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Molecular mechanistic model of plant heavy metal tolerance

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Abstract Plants being sessile are susceptible to heavy metals (HMs) toxicity and respond differentially to hostile environments. The toxicity of HM is governed by the type of ion and its concentration, plant physiology and stage of plant growth. Plants counteract the HMs stress by overexpressing numerous stress related proteins, glutathione mediated tolerance pathways and signaling proteins involving networks of various stress regulations. Though the response may vary and be specific in its stress networks regulation for each HM. The intricacy of HM tolerance response involves the set of molecular regulation, which demands to be understood to yield HM tolerant plant. Topical advancements in molecular biology and genomics have facilitated studies in transcriptomics and proteomics to identify regulatory genes implied in HM tolerance in plants. The integration of resources obtained through these studies will be of extreme significance, combining the diverse fields of plant biology to dissect the actual HM stress response network. In this review, we put an endeavor to describe the specific aspects of the molecular mechanisms of a plant response to HMs which may contribute to better understanding of the mode of HMs action and overlaps in metal sensing and signaling/crosstalk to other stresses.

Keywords Hyperaccumulator plants · HMs · Metal transporters · Thiols · *cis*-Regulatory elements · Phytochelatins · Phytoengineering

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Introduction

Influence of heavy metals has remained as a significant environmental predicament with a negative probable impact on human health and plant productivity. In spite of the natural occurrence as rare elements, diverse anthropogenic practices and refuse dumping has contributed to spread of HMs in the environment. The toxicity mechanisms of HMs vary from plant to plant, and several efforts to understand their mechanism fully at the molecular level have not been successful. In order to cope with stress signals, plants require a coordination of complex physiological and biochemical processes, gene expression, protein modifications and changes in metabolite's compositions leading to proper stress signal perception and tolerance (Urano et al. 2010). Though there has been a recent development in the HMs stress signaling cascades,

gene expression modulation and stress responsive non-coding RNA (Matsui et al. 2008; Borsani et al. 2005) may facilitate the understanding intricacies of stress response of plants and radically contribute towards development of HMs stress tolerant plants.

"Heavy metals" are elements with a specific gravity of at least five times higher than that of water (specific gravity of water is 1 at 4 °C). Some well-known toxic HMs with a specific gravity of five or more times that of water are arsenic 5.7, cadmium 8.65, iron 7.9, lead 11.34, and mercury 13.54 (Lide 1992). Transition metal as such bears all the characteristic properties of metals with incomplete d sub-shells leading to variable oxidation states (IUPAC 1997) and it has been reviewed for the use of term transition metal and d-block (Jensen 2003). Among all the HMs reported, Cd is considered to be the most phytotoxic because of its high solubility and absorption by plants and radical introgression into the food chain causing serious human hazards (Buchet et al. 1990; Lux et al. 2010; Gill et al. 2011). The elevated degree of negative effects on mineral nutrition and homeostasis in plant shoot and root growth and development even at low concentrations uptake makes Cd the most toxic HM to plants (Macek et al. 2002; Farinati et al. 2010). Hence, in this review, it is emphasized on Cd specifically to discuss the molecular mechanisms of HMs toxicity and signaling crosstalks. The mode of HM action, toxicity mechanisms, its accumulation in various subcellular organelles and different strategies followed by plants to combat HM stress has been discussed extensively in previous reviews but key factors at different signaling stages that lead to HM tolerance has not been studied well. In general, HMs stress in plants is characterized by synthesis of stress proteins, signaling molecules and chelators like organic acids and GSH mediated phytochelatins. Though the mode of action of each metal is specific, but some toxicity mechanisms may be shared. Correspondingly, cells may mount both customary and metal-specific responses to counteract HMs toxicity. Because of this reason only a relatively diminutive set of hyperaccumulator plants is capable of sequestering HMs in their shoot tissues at elevated concentrations. Therefore, in this review, we focus on the progresses made on the molecular key components of HM response and associated regulatory networks in plants viz., expression of sensors, kinases, *cis*-regulatory elements, transcription factors, aquaporins/transporters, nitrous oxide signaling, etc. to understand HM induced signaling network and crosstalk with other stresses.

Heavy metal stress response: from sensing to gene regulation

Plants possess a range of potential mechanisms of detoxification, which is involved in a response to HM stresses. Hence, a coordinated network of molecular processes such as reduced uptake or increased plant internal sequestration along with multiple metal-detoxifying mechanisms, repair capabilities and signaling molecules governs the process from HM sensing to plant HM stress tolerance.

Based on their solubility under physiological conditions, only 17 out of 53 heavy metals are of biological significance (Weast 1984). Under physiological conditions, most of these heavy metals with an incompletely filled δ-orbital are present as cations. The physiological redox range of aerobic cells is between -420 and $+800$ mV. Autoxidation of certain heavy metal ions which are redox-active results in O_2^- formation and subsequently production of reactive oxygen species (ROS) via the Fenton reaction causing cellular injury (Schutzendubel and Polle 2002). Heavy metals with lower redox potentials indirectly interfere with cellular redox reactions causing oxidative injury. The displacement and the release of redox-active metal ions in process from various biomolecules as well as the depletion of the antioxidant system disturb the redox balance of the cell leading to oxidative injury. Another important mechanism of heavy metal toxicity is their ability to bind strongly to oxygen, nitrogen and sulphur atoms and low solubility of these products (Nieboer and Richardson 1980). This binding affinity is related to free enthalpy of the formation of the product of metal and ligand, and therefore, 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 (Rao and Sresty 2000).

There are various reports of co-transportation of the metals ions by metal transporters in plants, which may be the prime cause of metal toxicity. Specific transporters might be involved in metal ion uptake, such as in the case of Cd uptake in Alpine Penny-cress

(*Thlaspi caerulescens*) (Lombi et al. 2001). The ZIP transporter family members, IRT1 (iron regulated transporter) expressed in *Arabidopsis* iron deficient plants (Eide et al. 1996) and ZRT1 and ZRT2 (zinc regulated transporter) high and low affinity zinc transporters respectively of yeast were found to uptake other HMs apart from transporting Fe(II) across the membrane (Zhao and Eide 1996). AtIRT1 was found to functionally complement yeast mutant (*fet3–fet4*) defective for iron uptake (Eide et al. 1996). Hence IRT1 through complementation and uptake studies in yeast confirmed the ability to transport both Mn and Zn along with Fe. Guerinot (2008) also reported that in Fe deficient plants, the IRT1 gene upon expression facilitates the transport of Mn and Zn along with Fe. Furthermore, a pea isolog of IRT1 called RIT1 expressed in roots showing 79 % similarity and 63 % homology to AtIRT1 when expressed in yeast iron transport deficient (*fet3–fet4*) and zinc transport deficient (*zrt1–zrt2*) mutants were found to have high affinity uptake of Fe and Zn along with low affinity Cd uptake (Cohen et al. 2004). Hence, the co-transportation of HMs may be one of the numerous reasons of HM toxicity in plants.

Plants have evolved a complex network of homeostatic mechanisms to minimize the damages due to HM ions. As primary defense to Cd stress, plant exudates such as malate or citrate bind to metal ions to avoid root absorption (Delhaize and Ryan 1995) or physical interaction of HM with cell wall may immobilize toxic ions and prevents their uptake into the cytosol (Sanit'a di Toppi and Gabbrielli 1999). Nevertheless, the high mobility and water solubility of Cd enable it to readily enter the roots through the cortical tissue and reach the xylem via an apoplastic and/or symplastic pathway (Salt et al. 1995). As soon as the concentration of the toxic element rises above the threshold limit in cytosol, production of chelators involved in the detoxification and sequestration of the HMs in specific cellular compartments become imperative. It is well known that GSH functions as an HM-ligand (Ca′novas et al. 2004) and an antioxidant. Upon HM exposure, GSH concentrations drop as a consequence of initiated phytochelatin (PC) biosynthesis. The constitutively elevated GSH biosynthesis in different *Thlaspi* species was reported to be the indicator of their tolerance to Ni stress via mitigation of Ni dependent oxidative stress (Freeman et al. 2004). The production of phytochelatins in response to As stress may play a significant role in As tolerance in plants but still needs a thorough study (Panda et al. 2010). It has been reported that the ability of high-rate PC synthesis is insufficient to cope with the metal load if the functionality of the antioxidant system is simultaneously hampered. Similarly, the GSH content was inversely linked to Cd sensitivity when comparing ten pea genotypes showing differing Cd sensitivity (Metwally et al. 2005). Moreover, as for other abiotic stresses, HMs especially Cd resistance involves the synthesis of stress-related proteins and signal molecules (Sanit'a di Toppi and Gabbrielli 1999) (Fig. 1). The signaling pathway involves complex interaction of genes in which transcription factors have essential roles since regulation of their expression may strongly affect plant stress response (Uno et al. 2000). HMs interfere with cell signaling pathways in all the biotic systems. Even in animals and humans, HMs activates the transcription factors nuclear factor kB (NF-kB) and activating protein 1 (AP-1), which control cell survival, differentiation, inflammation and growth (Valko et al. 2005). In plants, convincing evidence demonstrates interference of Cu, Pb, Zn and Cd with mitogen kinase signaling cascades. Plant jasmonic acid, salicylic acid and ethylene levels increase upon exposure to HMs (Ca′novas et al. 2004; Metwally et al. 2003; Maksymiec et al. 2005). These plant hormones play controversial roles in HM tolerance, and both beneficial effects- the counteraction of Cd and Mn toxicity by the activation of antioxidants (Metwally et al. 2003; Shi and Zhu 2008) and negative effects such as enhancement of H_2O_2 generation (Zawoznik et al. 2007) have been reported. By modulating components of the cellular signaling network, mostly through application of established inhibitors or effectors and overexpression of HM transporter, systematic effort needs to be made to enhance the HM tolerance of the plants.

The databank of numerous putative genes involved in response to Cd-stress has been increasing with the genomics studies: for example, in *Brassica juncea,* receptor like kinase protein induction as metal sensor was reported during Cd stress (Fusco et al. 2005a). Further, the activation of MAPK family kinases during Cd and Cu stress suggests the involvement of phosphorylation cascade in Cd signaling (Jonak et al. 2004). Cd stress was also found to stimulate Ca concentration leading to changes in calmodulin proteins that may at least regulates Cd tolerance in plants (DalCorso et al. 2008; Yang and Poovaiah 2003). In

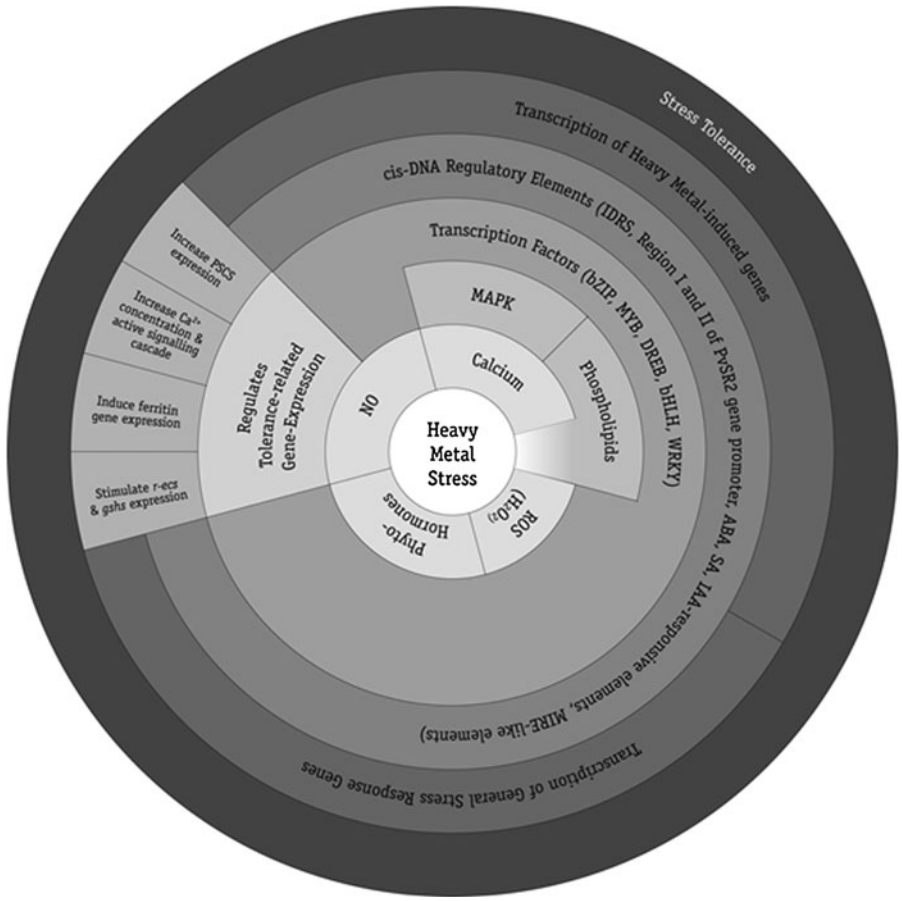


Fig. 1 Probable mechanisms of HM induced response in plants

fact, Ni and Pb tolerance has been achieved in transgenic plants overexpressing tobacco calmodulin like protein (Arazi et al. 2000). Although HM induced gene regulation has been known for decades, the microarray analysis done in various plants viz., *Arabidopsis* (Kovalchuk et al. 2005; Herbette et al. 2006; Weber et al. 2006), pea (Romero-Puertas et al. 2004), and barley (Tamas et al. 2008) indicates the mode of action of HM in different organs, depending on metal used and its concentration. The genes (*AtFer1–AtFer4*) which encode ferritin were found to be differentially expressed during high Fe treatment in *Arabidopsis* roots and leaves (Connolly and Guerinot 2002). The increased accumulation of jasmonates due to Cu and Cd exposure in mature leaves of *Arabidopsis* and *Phaseolus coccineus* suggests the transcriptional control of GSH biosynthesis genes during HM stress (Maksymiec et al. 2005). The specific upregulation of genes for zinc finger (C_2H_2) proteins, metal transporters (ZIP9), calmodulin-binding protein and ethylene receptor-related protein in *Arabidopsis* roots by Cd stress only not by Cu indicate specificity of plant response to organ and metal type (Weber et al. 2006). The transcriptome analysis of the rice roots during Cr(IV) stress suggested the vulnerability to short-term exposure of higher Cr concentration than environmentally relevant high concentration. Furthermore, it may be clear that GSH plays an important role for detoxification of Cr-stress as most of the genes related to glutathione metabolism, transport, and signal-transduction were differentially expressed upon Cr(VI) stress (Dubey et al. 2010). Recently, the Cd-responsive miRNAs were identified in rice upon Cd stress and the mRNA levels of several target genes were negatively correlated with the corresponding miRNAs under Cd stress suggesting their function in heavy metal tolerance in plants (Ding et al. 2011). Hence, HMs stress response in plants involves a number of pathways and stress signals which differs with organ, metal and concentration that overlap and transduce/crosstalk to impart HM tolerance (Fig. 2). The overall stress network is poorly

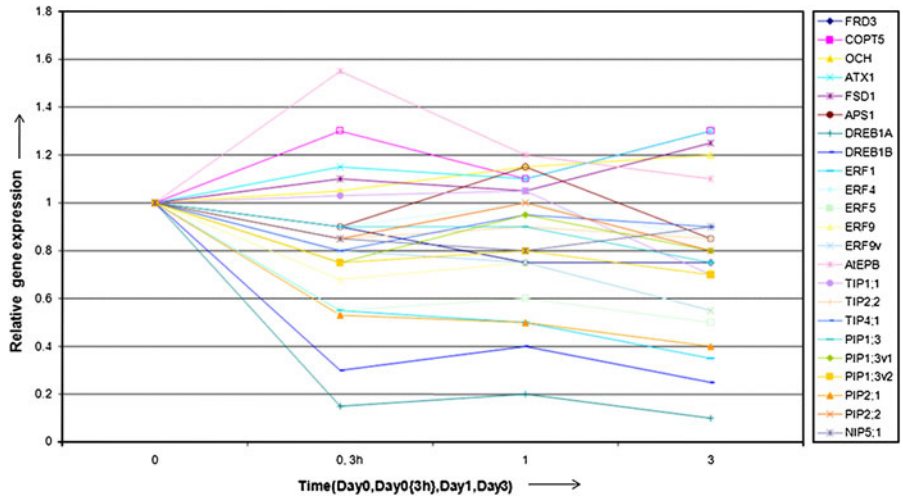


Fig. 2 Expression profile of the various unigenes showing the varied role in HM response in plant roots. The Cd treatments of 0.1 μM are depicted as 0 day, 0 day, 3 h, 1 day and 3 day

understood which needs further study to get a clear picture of plant HM response.

Metallomics: key players of HM tolerance/ sensitivity engineering

Plants respond to the HMs stress in a specific and discrete manner via its varied response mechanisms. Understanding the plant-based molecular mechanisms is important to isolate plant genes responsible for the expression of the remediating phenotype. The identification and isolation of these genes may open the opportunity to ameliorate plants for environmental cleanup. For example, transferring a single gene involved in metal transport, such as HMA4, from *A. halleri* to *A. thaliana* has enhanced the leaves metal uploading potential of *A. thaliana*, a non-accumulator species (Hanikenne et al. 2008). Regarding metal-conjugates transport, plants overexpressing specific transport proteins such as CDF proteins might acquire elevated detoxification and compartmentalization of GS-HM conjugates into the vacuoles (Kramer et al. 2007). Transgenic *B. juncea* plants engineered to produce more glutathione and phytochelatins accumulated significantly more Cd than wild-type plants (Bennett et al. 2003). *A. thaliana* and tobacco plants engineered with the MT gene developed Cd tolerance and accumulation (Eapen and D'Souza 2005). Furthermore, Cd tolerance and accumulation is also enhanced by overexpression of γ-glutamylcysteine synthetase, an enzyme with an important role in controlling glutathione synthesis and therefore metal chelation (Zhu et al. 1999). Another study revealed that the expression of the *AtPCS1* increased Cd and As tolerance and accumulation in *B. juncea* (Gasic and Korban 2007) and in tobacco (Pomponi et al. 2006). Recently it has been validated that a bZIP transcription factor isolated as differentially expressed in response to Cd treatment in *B. juncea* (Fusco et al. 2005a), enhances Cd accumulation and tolerance in transgenic *Arabidopsis* and tobacco plants. Moreover, the comparison between hyperaccumulator with non-accumulator species (e.g. *A. halleri* with *A. thaliana*) suggests that the hyper-accumulating features could be due to sequence mutations, gene copy number or due to varied expression levels of the metal stress proteins (Plaza et al. 2007; Hanikenne et al. 2008). This indicates that genetic potential for metal detoxification is already present in plant genomes and those mutations probably affects both metal sensing and activation of appropriate responses which make the difference. Based on the recent progresses made in the field of HM response mechanisms, we herewith discuss the upcoming key players of HM tolerance engineering.

Heavy metals sensors

Plant root as the main site of HMs access has been proved by electron microscopy localization study of majority of HMs getting accumulated in root cells than cytoplasm or other tissues (Andruini et al. 1996). Cd and Ni exposure has resulted in synthesis of proteins localized in apoplastic space suggesting cell wall as prime metal sensing site (Blinda et al. 1997). Hence,

primarily cell wall is the site of functional signaling molecule and metabolite in response to HMs (Dal-Corso et al. 2010). Study on HM sensing and signaling pathways in plants have been limited to extracellular signals receptor-like protein kinases. It has been reported that the gene coding for lysine motif receptor-like kinase in barley is shown to be induced by Cr^{2+}, Cd^{2+}, Cu^{2+} during leaf senescence (Gleba et al. 1999). Further the expression of metal induced barley receptor-like kinase was also reported to be facilitated by Ca^{2+} level. The proteomic study on Cd-treated rice roots indicates the induction of putative receptor protein kinase. However, more detailed study on the function of other Cd induced sensor has to be done.

Heavy metals induced signaling protein kinases

The HM stress responses in plants were reported to encompass calcium level changes, mitogen-activated protein kinases (MAPK) cascades and transcriptional modulation of the stress-responsive genes (Shao et al. 2008, 2009). It was hypothesized that some HMs (Cd, Ni, Co) may cause a perturbation in the intracellular Ca^{2+} level and interferes with calcium signaling by substituting Ca^{2+} in calmodulin regulation (Kim et al. 2007). Yeh et al. (2007) has reported that Cd^{2+} and Cu^{2+} induce ROS production and Ca^{2+} accumulation in rice roots and thereby activate the MAPK signaling pathways. The Cd^{2+} and Cu^{2+} induced MAP kinase activation required the involvement of Ca^{2+}-dependent protein kinase (CDPK) and phosphatidylinositol 3-kinase (PI3 kinase) as shown by the inhibitory effect of a CDPK antagonist, W7, and a PI3 kinase inhibitor, wortmannin, respectively. Hence, using a Ca^{2+} indicator, it was demonstrated that Cd^{2+} and Cu^{2+} induce Ca^{2+} accumulation in rice roots. Although CDPK and PI3 kinase may be involved in both Cd^{2+}- and Cu^{2+}-induced MAP kinase activities, Cd^{2+} and Cu^{2+} induced MAP kinase activation via distinct ROS-generating systems in rice roots, and their responsiveness may differ depending on the types of ROS generated due to associated stresses (Yeh et al. 2007). Taken together, these findings implicate that Cd^{2+} and Cu^{2+} induce MAP kinase activation through distinct signaling pathways may lead to HM tolerance in plants.

Hsu and Kao (2003) have reported that ROS, Ca^{2+} and abscisic acid (ABA) were significantly accumulated in japonica Tainung 67(TNG67) than in Cd^{2+} sensitive Taichung Native1 (TN1) variety during Cd^{2+} treatment and the accumulated ROS and Ca^{2+} subsequently might activate MAP kinase leading to Cd^{2+} tolerance to rice plants. The fact that ABA plays crucial role in MAP kinase activation in plants was further strengthen by the report on MAP kinase activation in barley aleurone and pea leaves (Knetsch et al. 1996; Burnett et al. 2000), Thus, low levels of MAP kinase activity of TN1 may also be due low levels of ABA content in TN1 and the HM induced Ca^{2+} accumulation and MAPK activation in tolerant TNG67 rice plants may be consistent as compared to transient accumulation in sensitive plants. Furthermore, it has been reported that the treatment of tobacco cells and Scots pine roots with Cd and lupine roots with Pb^{2+} caused the generation of H_2O_2 (Rucinska-Sobkowiak and Pukacki 2006). The Cd-producing oxidative burst in tobacco in response to Cd is mediated by calmodulin and/or calmodulin-dependent proteins (Olmos et al. 2003) suggest the involvement of Ca/calmodulin pathway in signaling of metal response in plants. The MAPK pathway is universally involved in the transduction of extracellular signals to intracellular targets in all eukaryotes (Li et al. 2006) and helps to pass on the cytoplasmic signal to nucleus, where they activate other protein kinases, specific transcription factors and regulatory proteins (McCully 1999; Shao et al. 2008). It was recently indicated that Cd and Cu activate four different MAPKs (SIMK, MMK2, MMK3, and SAMK) in *Alfalfa,* whereas Cd induces one such kinase (AtMEKK1) in *Arabidopsis* and one (OsMAPK2) in rice (Shao et al. 2010) suggesting the role of MAPKs in HM signalling. However, it is not clear if activation of MAPKs occurs by these HMs or ROS or it occurs via action of other mediators and therefore, the MAPK responsiveness may differ depending on the type of metals and ROS involved.

Heavy metals induced NO signaling

Xiong et al. (2010) reported that HM toxicity lead to changes in the endogenous nitric oxide (NO) content in plants and thereby NO plays various roles in alleviating the HM toxicity. In vitro, an increase in NO production has been observed in *Arabidopsis* cell suspension cultures under a 300 μM Fe stress (Arnaud et al. 2006). Similarly, soybean cells treated with 4 or 7 μM Cd^{2+} for 72 h exhibit a dose dependent and rapid production of NO, which may suggest that NO functions as a signal molecule involved in the

alleviation of the HM stress (Kopyra et al. 2006). Many authors have reported contradictory results like in cell suspension that a short-term HM treatment has been reported to promote a NO burst whereas a long treatment decreases NO generation. Groppa et al. (2008) propose that these opposite results could be explained by using different HM concentrations, variable age of sample and plant tissue and by varying time duration of HM treatment.

Recent transcriptomic studies indicate that genes regulated by NO modulate a diverse set of cellular functions, a pleiotropic role of this molecule in plant physiology (Zago et al. 2007). Although a detailed study has been reviewed on stress-related genes modulated by NO (Grün et al. 2006), little is known about heavy metal tolerance-related genes modulated by exogenous NO. Exogenous NO was thought to attenuate oxidative stresses by decreasing the H_2O_2 content enhancing the activity of antioxidant enzymes like SOD, APX, and CAT. Whether the enhanced activity follows from increased gene expression or from the post-translational modifications (PTM) of the respective proteins has not been investigated (Grün et al. 2006). HM-induced accumulation of NO also appears to be responsible for HM toxicity. These convicting results on the relationships between NO and heavy metal toxicity are attributed to the impacts of HM on NO content and the various pathways of NO production in plants. Further studies may facilitate to understand the networks involved in plant defenses against HM stress and the roles of NO in regulating both ion homeostasis and cellular responses to heavy metals (Xiong et al. 2010).

Heavy metals induced aquaporins/transporters

Aquaporins are membrane localized such as of tonoplast and plasma membranes, and function as water channels and play a role in water managements in plants (Kaldenhoff and Fischer 2006). The aquaporins namely TIPs, PIPs, SIPs, and a NIP respectively, were reported to be significantly down-regulated in Cd stress (Hirotaka et al. 2010). So far, little information is available regarding transcriptional regulation of aquaporins under Cd stress except for a report on induction of a PIP gene in barley roots by Cd (Tamas et al. 2008). In *A. thaliana*, the expression of TIP and PIP genes were reported to be down-regulated by salt and drought stresses (Alexandersson et al. 2005; Boursiac et al. 2005). The down regulation of aquaporin genes during HM stress possibly may be attributed to the fact that the unavailability of aquaporins will hamper the water and solutes exchange across the membranes which might lead to a potential dehydration stress in plant root. Consequently, this will mediate the dehydration induced expression of genes, aquaporins and transporters inhibiting water exchange across the membrane and hence the plant may strive to adapt in the HM polluted areas due to the other associated stresses. Hirotaka et al. (2010) reported diverse range of gene regulation in *Solanum torvum* Sw. cv. Torubamubiga (TB) roots at low Cd concentration, 0.1 μM (Fig. 2), showing down regulation of dehydration-related transcription factors and aquaporin isoforms. Together with the transcriptional regulation of dehydration-related transcription factors and other regulators, including DREB and ERF proteins, these implies that drought stress is one of the important constituents of the impediments caused by HM stress in roots (Nakashima and Yamaguchi-Shinozaki 2006). The inhibitory effect of HM on aquaporins may explain the transcriptional regulation of dehydration-related genes in plant roots during HM stress.

It has been reported that low loading of Cd into xylem in the roots is responsible for the reduced translocation of Cd to the shoots (Mori et al. 2009). The transcriptional regulation of metal transporter genes that might be involved in the reduced amount of Cd and acclimation processes during Cd treatment was examined. Members of the P1B-ATPase families are involved in the transport of HM ions in higher plants (Baxter et al. 2003; Williams and Mills 2005). In *Arabidopsis*, eight family members were identified, and HMA1–HMA4 and HMA5–HMA8 were predicted to transport Zn/Cd/Pb/Co and Cu/Ag, respectively. HMA2 and HMA4 are exporters of Zn and Cd, and their involvement in HM tolerance and metal hyperaccumulation has been characterized (Mills et al. 2003; Hussain et al. 2004; Courbot et al. 2007). It is expected that Cd ions are taken up via divalent cation transporters such as ZIP transporters. In addition, a member of the ABC transporter AtPDR8 has been reported to contribute to HM tolerance by mediating efflux of Cd from plasma membrane of root epidermal cell (Kim et al. 2007). Recently, the expression of another family class of metal transporters, natural resistance-associated macrophage protein (NRAMP), AtNRAMP6 in yeast resulted in Cd mobilization from

storage organelle or accumulating Cd to cellular compartment where it is toxic (Cailliatte et al. 2009). This suggests that NRAMP transporters contribute to Cd toxicity. Furthermore, HMs were also reported to induce cation diffusion facilitator (CDF) family associated with cytoplasmic efflux and vacuolar sequestration of Zn, Cd, Co, Ni and Mn (Devez et al. 2009).

Hormonal *cis*-DNA regulatory elements

The promoter analysis has revealed that the respective *cis*-DNA regulatory elements for abscisic acid (ABA), salicylic acid (SA), and auxin (IAA) were detected in HM-induced genes suggesting the involvement of such signaling in HM response. The auxin-responsive mRNA was detected in Cd-treated *Brassica juncea* plants (Minglin et al. 2005). Proteomic analysis of Cd-treated *A. thaliana* showed the induction of nitrilase protein, which is involved in auxin biosynthesis and the transcription activation of the gene (SAMT) involved in the biosynthesis of SA was detected in pea treated with Hg (Shao et al. 2010). It is known that Cd induces the biosynthesis of ABA and ethylene, which in turn evoke various stress responses (Polle and Schützendübel 2004). Furthermore there are reports in other systems where the interference between HMs and growth hormones affects gene expression. Vergani et al. (2009) reported that in fish RTH-149 cell line, the crosstalk between HMs and growth hormones regulate metallothionein expression, which does not strictly depend on Ca^{2+} signaling but activates ERKs which enhances metallothionein because of differential recruitment of transcription factors. These results suggest that phytohormones may have a role to play in plant response network to HM. However, further endeavours need to be made in this direction to dissect out whether these hormones helps in signaling for the activation of HM-responsive genes, or serve as effectors of certain HM-obligatory reactions or participate in both processes (Cobbett 2002; Gasic and Korban 2007; Doty 2008).

Heavy metals responsive transcription factors and *cis*-regulatory elements

The modulation of transcriptional processes in plants in response to HMs and its functional link between signaling pathways and responses has not been extensively studied. The transcriptome analysis of plants treated with various HMs has revealed that HMs can induce transcription factors that regulate corresponding transcriptional processes (LeDuc et al. 2006). The Cd-induction of transcripts for basic region leucine zipper (bZIP) and zinc finger transcription factors has been detected in *A. thaliana* and *Brassica juncea* (Ramos et al. 2007). Members of the ERF subfamily are reportedly involved not only in ethylene signaling but also in plant responses to various biotic and abiotic stresses. The AP2/ERF superfamily is a large gene family of transcription factors characterized by a unique AP2/ERF domain (Nakano et al. 2006). It has been reported that ERF1 and ERF5 were induced by Cd in *A. thaliana* (Herbette et al. 2006). Induction of ERF genes by Cd in *A. thaliana* and *A. halleri* has also been reported (Weber et al. 2006). Diverse patterns of ERF gene expression induced by Cd suggest that each of the ERF proteins might be specific to different genes that respond to various levels of Cd stress. It has been reported that dehydration-responsive element-binding protein (DREB) transcription factors gets down-regulated after HM treatment. DREB1A, DREB1B, and DREB1C are involved in induction of cold stress-responsive genes, and DREB2 is involved in osmotic stress-responsive gene expression in *A. thaliana* (Nakashima and Yamaguchi-Shinozaki 2006). Although it is unclear why these stress-related transcriptional regulators were down-regulated under Cd-stressed environments, they might play an important role in transcriptional regulation of stress- responsive genes during the Cd acclimation process in plant roots. This phenomenon may be explained as when the plant senses the HM stress, the DREB gene family gets down regulated to maintain a normalized osmotic potential across the cell membrane so as to reduce the inflow of HM contaminated water. This will mitigate the HM concentrations at the cellular level and the loading of HM to transport vessels to avoid phytotoxic affects. This may provide a preliminary line of defense of plants to sustain in hostile HM contaminated environments.

Screenings of Cd-responsive genes in *A. thaliana* indicate that DREB2A gene is upregulated by Cd (Bennett et al. 2003; Curie and Briat 2003). Qi et al. (2007) reported Cd-induced transcription factors (OBF5) which binds to the promoter region of the glutathione transferase gene (GST6) in *Arabidopsis* which was known to be induced by auxin, SA and oxidative stress. Despite existing data on the HM-

induction of different transcription factors, it is still not clear if these activations are specific to a particular HM, common to most of the metals, related to oxidative stress. The process of ROS mediated transcription activation of factors is thought to be a common link in different stress responses in plants. Therefore, among all possible pathways, ROS seems to play a key role, but not the only role, in activation of HM induced transcription factors in plants. Other organisms, such as yeast and animals, contain specific HM induced transcription factors which bind to the HM responsive element present in promoters of HM responsive genes. It has been reported that MTF-1 controls heavy metal load by binding with MREs (TGCRCNC) present in metallothioneins and other HM genes from insects to mammals. In contrast, it has been reported that copper starvation has lead to evolution of *Drosophilidae* specific regulation of the *Ctr1B* copper importer via MREs/MTF-1 (Selvaraj et al. 2005). This indicates to a regulatory mechanism where the same transcription factors exert two diverse functions in response to opposite environmental situation (Selvaraj et al. 2005). Whereas in *Saccharomyces cerevisiae*, it has reported that the two extremes of Cu availability require different transcription factors. The homologs of Ctr1 that import copper are activated upon copper starvation by the Mac1 transcription factor (Yamaguchi-Iwai et al. 1997); the activation of metallothionein genes upon copper load is driven by the transcription factor Ace1. Furthermore in mammals, there exists Ctr1 and Ctr2 (Rutherford and Bird 2004); neither of which is regulated at transcriptional level by Cu availability, and there are no MREs in their promoter region (Selvaraj et al. 2005). Dormer et al. (2000) presented evidence that the transcription factors Met-4, Met-31, and Met-32 are involved in Cd induced gene regulation whereas the DNA-binding protein Cbf1 acts as negative regulation of *GSH1* expression. It is seen here that HM responsive transcription factors is involved in various gene expression and other cellular functions. Furthermore, Rutherford and Bird (2004) described the varied roles of HM induced transcription factors in his excellent review that focuses on the role that HM-responsive transcription factors in regulating trace metal metabolism, sensing HM and coordinating the expression of genes that are involved in the HM stress response.

The *cis*-acting elements related to HM responsive elements have been found within promoters of a few plant genes, including metallothionein-like genes; however, there is no evidence that these sequences confer HM responsiveness on these genes. So far Deckert (2008) reported existence of *cis*-DNA elements in plants, which may be functional in HM response viz., iron-dependent regulatory sequences (IDRS) responsible for the iron-regulated transcription of genes and recently identified element in the promoter region of the *PvSR2* gene from *Phaseolus vulgaris*, whose expression is upregulated by Hg, Cd, As and Cu. The HM-responsive elements were localized within two regions of the *PvSR2* gene promoter-region I (similar to MRE of metallothionein genes) and region II (represents a novel HM responsive element in plants and has no similarity to other *cis*-acting DNA elements) (Cunningham et al. 1995; Fox and Guerinot 1998; Shao et al. 2009). It seems obvious that plants employ a wide array of mechanisms to activate the genes required to cope with the HM stress and to confer other regulatory functions. Depending on the available information of possible molecular mechanisms of plant response to HM signaling pathways and transcription regulation, a conceptual model have been postulated (Fig. 3). This proposed model simply put forward the putative targets where the further experimental endeavour may be directed to develop HM tolerant plants.

Heavy metal stress signaling and crosstalks

The plant prefers to change gene activation patterns after sensing the HM stress stimuli through various signaling cascades (DalCorso et al. 2010). Several molecular techniques have been established to study the response mechanisms in a global view against abiotic stress in plants. Studies on HM responses have also seen the emergence of proteomic analysis as a promising tool. The recent findings of proteomic studies on the effects of metal ions showed the importance of transporter proteins in roots, the effect on primary metabolism, most noticeably the importance of the sulfur assimilation and metabolism in roots, as well as phytochelatin and glutathione synthesis (Aina et al. 2007; Kieffer et al. 2008; Roth et al. 2006; Sarry et al. 2006). The plants response to combined environmental stresses is unique and need thorough understanding to predict and avoid underscoring of the effects of crosstalks (Zurbriggen et al. 2010).

To discuss the HM induced proteomic changes in the plant system, we would like to corroborate with the excellent study done by Kieffer et al. (2009a) in poplar against Cd stress. Their study reveals the early accumulation of many typical stress-related proteins like HSPs, or glutathione-*S*-transferases, while most proteins from the primary metabolism (glycolysis, TCA cycle, nitrogen and sulfur metabolism) were severely decreased in abundance due to Cd stress, and the important metabolic proteins regulation has been depicted in Fig. 4. The upregulation of the molecular chaperones and phytochelatins in the early stage may be due to avoid the misfolding of crucial proteins and enzymes and to repair the HM degraded proteins. Further, the increased synthesis of phytochelatins may lead to faster chelation of the HMs to mitigate the cellular damage. Furthermore, the enhanced abundance of the TCA cycle proteins at later stages of HM stress may be to provide energy supplement to help plants acclimatize in hostile environments. Thus, it seems that plants follow a definite pattern of prophylactic and avoidance measures in the early stages of

Fig. 4 Varied proteomic regulatory expression profile of Cd▶ treated poplar plant showing the evidence HM stress affects different functional networks (Kieffer et al. 2009a)

HM stresses but when the HM stress prolongs, the plants preferred to opt for survival and acclimatization by conserving energy and switching off large fraction of proteins. This varied expression of proteins and gene regulation suggests that HM stress response is a coordinated complex process which brings together the coherent action of different cellular processes. Since roots are the first tissue system to counter the HM ions and consequently proteomic changes may be seen earlier than in leaves (Kieffer et al. 2009a). Furthermore, the energy conserving phenomena in Cd treated plants may be due to Cd stress, where Cd affects leaves later by blocking CO_2 uptake or by interfering with the guard cell regulation via Ca^{2+} channels (Perfus-Barbeoch et al. 2002), and it also has direct impact on electron transport in chloroplasts by damaging photosystems I and II (Sanit'a di Toppi et al. 2002). This would greatly increase the ROS production in chloroplasts even though the overall Cd content

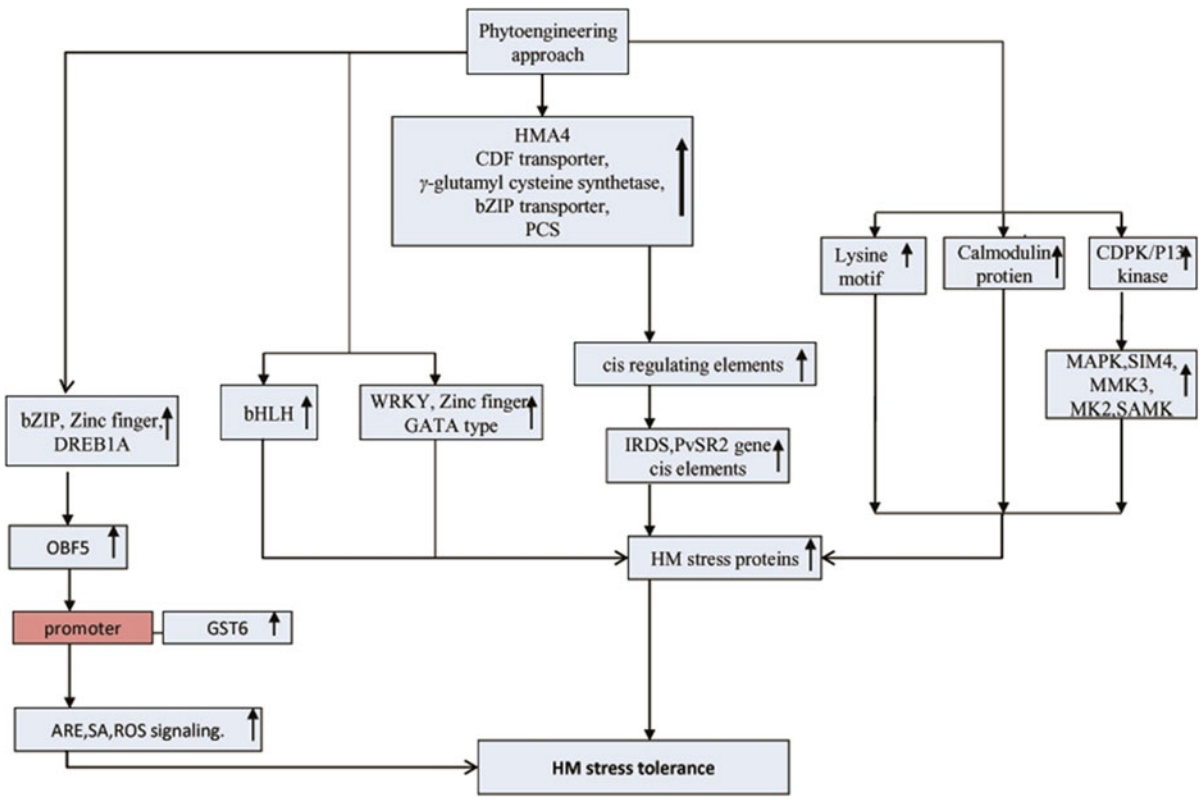
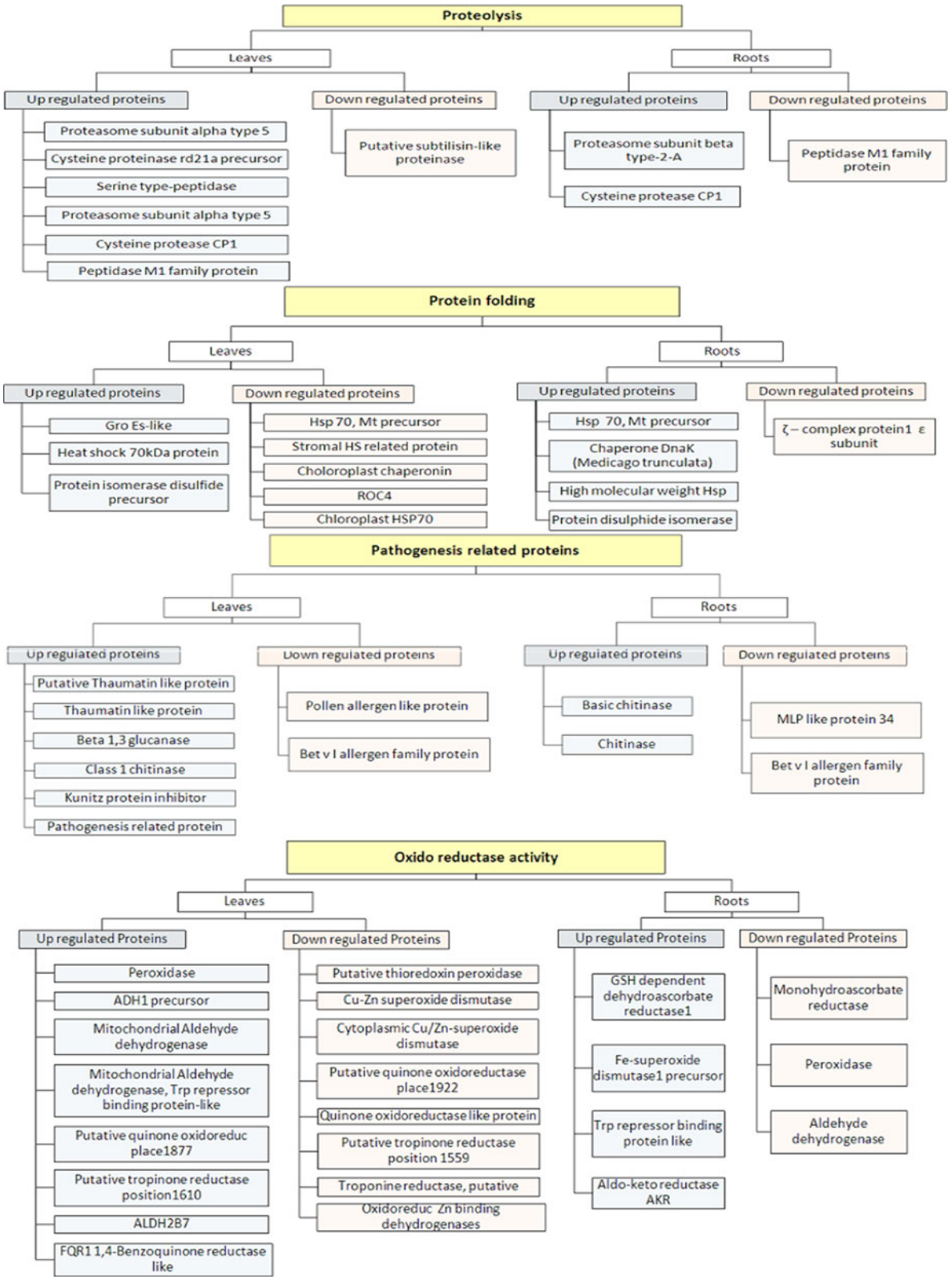


Fig. 3 A regulatory target based conceptual model for HM tolerance phytoengineering (↑ indicates upregulation of the target genes)


Proteolysis
Leaves
Roots
Up regulated proteins
Down regulated proteins
Up regulated proteins
Down regulated proteins
Proteasome subunit alpha type 5
Cysteine proteinase rd21a precursor
Serine type-peptidase
Proteasome subunit alpha type 5
Cysteine protease CP1
Peptidase M1 family protein
Putative subtilisin-like proteinase
Proteasome subunit beta type-2-A
Cysteine protease CP1
Peptidase M1 family protein
Protein folding
Leaves
Roots
Up regulated proteins
Down regulated proteins
Up regulated proteins
Down regulated proteins
Gro Es-like
Heat shock 70kDa protein
Protein isomerase disulfide precursor
Hsp 70, Mt precursor
Stromal HS related protein
Choloroplast chaperonin
ROC4
Chloroplast HSP70
Hsp 70, Mt precursor
Chaperone DnaK (Medicago trunculata)
High molecular weight Hsp
Protein disulphide isomerase
ζ – complex protein1 ε subunit
Pathogenesis related proteins
Leaves
Roots
Up regulated proteins
Down regulated proteins
Up regulated proteins
Down regulated proteins
Putative Thaumatin like protein
Thaumatin like protein
Beta 1,3 glucanase
Class 1 chitinase
Kunitz protein inhibitor
Pathogenesis related protein
Pollen allergen like protein
Bet v I allergen family protein
Basic chitinase
Chitinase
MLP like protein 34
Bet v I allergen family protein
Oxido reductase activity
Leaves
Roots
Up regulated Proteins
Down regulated Proteins
Up regulated Proteins
Down regulated Proteins
Peroxidase
ADH1 precursor
Mitochondrial Aldehyde dehydrogenase
Mitochondrial Aldehyde dehydrogenase, Trp repressor binding protein-like
Putative quinone oxidoreduc place1877
Putative tropinone reductase position1610
ALDH2B7
FQR1 1,4-Benzoquinone reductase like
Putative thioredoxin peroxidase
Cu-Zn superoxide dismutase
Cytoplasmic Cu/Zn-superoxide dismutase
Putative quinone oxidoreductase place1922
Quinone oxidoreductase like protein
Putative tropinone reductase position 1559
Troponine reductase, putative
Oxidoreduc Zn binding dehydrogenases
GSH dependent dehydroascorbate reductase1
Fe-superoxide dismutase1 precursor
Trp repressor binding protein like
Aldo-keto reductase AKR
Monohydroascorbate reductase
Peroxidase
Aldehyde dehydrogenase

in leave is less. Just to summarize the importance of validating observed protein changes, it can be said that the changes can be related to an increase or decrease in abundance, but they can also result from PTM or may be related to degradation processes. Having said these, the detailed proteomic study during HM stress will definitely provide new leads to tackle HM stress and it definitely seeks attention for further study.

The HM stress was reported to alter cellular level of Ca and calmodulin proteins which regulates ion uptake, gene activation, metabolism apart from contributing to Cd tolerance suggesting a link to crosstalks (Yang and Poovaiah 2003). Furthermore, the reduced glutathione level increases during Cd stress with parallel upregulation of antioxidant enzymes and response genes (Romero-Puertas et al. 2007). The increased accumulation of the jasmonic acid, ethylene and salicylic acid (SA) in roots of Cd stressed roots indicate towards the existence of crosstalk between biotic stress and HM stress (Maksymiec 2007). The induction of the phytoalexins biosynthesis in response to both HM stress and ROS-mediated biotic stress indicates the sharing of common signaling pathways (Mithoefer et al. 2004). It is important to mention here that HM signaling in plants is specific for each HM and it differs for each metal. Hence, the elevation of NO level in response to HM stress suggests the HM signaling share common pathway in plant defense response alone (Xiong et al. 2010).Taken together all, elevation of Ca level, calmodulin and MAPK activation, GSH mediated regulation, ROS and NO signaling and stress related hormone signaling all culminate through diverse pathways to activate transcription factors to activate stress responsive genes to imparts HM tolerance.

Conclusion

There is a dearth of data in heavy metal extracellular signal perception, and now it is believed to be mediated by receptor like protein kinase during HM stress. The induction of putative receptor like kinase during Cd treated rice root proteomic study points towards its role as metal sensors (Yeh et al. 2007). The plant HM response and accumulation seem to vary with each HM type, time and exposure of HM stress, organs and transport process to shoots, which signifies role of various HM response mechanisms in plants (Savenstrad and Strid 2004; Haydon and Cobbett 2007). The up regulation of an array of stress-related proteins like HSP, proteinases and PR-related proteins and glutathione metabolism related proteins, signaling proteins upon HM stress suggest the involvement of diverse networks of stress pathways ultimately leading to plant HM tolerance. Further, some crucial questions still remain to be answered viz., which mechanisms link HM stress intimately to redox imbalances and oxidative stress? Why during HM stress, even in the presence of effective scavenging and export mechanisms, metals conversely bind to high-affinity targets? How HM-binding can disturb cellular metabolism? What governs the plant organs to behave in a specific way to HM stress? Does the plant conserve energy during HM stress or is it due to the HM stress? Does the HM tolerance engineering of plants demands modulation of single regulatory gene or a family of multigene in a coordinated network? A systematic understanding of implicated mechanisms is unavailable, and it certainly should be a major goal for future research prospects to answers the raised questions.

Further we need to look deep into the HM signaling and crosstalks with other stresses and its eventual effects. It has been reported recently that the exogenous NO plays role on alleviating heavy-metal toxicity in plants. However, the understanding of how the NO alleviates heavy-metal toxicity and its role in plant defense mechanisms has to be studied. To explain such differences, a better understanding of the redox network of the plant cell is mandatory. We need to answer the question that why do the signaling cascades fail to activate appropriate compensation mechanisms when subjected to HM stress? Further research directions might concern how the plants redox mechanism behaves during multi metals stress as practically there exists numerous HMs in soils? The varied HM stress induced proteins expression suggests that a coordinated network of proteins and genes work together to finally reach at HM response. This require effort from all the specialized laboratories and institutes throughout the world to work as unit to converge at a point of the master regulator, which can be modulated to achieve HM stress tolerance.

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