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RESEARCH ARTICLE

Physiological, Biochemical and Molecular Response of Plants Against Heavy Metals Stress

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ABSTRACT

Heavy metals pollution to water and soils due to anthropogenic activities are threats to living organisms which needs to be tackled with a new emerging technology, referred as phytoremidiation, offers a cost-effective and environmental friendly way for clean up of contaminated areas with exploitation of green plants, which either accumulates or converts it to non toxic forms, but that incurred serious physiological and metabolic constraints in plants. This review is focused on promising aspects of physiological, biochemical and molecular level to know the mechanism of plant response towards heavy metal stress.

INTRODUCTION

Natural processes such as volcanic eruptions, continental dusts and anthropogenic activities like mining, combustion of fossil fuel, phosphate fertilizers, military activities, metal working industries etc. lead to emission and accumulation of heavy metals in ecosystem. Since the beginning of the industrial revolution, pollution of the biosphere with toxic metals has accelerated dramatically. Elements such as Cu, Zn, Ni, Co, Fe, Mo and Mn are essential mineral nutrients, play a significant role in gene expression, biosynthesis of proteins, nucleic acids, growth substances, chlorophyll and secondary metabolites, and carbohydrate and lipid metabolism required in trace amounts[1]. Metal contaminated soil with excess amount of any or all type of heavy metals like Lead (Pb), Cadmium (Cd), Chromium (Cr), Arsenic (Ar) etc. in varying combinations and concentrations adversely affect the health of millions of people world wide. More than 400 million people are at the risk of arsenic poisoning in Bangladesh and West Bengal of India due to high level of arsenic in drinking water and soil. These heavy metals are toxic because they cause DNA damage and their carcinogenic effects in animals and humans are probably caused by their mutagenic ability [2,3]. At high concentrations, nickel reduce or inhibit shoot and root growth [4,5], though low concentrations of nickel may also stimulate the germination and growth of various crop species [6]. High on the list of heavy metal pollutants from man-made sources are lead, mercury, cadmium, copper and arsenic. Cadmium (Cd) is a common environmental contaminant introduced into the soil through anthropogenic activity. While some metals are required for life, their excessive accumulation in living organisms is always toxic. The danger of Cd is aggravated by their almost indefinite persistence in the environment. For example lead, which is one of the more persistent metals, was estimated to have a soil retention time for 150–5000 years [7]. Potential threat is that heavy metals are not degradable and without intervention stay in soil for centuries. Heavy metals contamination has reached toxic levels in the air, land and water of many parts of the world [8], and clean up has become an urgent problem to minimize the entry of toxic elements into the food chain. The conventional remediation technologies (other than bioremediation) used for in situ and ex situ remediation are typically expensive and destructive. They include solidification and stabilization, soil flushing, electro kinetics, chemical reduction/ oxidation, soil washing, low temperature thermal desorption, incineration, vitrification, pneumatic fracturing, excavation/retrieval, landfill and disposal. Plants are one pathway for toxic metal mobilization into the human food chain, and paradoxically they may also provide an elegant means of reducing this spread. Phytoremediation, the use of plants to extract, sequester and/or detoxify heavy metals and other pollutants, may offer a cost-effective and ecologically sound alternative [9]. Phytoremediation appears to be a relatively less expensive, less invasive and potentially more effective means of addressing existing heavy metal contamination than those currently practiced [10,11]. The survival of plants under exposure to heavy metal salts is ensured by a complex of cell-defense mechanisms, the most important of which are (1) the de novo synthesis of phytochelatins and metallothioneins, which bind heavy metals and thus withdraw them from active cell metabolism; (2) the synthesis of molecular chaperons, homologues of HSP70, HSP90, etc.; and (3) the formation and function of antioxidant systems [12].

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Physiological response

Majority of the response in relation to environmental stresses in plants are linked with growth, differentiation and physiological aspects such as photosynthesis, ions uptake and transport [13,14]. Under Cd stress plant shows no of symptoms like chlorosis, leaf rolling and stunting. In certain species it resulted in structural disorders and thereby growth restriction [15,16,17,18, 19,20]. Its effect on metabolism have also been reported, such as decreased nutrient uptake [21], changes in nitrogen metabolism [22], altering of water balance, and inhibition of stomatal opening [23]. Moreover, Cd^{2+} ions might cause alterations in permeability of membranes by affecting lipid composition [18] and certain enzymes associated with membranes, such as H^{+}-ATPase [24]. Net photosynthesis is also sensitive to Cd, which directly affects chlorophyll biosynthesis

[25,26] and proper development of chloroplasts [18,20]. It lead to decrease in shoot dry weight which was concomitant with a significant reduction in both length and diameter of the internodes as well as in leaf area and thickness but it is dependent on tissue age. The reduction of internode diameter is associated with significantly lower cortical and medullar tissue extends because of a disturbance of the cellular expansion, leading to organ contour distortions due to irregular cell division [27]. In certain spp, Cd interfered with the formation of regular cell rows and caused, in the most external layers, an enlargement of cells, which appeared the result of anomalous division planes. Simultaneously, Cd caused a significant reduction of the number and diameter of the xylem vessels. This response, commonly reported in other plant species like bean [16], could involve a restriction of water flow translocation to shoots, and thus contribute to the perturbation of the plant water balance. Among the most spectacular effects generated by metal stress at the stem level, was the appearance of "peculiar" formations, characterized by the presence of particular cells, delimiting a dense space, whose induction and localization remain tributary to the organ developmental stage and stress intensity [28]. Cadmium that reached the leaves resulted in further physiological and structural damages. Leaf growth was inhibited and blade thickness was diminished owing to the reduced enlargement of mesophyll cells, resulting in increasing tissue dry weight / fresh weight ratio, especially in young (third) leaves. Under Cd stress, stomatal conductance was found to sharply decline suggesting that stomatal functionality may be compromised [29]. Changes in plant water relations with a decline in the transpiration rate have been observed in other Cd-exposed species [30,27] and were ascribed to decreased leaf blade expansion [31]. Although the reduction in leaf surface area is a rather common consequence of exposure to Cd, data regarding Cd effects on leaf cell enlargement are somewhat contradictory. In *Phaseolus vulgaris*, Cd caused a decrease in cell size [32], whereas in both *Brassica napus* and *Pisum sativum* it led to an increase of the mesophyll cell dimensions [27,23]. Exposure of submerged leaves to Cd in the aquatic plant *Elodea canadensis* was found to inhibit cell division but induced a significant enlargement of only one of the two cell layers constituting the leaf blade [33]. This suggests that the changes in leaf cell enlargement caused by Cd may actually be due to specific morphogenetic effects rather than to impaired water balance [34]. *Pisum sativum* further showed an impaired photosynthesis due to lower PSII activity under metal stress conditions [35]. It is well-known that leaves with high cell density have less efficient photosynthesis, and it affects the intensity of gas exchanges. Niinemets *et al*.[36] reported that leaf photosynthetic capacity scaled negatively with cell density. Furthermore, leaves with high cell density have a low intercellular space rate, which increases the resistance to gas diffusion into the tissues [37]. On the other hand, thick leaves have high photosynthetic potential [36] and an ample lacunar tissue, which favours the diffusion of gases in the mesophyll. Cell density can provide further information about the importance of the apoplastic fraction and, as an indirect result, on the detoxifying potential [38].

Anatomical response

Copper or cadmium applied alone or in combination caused significant reduction in root diameter, width and thickness of leaf midrib, diameter of xylem vessels of all seedling organs, parenchyma cell area in the stem, leaf midrib and pith and cortex of root, dimensions of stem vascular bundles, number of xylem arms in root, and frequency of stomata on abaxial leaf surface and reduction in grain yield due to the heavy metal-induced changes in the structure and consequently the function of the vascular and stomatal apparatus [39]. The increase in number of xylem tissues due to increased rate of transpiration and thickening of cell wall with reduction in number of cells in the cortical region of the roots due to accumulation of heavy metals in the vacuoles of the cortical cells and the cell wall of the root was also reported under heavy metal stress [40].

In plant cells, actin forms various structures that may undergo mutual transitions; it is present in the form of tight bundles of longitudinal filaments linked to one another and the cell membrane into a single network, a thin near membrane network formed by polymeric actin, and the pool of monomeric (unpolymerized) actin. Rearrangements in cell metabolism are often accompanied by changes in the organization of actin filaments and in the proportion of polymeric and monomeric forms of actin. The studies of the effect of heavy metals on the cytoskeleton of plant cells that have been performed so far are quite scanty. It was shown that cadmium caused disorganization and destruction of microfilaments and microtubules in the green alga *Spirogyra* and that aluminum induced formation of tight bundles of actin filaments in meristematic cells of *Triticum turgidum* root. It was also established that the resistance to aluminum of tobacco mutants was associated with a considerable increase in the total content of actin and especially in the proportion of tight bundles of actin filaments in leaf cells. It is well known that HSPs (or their analogues) are molecular chaperons that are involved in maintenance of the native structure of proteins and prevent their denaturation and aggregation. The interaction between HSP and actin may be of principal importance for maintenance of the cytoskeleton structure and survival of plants under exposure to heavy metals. Short term treatment of rape plants with 50 μM $CuSO_4$ resulted in a considerable increase in the amount of polymeric actin in root cells and an increase in the amount of HSP70 and HSP60 associating with actin filaments. It assumed that HSP70 and HSP60, which interact with the polymeric actin under exposure to heavy metals, function as molecular chaperons and protect the actin cytoskeleton from damage [12].

Biochemical response

As and when the plant encounters with either biotic or an abiotic stress the preliminary response of the all plant spp is generation of ROS which is due to the auto oxidation of redox-active heavy metal or cellular damage or may be due to displacement of ions. Reactive oxygen species (ROS) are continuously produced at low level during normal metabolic processes [41]. But in biological systems, increasing the synthesis of ROS is one of the initial responses to different stress factors [42]. ROS induce damage to the biomolecules through peroxidation of membrane lipids, alteration of protein functions, DNA mutation, damage to chlorophyll and disruption some of metabolic pathways (electron transport chain and ATP production) [43,44]. Plants have complex ROS scavenging mechanisms at the molecular and cellular levels. These mechanisms with inhibition or slow the oxidation of biomolecules and oxidative chain reactions [45] decrease the cellular oxidative damage and increase resistance to heavy metals. The plant antioxidant defense systems include antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) and low-molecular weight quenchers (cycteine, ascorbic acid, thiols, proline [42], α-tocopherol, glutation, carotenoids, phenolic and nitrogen compounds [45].

Proline

Proline is a heterocyclic amino acids found abundantly in basic proteins. Proline accumulation is an indicator of heavy metal tolerance under stress as it plays an essential role. The amino acid acts as an osmolyte by antioxidative, osmoprotection properties and metal chelator [46], takes part in reconstruction of chlorophyll [47], regulation of cytosolic acidity [48] tolerance to stress by osmoregulation and stabilization of protein synthesis [49], stabilize the macromolecules and organelles [50] the protection of enzymes from denaturation [48] and also serve as source of nitrogen and energy in recovery growth [51].The increment in proline level under abiotic stress may occurred due to increase of de novo synthesis or decrease degradation [52] and the effect of proline on the permeability of membrane [53]. It was also correlated that decrease

in the mitochondrial electron transport activity accompany increase in proline accumulation under environmental stresses [54].

Ascorbic acid

It is a sugar acid and an antiascorbutic required for formation of connective tissue collagen, hydroxylation of certain proline and lysine residues and for normal iron metabolism. It is a strong reducing agent losing hydrogen atom readily to become dehydroascorbic acid which has vitamin C activity in turn protects the cell membrane from the toxic action of powerful oxidizing agents. Ascorbic acid is universally present in plants as a constituent of oxidation –reduction systems and is actively involved in the plant growth, differentiation and development [55]. Ascorbic acid acts as potent antioxidant and the highly reactive oxyradicals promote the oxidation of ascorbic acid to dehydroxy ascorbic acid, leading to the reduction of ascorbic acid [56]. The retention of ascorbic content in the cotyledons may be due to its reduced translocation. Ascorbic acid oxidase is unlikely to be involved in scavenging system, since the reaction consumes molecular oxygen, rather than hydrogen peroxide [57]. Elevated level of ascorbic acid on exposure to the heavy metal stress in roots suggest that it plays an important role of detoxifying the ROS generated under heavy metal stress with scavenging of H_2O_2, a reaction catalyzed by APX [58].

Chlorophyll

An essential component of photosynthesis present in chloroplast with porphyrin (tetrapyrrole) nucleus with a chelated magnesium atom at the centre and a long chain hydrocarbon (phytol) side chain attached through a carboxylic acid group. Regarding the green pigment content, heavy metal-treated plants showed a remarkable decrease of chlorophyll that causes photosynthesis rate enormously decrease in response to elevated heavy metal concentration. Reduction in chlorophyll content as a consequences of many metabolic reactions like inhibition of enzymes activity such as δ-aminolevulinic acid dehydratase (ALAdehydratase) [59] and protochlorophyllide reductase [60], replacement of Mg with heavy metals in chlorophyll structure [61], decrease in the source of essential metals that involved in chlorophyll synthesis such as Fe^{2+} and Zn^{2+} [60,62], destruction of chloroplast membrane by lipid peroxidation due to increase in peroxidase activity and lack of antioxidants such as carotenoids [63], decrease in density, size and the synthesis of chlorophyll and inhibition in the activity of some enzymes of Calvin cycle [27,64]. In another word, chloroplast contains many different parts that respond to heavy metal stress therefore any changes in chlorophyll synthesis and activity used as the index of direct toxic effects of heavy metals. In contrary to above findings an increase in chlorophyll ratio (a/b) in Co and Ag stress shows that chlorophyll 'b' is more sensitive to Co and Ag that disrupt the balance between energy trapping in photosystem II and cause a decrease in electron transport [65]. While decrease in chlorophyll ratio (a/b) in response to Cd and Pb treatments suggest that chlorophyll 'a' is more sensitive to Cd and Pb.

Carotenoid

Caratenoids are tetraterpeniod (C_{40}) compounds widely distributed in plants, function as accessory pigments in photosynthesis and as colouring matters in flowers and fruits. Some of the commonly occurring carateniods are simple unsaturated hydrocarbons based on lycopene and their oxygenated derivatives known as xanthophylls. B-carotene is the most common pigment in this group found in higher plants. Carotenoid is a non-enzymatic antioxidant pigment that protects chlorophyll, membrane and cell genetic composition against ROS under heavy metals stress [66]. In plant cell protective role of this pigment might be due to quenching triplet chlorophyll, replacing peroxidation and destruction of chloroplast membrane [67]. Decrease in carotenoid content is a common response to metal toxicity [68], but increase is due to important role of this pigment in detoxifying ROS [69, 70]. The carotenoid content decreased in response to heavy metals indicate a severe effect on cell and its component parts at first carotenoid content increased to protect the cell against these heavy metals, but in high concentration (100 µM) these heavy metals activate some mechanisms and degrade carotenoid pigments.

Phenolic Compounds

Plants contain a large no of aromatic compounds with hydroxyl groups which are collectively referred as phenolics or phenols. They are the derivatives of phenol molecule and are quite diverse in their chemical structure. It includes wide range of compounds such as catechol, cyaniding, caffeic acid, ferulic acid, tannins, flavonols, chlorogenic acid, lignins and capsaicin. The increase in phenolic content may be due to protective function of these compounds against heavy metal stress by metal chelation and ROS scavenging [71,72]. Under heavy metal stress the level of phenol content gets increased due to its antioxidative activity govern by their ability to chelating transition metal ion, the inhibition of superoxide-driven Fenton reaction [73,74] and membranes stability by decreasing membrane fluidity [75]. Phenolic compounds beside ascorbate can protect cell against oxidative stress by phenol-coupled APX reaction [76].

Total Soluble Protein Content

Lipids and proteins are important constituents of the cell that easily damage in environmental stress condition [77]. Hence, any change in these compounds can be considered as an important indicator of oxidative stress in plants It is thought that decrease in total soluble protein content under heavy metals stress may be due to increase in protease activity [78], various structural and functional modifications by the denaturation and fragmentation of proteins [79], DNA-protein cross-links [80], interaction with thiol residues of proteins and replacement them with heavy metals in metalloproteins [81]. It has been reported that cadmium is able to decrease protein content by inhibiting the uptake of Mg and K and promote posttranslational modification [82,83], decrease in synthesis or increase in protein degradation [84] and the prevention of Rubisco activity [85,86]. The increase in total soluble protein content under heavy metal stress may be related to induce the synthesis of stress proteins such as enzymes involved in Krebs cycle, glutathione and phytochelatin biosynthesis and some heat shock proteins [87,88].

SOD Activity

Superoxide dismutase (SOD) is an essential component of plant antioxidation system that can be used as biomarker of environmental stress [89]. Superoxide dismutase is the first enzyme in ROS detoxifying process that with converting O^{2-} to H_2O_2 in cytosol, chloroplast and mitochondria plays an axial role in cellular defense mechanisms against the risk of OH formation [90,91]. Increase in SOD activity under metal stress indicates high accumulation of ROS in order to activate antioxidative defense enzymes to inhibit oxygen radical accumulation. Increase in SOD activity appears to be probably attributed to superoxide radical accumulation, de-novo synthesis of the enzymatic proteins[87] and induction the expression of genes encoding SOD [92]. Possible explanations for the decrease in SOD activity under 100 µM Ag treatments may be linked to inactivation of enzyme by the production of excess ROS and unspecific enzyme degradation [93] or the binding of nonessential heavy metals to the active center of the enzyme [94].

GPX Activity

Peroxidases (POX) with large number isoenzymatic forms participate in a variety of cellular functions such as growth, development, differentiation, senescence, auxin catabolism, and lignifications [95]. SOD activity results in H_2O_2 production that should be detoxified by some other oxidative enzymes such as APX, GPX and CAT to H_2O and O_2 [96]. Although any change in GPX activity can consider as a

typical response to oxidative stress, but diversity in peroxidase activity under heavy metals stress depends on plant species (physiological status and genetic potential of plant), time of treatment and metal concentration [97,98]. The decrease in GPX activities may result in the cytotoxicity due to blocking of essential functional groups, replacement of essential metals with heavy metals, changes in structure or the integrity of proteins and the interruption of signal transduction pathways of antioxidant enzymes because of poisonous active oxygen species (AOS) derivatives [92,94,97]. In comparison with control, GPX activity increased in both levels of Cd treatments. Van Assche and Clijsters [60] demonstrated that increase in GPX activity might be a result of increase in de novo protein synthesis or the activation of enzymes already present in plant cells to diminish ROS deleterious effects.

CAT Activity

Increase in CAT activity was expected because increase in SOD activity lead to H_2O_2 generation that will be detoxified in further steps by CAT or POX to maintain the cellular redox state. There are two pathways in ROS scavenging: SOD/CAT and ascorbate-glutathione cycle [99]. CAT is one of the most important component of plant protective mechanisms that exist in mitochondria and peroxisomes [100] and has important role in scavenge free radicals specially H_2O_2 generated during photorespiration [101] and stress condition [102,103]. This enzyme by catalyzing H_2O_2 to H_2O and O_2 via two-electron transfer [104] prevent the generation of OH and protect proteins, nucleic acids and lipids against ROS [105]. CAT activity increased in all treatments except Pb and Ag (100 µM) which can be considered as a circumstantial evidence for role of CAT in detoxification of H_2O_2 that induced under heavy metals stress. Under Pb stress a significant dose-dependent decrease in activity was observed and enzyme activity at higher concentration (100 µM) was less than 50 µM and this indicate that increase in metal concentration cause the inhibition of enzyme activity because in high concentration of metal CAT is not properly able to protect cell against ROS. These results are in agreement with results of Verma and Dubey [87]. It has been reported that decrease in CAT activity under Pb treatment resulted in increase in lipid peroxidation because of decrease in H_2O_2 detoxification [106]. It also reported that decrease in SOD and CAT activities cause increase in lipid peroxidation by convert Fe^{+3} to Fe^{2+} for generation OH. Some of the reasons for decrease in CAT activity under stress conditions are changes in the assembling of CAT subunits and enzyme inactivation or proteolytic degradation by peroxisomal protease [107,108], changes in enzyme structure due to binding non-essentional metals to them [109]. Also increase in GPX and decrease in CAT activity in Pb 50 µM support this hypothesis that most H_2O_2 produced by SOD, detoxified by peroxidase in oxidation processes.

Oxylipin

The lipid derived compounds have been identified very recently as signaling molecules in plants elicited by pathogens and are probably also responsible for heavy metal-induced defense responses as well. However, abiotic stress such as heavy metals or membrane integrity disturbing compounds can only partially mimic biotic interactions with respect to the activation of secondary metabolism initialized by ROS generation but still it has to be characterized for its role in signal transduction [110].

Molecular response

Heavy metal ions play essential roles in many physiological processes. In trace amounts, several of these ions are required for metabolism, growth, and development. However, problems arise when cells are confronted with an excess of these vital ions or with non nutritional ions that lead to cellular damage [111,97,112,113]. Heavy metal toxicity comprises inactivation of biomolecules by either blocking essential functional groups or by displacement of essential metal ions [114]. In addition, autoxidation of redox-active heavy metals and production of reactive oxygen species (ROS) by the Fenton reaction causes cellular injury [115]. In response to toxic levels of heavy metals, plants synthesize Cys-rich, metal-binding peptides including phytochelatins and metallothioneins. Therefore, heavy metals can be detoxified by chelation and sequestration in the vacuole [116,117] and various membrane transport systems play an important role in metal ion homeostasis and tolerance [118]. Molecular response is also observed when plants encounter excessive amounts of heavy metals which are listed below.

Metal transporters

Metal cation homeostasis is essential for plant nutrition and resistance to toxic heavy metals. Therefore, heavy metal transport is a very exciting and developing field in plant biology**.** Although there is no direct evidence for a role for plasma membrane efflux transporters in heavy metal tolerance in plants, recent research has revealed that plants possess several classes of metal transporters that must be involved in metal uptake and homeostasis in general and, thus, could play a key role in tolerance. These include heavy metal (or CPx-type) ATPases that are involved in the overall metal ion homeostasis and tolerance in plants, the natural resistance- associated macrophage protein (Nramp) family of proteins, cation diffusion facilitator (CDF) family proteins [119] and the zinc-iron permease (ZIP) family [120]. Of course, many plant metal transporters remain to be identified at the molecular level. The CPx-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of essential, as well as potentially toxic, metals like Cu, Zn, Cd, and Pb across cell membranes [119]. Responsive-to-antagonist 1 (RNA1), a functional CPx-ATPase, plays a key role in the operation of the ethylene signaling pathway in plants. Hirayama *et al*.[121] identified an *Arabidopsis* mutant RNA1 that shows ethylene phenotypes in response to treatment with *trans*-cyclooctene, a potent receptor antagonist. Genetic epistasis studies revealed an early requirement for RAN1 in the ethylene pathway. Functional evidence from yeast complementation studies suggested that RAN1 transports copper and it was proposed that this CPx-ATPase may have a role in delivering copper to the secretory system, which is required in the production of functional hormone receptors. The CPx-ATPases are thought to be important not only in obtaining sufficient amounts of heavy metal ions for essential cell functions, but also in preventing the accumulation of these ions to toxic levels. The Nramp family defines a novel family of related proteins that have been implicated in the transport of divalent metal ions. Thomine *et al*.[122] reported that Nramp proteins play a role in Fe and Cd uptake; interestingly, disruption of an *AtNramps3* gene slightly increased Cd resistance, whereas overexpression resulted in Cd hypersensitivity in *Arabidopsis*. The CDF proteins are a family of heavy metal transporters implicated in the transport of Zn, Cd, and Co that have been identified in some plants. Certain members of the CDF family are thought to function in heavy metal uptake, whereas others catalyse efflux, and some are found in plasma membranes whereas others are located in intracellular membranes. The recent study by van der Zaal *et al.*[123] suggests that the protein zinc transporter of *Arabidopsis thaliana* (*ZAT1*) may have a role in zinc sequestration. Enhanced zinc resistance was observed in transgenic plants overexpressing *ZAT1* and these plants showed an increase in the zinc content of the root under conditions of exposure to high concentrations of zinc. However, this transporter is not confined to root tissue; northern blotting analysis indicated that *ZAT1* was constitutively expressed throughout the plant and was not induced by exposure to increasing concentrations of zinc. Until now, 15 members of the ZIP gene family have been identified in the *A. thaliana* genome. Various members of the ZIP family are known to be able to transport iron, zinc, manganese, and cadmium. Pence *et al.*[124] cloned the transporter *ZNT1*, a ZIP gene homolog, in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. They found that *ZNT1* mediates high-affinity Zn uptake as well as low-affinity Cd uptake. Northern blot analysis indicated that enhanced Zn transported

in *T. caerulescens* results from a constitutively high expression of *ZNT1* in the roots and shoots. Sequence analysis of *ZNT1* indicated that it is member of a recently discovered micronutrient transport gene family, which includes the *Arabidopsis* Fe transporter IRT1 and the ZIP Zn transporters [124]. Working with *T. caerulescens* from a different source population, Assuncao *et al*.[125] have also cloned two ZIP cDNA (*ZNT1* and *ZNT2*) and, similarly, have found them to be highly expressed in root tissue. The fact that downregulation of transcript levels was not observed in response to high concentrations of zinc suggests that a constitutively high level of expression of these transporters may be a distinctive feature of hyperaccumulator plants. Lombi *et al*.[126] have also cloned an ortholog of the *A. thaliana* iron transporter IRT1 from *T. caerulescens* that also belongs to the ZIP gene family. Of course, many plant metal transporters remain to be identified at the molecular level and the transport function, specificity, and cellular location of most of these proteins in plants remains unknown.

Amino acids and organic acids

Plants produce a range of ligands for Cd, Cu, Ni, and Zn. Carboxylic acids and amino acids, such as citric, malic, and histidine (His), are potential ligands for heavy metals and, so, could play a role in tolerance and detoxification [127,128]. The Cd-and Zn-citrate complexes are prevalent in leaves, even though malate is more abundant. In the xylem sap moving from roots to leaves, citrate, and His are the principal ligands for Cu, Ni, and Zn. Recently, Salt *et al*.[129] identified putative Zn-His complexes in the roots of the closely related Zn hyperaccumulator *T. caerulescens*. Kramer *et al*.[130] observed a 36-fold increase in the concentration of free His in the xylem exudate of the Ni-hyperaccumulator *Alyssum lesbiacum* after exposure to Ni. However, no significant change was observed in the non-accumulator *Alyssum montanum* and a significant linear correlation in the xylem exudate concentrations of free His and Ni in several Ni-hyperaccumulators in the genus *Alyssum* was also observed [130]. Furthermore, the addition of equimolar concentrations of exogenous *L*-His to an Ni-amended hydroponic rooting medium enhanced Ni flux into the xylem in the non-accumulator *A. montanum*, as well as in the non-accumulator *Brassica juncea* L. cv. *vitasso*. In *B. juncea*, reducing the entry of *L*-His into the root by supplying *D*-His instead of *L*-His or *L*-His in the presence of a 10-fold excess of *L*-alanine did not affect root Ni uptake, but reduced Ni release into the xylem. Compared with *B. juncea*, root His concentrations were constitutively approximately 4.4-fold higher in the hyperaccumulator *A. lesbiacum* and did not increase within 9 h of exposure to Ni [131]. However, no increase was observed in the concentration of free His in root, shoot, or xylem sap in the other Ni-hyperaccumulator *Thlaspi goesingense* in response to Ni exposure [132].

Phytochelatins

Unique to plants and certain fungi, such as *Schizosaccharomyces pombe,* metal stress induces the synthesis of metal-binding peptides commonly known as phytochelatins (PCs). Derived from glutathione, PCs have the general structure of (g- Glu-Cys)nGly, where the number of g-Glu-Cys units extends up to 11 [133]. Some PC related peptides lack the carboxyl-terminal Gly or have instead b-Ala, Ser, or Glu [134]. However, these variant peptides are usually found in lower abundance. Many metals induce PC synthesis, but formation of PC-metal complexes has largely been examined with Cd^{2+} and Cu^{2+}. Several reports show that PCs also chelate Ag^{+}, Hg^{2+}, Pb^{2+} and Zn^{2+} [135,136,137,138]. In the case of Cd^{2+}, two PC-metal complexes have been described: a low molecular weight (LMW) PC-Cd complex, and a more stable high molecular weight (HMW) PC-CdS complex that contains additional acid-labile sulfide [139,140]. Formation of the HMW complex reduces the Cys:Cd ratio from 4:1 to a more economical ~2:1 [141]. The appearance and the location of the two complexes suggest that PC is a cytoplasmic Cd/metal scavenger that targets metals to the vacuole for storage as a stable HMW PCCdS complex. In addition to PCs, plants can synthesize small cysteine-rich proteins known as metallothioneins [142]. The role of plant metallothioneins in metal tolerance is not entirely elucidated, but the animal and fungal proteins appear to play an important role in metal tolerance [143]. There have been numerous reports of engineering animal metallothionein production in plants [144,145,146,147,148]. These proteins increase metal tolerance to varying degrees, but not with substantial increase in metal uptake. In addition, the tolerance effect depends on whether the PCs are given the chance to be synthesized. When a high amount of Cd is suddenly introduced to the seed or the plant, the metallothionein producing seedling or plants are better able to respond to the toxic metal. This is because of the already accumulated metallothioneins, whereas the control plants would have to induce *de novo* synthesis of the PC peptides. In field situations, however, plants rarely are challenged with a sudden influx of toxic metals. Rather, they slowly accumulate the metals as their roots extend into the rhizosphere. When given adequate induction of PCs, the additional synthesis of metallothioneins does not confer a significant advantage. The existence of metal hyperaccumulators reveals that two critical genetic traits exist [149]. First, there is genetic potential for overaccumulating toxic metals**.** For example, this has been shown for the Zn and Cd hyperaccumulator *Thlaspi caerulescens,* which has a higher Zn influx rate and more ZNT1 Zn-transporter RNA than the non accumulator *Thlaspi arvensae* [150]. Second, these plants must also have evolved hypertolerance mechanisms that accommodate their high metal content. Plants can produce cysteine-rich peptides such as GSH, PCs, or metallothioneins (MTs) for detoxification or homeostasis of heavy metals. PCs include a family of small enzymatically synthesized peptides having a general structure of (γ-Glu-Cys)n-Gly, and these peptides are rapidly synthesized in response to toxic levels of heavy metals in all tested plants

Metallothioneins

Detoxification of metals by the formation of complexes is used by most of the eukaryotes against heavy metal stress. Metallothioneins (MTs) are low molecular weight (6–7 kDa), cysteine-rich proteins found in animals, higher plants, eukaryotic microorganisms, and some prokaryotes [151]. They are divided into three different classes on the basis of their cysteine content and structure. The Cys-Cys, Cys-X-Cys and Cys-X-X-Cys motifs (in which X denotes any amino acid) are characteristic and invariant for metallothioneins. No aromatic amino acids or histidines are found in MTs. MTs found in a few higher plants are also low molecular weight proteins with a high cysteine content, but the cysteines distribute differently than they do in mammalian MTs; therefore, these proteins are designated class II (mammalian MTs comprise class I). The biosynthesis of MTs is regulated at the transcriptional level and is induced by several factors, such as hormones, cytotoxic agents, and metals, including Cd, Zn, Hg, Cu, Au, Ag, Co, Ni, and Bi [151]. Although it is believed that MTs could play a role in metal metabolism, the role of MTs in plants remains to be determined owing to a lack of information and their precise function is not clear [128].

Activation of Distinct Mitogen-Activated Protein Kinase Pathways

To elucidate signal transduction events leading to the cellular response to heavy metal stress, analysis of protein phosphorylation induced by elevated levels of copper and cadmium ions as examples for heavy metals with different physiochemical properties and functions was done. Exposure of alfalfa (*Medicago sativa*) seedlings to excess copper or cadmium ions activated four distinct mitogen-activated protein kinases (MAPKs): SIMK, MMK2, MMK3, and SAMK. Comparison of the kinetics of MAPK activation revealed that SIMK, MMK2,MMK3, and SAMK are very rapidly activated by copper ions, while cadmium ions induced delayed MAPK activation. In protoplasts, the MAPK kinase SIMKK specifically mediated activation of SIMK and SAMK but not of MMK2 and MMK3.

Moreover, SIMKK only conveyed MAPK activation by $CuCl_2$ but not by $CdCl_2$. These suggest that plants respond to heavy metal stress by induction of several distinct MAPK pathways and that excess amounts of copper and cadmium ions induce different cellular signaling mechanisms in roots [152].

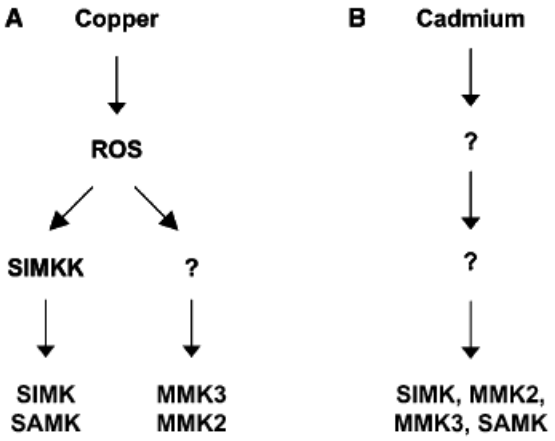

Figure: Copper and cadmium-induced MAPK signaling pathways. Excess copper and cadmium ions induce distinct MAPK pathways with different kinetics. A, As a redox-active metal ion, copper leads to the production of ROS that might trigger SIMK and SAMK activation via SIMKK. B, Cadmium activates SIMK, MMK2, MMK3, and SAMK. The upstream components mediating MAPK activation by cadmium remain to be identified.

brevicula, which is highly resistant to a wide range of heavy metal concentrations and has its metal-binding protein(s) induced in the presence of Cd and An. In their study, following purification by Sephacryl S-100 chromatography, Ryu *et al.* [156] found that Cu-BP contained an equal amount of Zn in non-exposed physiological conditions. However, Zn is replaced by Cu at the binding site upon the addition of excess Cu (100 µmol/L $CuCl_2$) to the cytosol or after a long period (60 d) of exposure of the periwinkles to the metal ion (150 µg/L CuCl2). Ryu *et al.* [156] also determined the molecular weight of the purified protein as 11.38 kDa using MALDI-TOF MS analyses. This Cu-BP is distinct from common mollusc MT in that it contains a significantly lower number of Cys (eight residues) and high levels of the aromatic amino acids Tyr and Phe. In addition, the protein contains His and Met, which are absent in the MT-like Cd-BP of *L. brevicula*. The Cu- BP of *L. brevicula* functions in the regulation of Zn as well as Cu, which is an essential component of hemocyanin under physiological conditions. This protein is possibly involved in the detoxification mechanism against a heavy burden of Cu (Table 1).

Conclusion

Under heavy metal stress plant adept itself by physiological, biochemical and molecular levels to combat the toxic effect which is important to be studies for the understanding of mechanism of plant response under stress to heavy metals, the present review is compilation of the heavy metal stress changes occurred in plants spp, which are worth to know for the engineering of the plant spp for the improvement of plant spp in terms of accumulation and its use for phytoremediation of heavy metals contaminated sites.

Table 1 Peptides and proteins contributing to heavy metal tolerance or accumulation

Peptides and proteins	Related heavy metals	References
Phytochelatins	Cd, Zn, Hg, Cu, Ag, Ni, Au, Pb, As	Grill *et al.* 1987, 1989; Grill *et al.* 1988; Thumann *et al.* 1991; Howden and Cobbett 1992; Mehra *et al.* 1995, 1996a, 1996b; Maitani *et al.* 1996; Raab *et al.* 2004
Metallothioneins	Cd, Zn, Hg, Cu, Au, Ag, Co, Bi	Kägi 1991; Hall 2002
Heat shock proteins	Cu	Neumann *et al.* 1995
Cpx-type heavy metal ATPases	Cu, Zn, Cd, Pb	Hirayama *et al.* 1999
Nramp	Cd	Thomine *et al.* 2000
CDF family proteins	Zn, Co, Cd	van der Zaal *et al.* 1999
ZIP family	Cd, Zn, Mn	Pence *et al.* 2000; Assuncao *et al.* 2001; Lombi *et al.* 2002
Metal-binding protein	Zn, Cu, Cd	Ryu *et al.* 2003

CDF, cation diffusion facilitator; Nramp, natural resistance-associated macrophage protein; ZIP, zinc-iron permease.

Heat shock proteins

Heat shock proteins (HSPs) characteristically show increased expression in response to the growth of a variety of organisms at temperatures above their optimal growth temperature. They are found in all groups of living organisms, can be classified according to molecular size, and are now known to be expressed in response to a variety of stress conditions, including heavy metal stresses [153,154]. HSPs act as molecular chaperones in normal protein folding and assembly, but may also function in the protection and repair of proteins under stress conditions. Today, there have been a couple of reports of increased HSP expression in plants in response to heavy metal stress. Neumann *et al.*[155] observed that HSP17 is expressed in the roots of *Armeria maritime* plants grown on Cu-rich soils. It was also reported that a short heat stress given prior to heavy metal stress induces a tolerance effect by preventing membrane damage.

Other metal-binding proteins

Metal-binding proteins and peptides in plants can enhance metal tolerance/accumulation. These metalbinding peptides or proteins should be preferentially metal specific such that only the toxic metals (e.g. Cd, Hg, and Pb) are sequestered rather than essential trace metals, such as Zn and Cu. Ryu *et al.*[156] isolated and characterized a novel Cu-binding protein (BP) in the Asian periwinkle *Littorina*

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