phytotoxicity induced by AgNPs. AM fungus also decreased the Ag content and uptake in roots and shoots. At molecular levels, this fungus decreased the expression of Ag transporters as well as genes encoding MT known for metal detoxification and homeostasis, as mycorrhizal maize plants possess lower metal concentration.

Metal oxide nanoparticles (MONPs) are creating unimaginable health issues and lethality to both animals and plants exposed to the environment. These include TiO_2, NiO, Al_2O_3 and many more. The study was conducted on radish, tomato, alfalfa, and cucumber (Ahmed *et al*. (2019). Statistical insignificance could be observed under exposure to $Al_2O_3$and TiO_2whereas the effect of CuO and ZnO were stimulatory with MTs, indicating metal-binding affinity of MTs. *Lupin luteus* was studied for validating the effects of silver nanoparticles with changes in expression on MTs, the stress markers. Though increase has been observed in the expression of MTs when subjected to different heavy metal stresses, no such significant statistical changes in expression levels were observed with silver nanoparticle treatment compared to untreated samples (Jaskulak *et al*., 2019).

CONCLUSIONS AND FUTURE PERSPECTIVES

Molecular analysis of stress regulatory pathways, including genes, can improve stress tolerance even in susceptible cultivars. MTs have been identified in numerous plants; yet their functions are still not fully explored, like ROS scavenging roles of MTs in different plants. Hence, more profound knowledge of MTs in terms of stress modulation must be investigated with their structural diversity. The general mechanism of action includes the compartmentalization of metals through vacuolar transporters in vacuoles in the cytosol as well as metal chelation, activated by the cells through complex machinery. But the actual mechanism of action is still behind the doors.

Genome-wide analysis from tolerant genotypes in abiotic stress unravels the tolerant gene network pathways to be used further for crop improvement. Physiological roles regarding the regulatory machinery of their activation are still not much explored. Bioinformatics methods, as well as *MT* gene expression analysis, may help in identifying the candidate genes for stress tolerance. The biological roles of these candidate genes could be validated through overexpression, gene editing (knockdown) in the crop, and model plants. To improve stress tolerance in crop plants, transgenics with overexpression of *MT* genes could be a promising approach.

Hence, functional characterization of such crucial genes is essential for progressive sustainable development. What are the specific functions of isoforms of MTs? Development of transgenic lines should be promoted to use MTs as tools for bioremediation and study the capability of such genetic molecular tools for chelation and elimination of toxic metals from the environment. Finding candidate genes for abiotic stress tolerance and metal detoxification requires the hour for sustainable development and food security globally. Their roles in light stress management and UV-B stress are still not fully explored hence is an important focus of research. Isolation and purification of MTs from plants is an exciting topic of research that could lead to improved use of such proteins, heterogeneously.

Various techniques for their functional validation include tissue-specific expression along with metal-regulated expression, overexpression, CRISPR-*Cas*9 based editing technologies with knockdown mutants with transgenics development, and metal-MT complexes' characterization of these tools and techniques, some are still untouched and needs future research attention.

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