



Heavy Metal Tolerance in Plants: Role of Transcriptomics, Proteomics, Metabolomics, and Ionomics

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Specialty section:

This article was submitted to
Plant Physiology,
a section of the journal
Frontiers in Plant Science

Received: 14 August 2015

Accepted: 02 December 2015

Published: 08 February 2016

Citation:

Singh S, Parihar P, Singh R, Singh VP
and Prasad SM (2016) Heavy Metal
Tolerance in Plants: Role of
Transcriptomics, Proteomics,
Metabolomics, and Ionomics.
Front. Plant Sci. 6:1143.
doi: 10.3389/fpls.2015.01143

Heavy metal contamination of soil and water causing toxicity/stress has become one important constraint to crop productivity and quality. This situation has further worsened by the increasing population growth and inherent food demand. It has been reported in several studies that counterbalancing toxicity due to heavy metal requires complex mechanisms at molecular, biochemical, physiological, cellular, tissue, and whole plant level, which might manifest in terms of improved crop productivity. Recent advances in various disciplines of biological sciences such as metabolomics, transcriptomics, proteomics, etc., have assisted in the characterization of metabolites, transcription factors, and stress-inducible proteins involved in heavy metal tolerance, which in turn can be utilized for generating heavy metal-tolerant crops. This review summarizes various tolerance strategies of plants under heavy metal toxicity covering the role of metabolites (metabolomics), trace elements (ionomics), transcription factors (transcriptomics), various stress-inducible proteins (proteomics) as well as the role of plant hormones. We also provide a glance of some strategies adopted by metal-accumulating plants, also known as “metallophytes.”

Keywords: crop, heavy metal, ionomics, metabolomics, metallophytes, proteomics, transcriptomics, yield

INTRODUCTION

During the last few decades, increased anthropogenic activities, rapid industrialization, and modern agricultural practices have resulted in increased heavy metal contamination in the environment, which causes toxicity to the living organisms (Eapen and D’Souza, 2005; Kavamura and Esposito, 2010; Miransari, 2011). Large areas of land have been contaminated with heavy metals due to the use of pesticides, fertilizers, municipal and compost wastes, and also due to heavy metal release from smelting industries and metalliferous mines (Yang et al., 2005). Although many heavy metals occur naturally in the earth’s crust at various levels, the problem arises when they are released in excess into the environment due to natural and/or anthropogenic activities. The 53 elements belonging to the d-block have been categorized as “heavy metals” based on their density (>5 g/cm³) (Jarup, 2003). During evolution of angiosperms, only 19 elements such as C, O, H, Mg, S, N, Ca, P, and K (macronutrients) and Cu, Zn, Mn, Fe, Mo, B, Ni, Co, Cl, and B (micronutrients) were selected for basic metabolism (Ernst, 2006). In addition, Si is also considered as a beneficial element, and it has been reported to be involved in the maintenance of plant structures in some plants (Epstein, 1999). Macro and micronutrients play an important role in physiological and biochemical processes of plants such as chlorophyll biosynthesis, photosynthesis,

DNA synthesis, protein modifications, redox reactions in the chloroplast and the mitochondrion, sugar metabolism, and nitrogen fixation. For example, Zn is a cofactor for more than 300 enzymes and 200 transcription factors associated with the maintenance of membrane integrity, auxin metabolism, and reproduction (Marschner, 1995; Barker and Pilbeam, 2007; Briat et al., 2007; Williams and Pittman, 2010; Prasad, 2012; Ricachenevsky et al., 2013). However, at elevated concentrations, heavy metals produce severe toxicity symptoms in plants, and therefore, their uptake and utilization are tightly controlled by the plant cells (Janicka-Russak et al., 2008; Saito et al., 2010; Singh et al., 2012; Srivastava et al., 2012; DalCorso et al., 2013a; Farias et al., 2013; Fidalgo et al., 2013). Some heavy metals, such as Cd, Cr, Pb, Al, Hg, etc., although being non-essential and without physiological function, are very toxic even at very low concentrations (Ernst et al., 2008; Janicka-Russak et al., 2008; Garzón et al., 2011; Hayat et al., 2012; Shahid et al., 2012; Chong-qing et al., 2013; Gill et al., 2013). Essential and non-essential heavy metals generally produce common toxic effects on plants, such as low biomass accumulation, chlorosis, inhibition of growth and photosynthesis, altered water balance and nutrient assimilation, and senescence, which ultimately cause plant death.

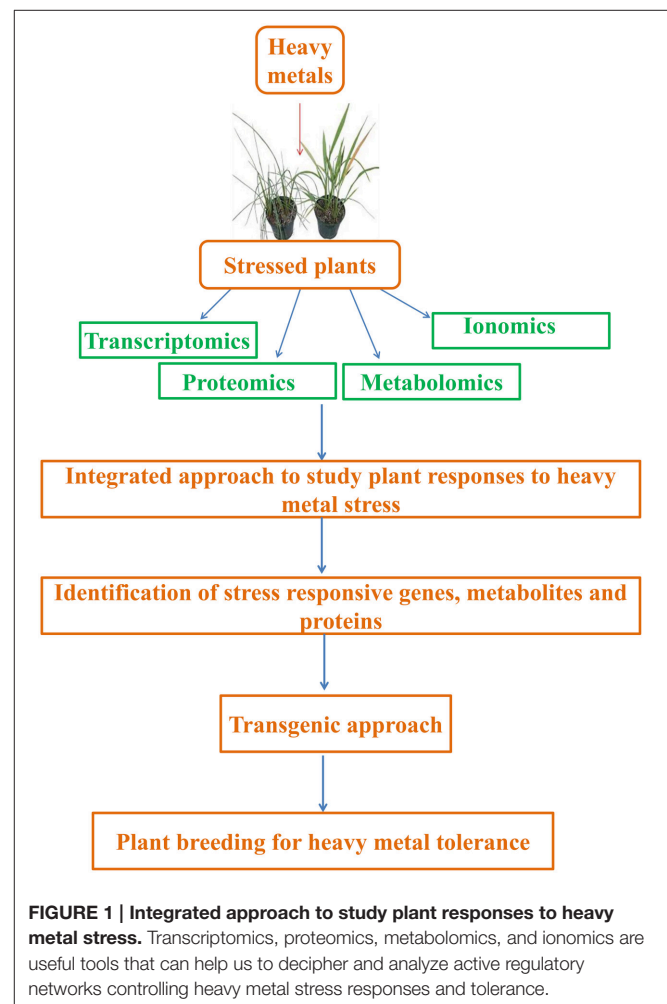
In addition to adverse impacts on plants, heavy metals pose threat to human health due to their persistence in nature. For instance, Pb is one of the most toxic heavy metals that has soil retention time of 150–5000 years and reported to maintain its concentration high for as long as 150 years (NandaKumar et al., 1995; Yang et al., 2005). Plants growing in heavy metal-contaminated sites generally accumulate higher amounts of heavy metals, and thus, contamination of food chain occurs. Contaminated food chain acts as a primary route for the entry of heavy metals into animal and human tissues, making them prone to several diseases that range from dermatitis to various types of cancers (McLaughlin et al., 1999). This problem might become even worse if sufficient measures are not taken at the right time. Therefore, research in this area is driven by the hope to decrease the entry of heavy metals in crop plants, thereby reducing the risk of contamination in animals and human beings.

Abiotic stresses are estimated to be the main cause for global crop yield reduction of ca. 70%, and thus, are considered a great constraint to crop production (Acquaah, 2007; Jewell et al., 2010). This situation has worsened due to disturbed equilibrium between crop productivity and population growth. Therefore, it is especially important to understand plants' responses to such stressors, particularly heavy metals, in order to find new methods for improving crops quantitatively and qualitatively. Currently, studies are being performed to address the above mentioned problems and have majorly focused on “omic” tools that take into consideration of ionomics (trace elements), metabolomics (metabolome), transcriptomics (transcriptome), and proteomics (proteome). The data obtained will provide insights that might help in enhancing stress tolerance and be employed in breeding and engineering programs aiming at developing plants with new and desired agronomical traits (Lee et al., 2007; Atkinson and Urwin, 2012). In this context, this review is focused on several aspects, from plant responses to heavy metals (considering sensitive as well as metallophytes) to the role of ionomics,

metabolomics, transcriptomics, and proteomics in the regulation of heavy metal tolerance (Figure 1).

PLANT RESPONSES TO HEAVY METAL STRESS

Being sessile organisms, plants cannot escape unwanted changes in the environment. Exposure to heavy metals triggers a wide range of physiological and biochemical alterations, and plants have to develop and/or adopt a series of strategies that allow them to cope with the negative consequences of heavy metal toxicity. Plants respond to external stimuli including heavy metal toxicity *via* several mechanisms. These include (i) sensing of external stress stimuli, (ii) signal transduction and transmission of a signal into the cell, and (iii) triggering appropriate measures to counterbalance the negative effects of stress stimuli by modulating the physiological, biochemical, and molecular status of the cell. At the whole plant level, it is difficult to measure sensing and changes in the signal transduction after exposing plants to heavy metal stress. However, monitoring early responses, such as oxidative stress, transcriptomic and proteomic changes, or accumulation of metabolites, might be useful to



study sensing and signal transduction changes that take place after plants' exposure to stress. For instance, Tamás et al. (2010) reported that early signs of metal toxicity in barley were similar to water deficiency signs, and thus, overexpression of genes related to dehydration stress in barley was found after exposure to Cd and Hg. Similar to this, Hernandez et al. (2012) reported oxidative stress and glutathione depletion in alfalfa roots as early signs of sensing and signal transduction after exposure to heavy metals. In another study by Zhang et al. (2002), seed germination and seedling growth of wheat was found to be inhibited due to high concentration of As. Similarly, Imran et al. (2013) reported reduction in plumule and radicle length of *Helianthus annuus* L. seedlings when exposed to As. In addition, As has also been reported to decrease the photosynthetic pigment, damage chloroplast membrane, and decrease enzyme activity by reacting with the sulfhydryl group of proteins and also reported to alter nutrient balance and protein metabolism (Li et al., 2006; Singh et al., 2009; Ahsan et al., 2010).

Heavy metals exert toxicities in plants through four proposed mechanisms. These include (i) similarities with the nutrient cations, which result into a competition for absorption at root surface; for example, As and Cd compete with P and Zn, respectively, for their absorption; (ii) direct interaction of heavy metals with sulfhydryl group (-SH) of functional proteins, which disrupts their structure and function, and thus, renders them inactive; (iii) displacement of essential cations from specific binding sites that lead to a collapse of function; and (iv) generation of reactive oxygen species (ROS), which consequently damages the macromolecules (Sharma and Dietz, 2009; DalCorso et al., 2013a).

The roots of sessile plants are the first organ that encounters heavy metals, and thus, roots have been widely studied to assess the impact of a stressor. Plants growing on heavy metal-rich soils suffer from both decreased growth and yield (Keunen et al., 2011), indicating an implication of heavy metal toxicity in hampering the overall growth performance of the stressed plants (Kikui et al., 2005; Panda et al., 2009; Buendía-González et al., 2010; Gangwar et al., 2010, 2011; Gangwar and Singh, 2011; Eleftheriou et al., 2012; Hayat et al., 2012; Silva, 2012; Anjum et al., 2014). Root growth is a combination of cell division and elongation. In this context, a decrease in mitotic activity has been reported in several plant species after exposure to heavy metals, which consequently results into a suppressed root growth (Fontes and Cox, 1998; Doncheva et al., 2005; Sundaramoorthy et al., 2010; Hossain et al., 2012a,b; Thounaojam et al., 2012). A study by Liu et al. (1992) showed that Cr(VI) has greater toxic effect on cell division than Cr(III). Furthermore, Sundaramoorthy et al. (2010) have also observed that Cr(VI) caused an extension in cell cycle that leads to the inhibition in cell division, thereby reducing root growth.

Pena et al. (2012) have reported that Cd toxicity affects the cell cycle G1/S transition and progression through S phase via decreased expression of a cyclin-dependent kinase (CDK), suggesting that ROS might be involved in such alterations. Yuan et al. (2013) have reported that excess Cu affects both elongation and meristem zones by altering auxin distribution through PINFORMED1 (PIN1) protein, and that Cu-mediated

auxin redistribution is responsible for Cu-mediated inhibition of primary root elongation. Similarly, Petö et al. (2011) have also demonstrated that excess Cu inhibits root length and alters morphology by inducing alterations in auxin levels, which antagonizes nitric oxide function. It has also been demonstrated that inhibition in root growth is accompanied by an increase in root diameter, suggesting that plant cytoskeleton might also be a target of heavy metal toxicity (Zobel et al., 2007). Therefore, these studies suggest that heavy metals might cause an inhibition in root growth that alters water balance and nutrient absorption, thereby affecting their transportation to the aboveground plant parts and thus negatively affecting shoot growth and ultimately decreasing biomass accumulation. Roots utilize several mechanisms such as synthesis and deposition of callose to reduce and/or avoid heavy metal toxicity. These mechanisms create a barrier for the entry of heavy metals and enhance plasticity of root anatomy. Apart from barricading the entry of heavy metals, roots also allow their transportation to aboveground plant parts (in the case of metallophytes or hyperaccumulator plants: plants that can grow in heavy metal-contaminated soil; Fahr et al., 2013) for sequestration into the vacuoles rendering them inactive, and thus non-reactive.

Plasma membranes serve as a highly regulated checkpoint for an entry of unwanted substances inside the cell and protect the cell from negative consequences of many stressors. It has been reported that *Arabidopsis halleri* and *Arabidopsis arenosa* were more tolerant to heavy metal stress than *Arabidopsis thaliana* due to the lowest membrane depolarization, indicating that rapid membrane voltage changes might be an excellent tool for monitoring the effects of heavy metal toxicity (Kenderešová et al., 2012). Once inside the cell, heavy metals alter metabolism that results into a reduction of growth and lower biomass accumulation (Nagajyoti et al., 2010). Heavy metal toxicity might also cause stunted stem and root length, and chlorosis in younger leaves that can extend to the older leaves after prolonged exposure (Israr et al., 2006; Guo et al., 2008a,b; Warne et al., 2008; Gangwar and Singh, 2011; Gangwar et al., 2011; Srivastava et al., 2012). At the cellular and molecular levels, heavy metal toxicity affects plants in many ways. For instance, it alters the key physiological and biochemical processes such as seed germination, pigment synthesis, photosynthesis, gas exchanges, respiration, inactivation and denaturation of enzymes, blocks functional groups of metabolically important molecules, hormonal balance, nutrient assimilation, protein synthesis, and DNA replication (Nagajyoti et al., 2010; Yadav, 2010; Keunen et al., 2011; He et al., 2012; Hossain et al., 2012a,b; Silva, 2012; Wani et al., 2012; Singh et al., 2013). Under Cd stress, severe deleterious effects on various photosynthetic indices such as photosynthetic rate (Pn) and intracellular CO₂ concentration (Ci) have been reported in tomato seedlings (Dong et al., 2005). Maleva et al. (2012) have observed that Mn, Cu, Cd, Zn, and Ni caused a significant decline in the levels of chlorophyll contents, accompanied by a decrease in the photochemical efficiency of photosystem II (PS II) in *Elodea densa*. Similarly, Li et al. (2012) have also reported that Cu, Zn, Pb, and Cd depressed chlorophyll and carotenoids levels and the quantum yield of PS II in *Thalassia hemprichii*,

indicating that heavy metals have negative consequences on photosynthesis. Apart from affecting light reactions, heavy metals decrease CO₂ assimilation by either diminishing RUBP carboxylase activity or by reacting with the thiol group of RUBISCO. For instance, Zn has been reported to inhibit RUBISCO activity in *Phaseolus vulgaris* by replacing Zn⁺² for Mg⁺², as both are bivalent cations (Monnet et al., 2001). In another study on *Erythrina variegata* by Muthuchelian et al. (2001), decreased RUBISCO activity was observed under Cd stress, and this decrease in RUBISCO activity might be due to the formation of mercaptide by Cd with thiol group of RUBISCO (Siborova, 1988). These researchers also reported decreased CO₂ fixation, which is possibly due to a decrease in ATP and reductant pool (Husaini and Rai, 1991), as Cd ions decrease the proton source for reduction reactions (Ferretti et al., 1993). Similarly, Cu, a well-known inhibitor of carboxylase and oxygenase activities of RUBISCO (Lidon and Henriques, 1991), was found to decrease RUBISCO activity in *Chenopodium rubrum* (Schafer et al., 1992) by interacting with the essential cysteine residue of the enzyme (Siborova, 1988). Such reduction in pigments, photosynthetic rate, quantum yield of PS II, gas exchange, stomatal conductance, and CO₂ assimilation might be linked to the ultrastructural changes (changes at cellular and tissue levels) induced by heavy metal stress. The effects arising due to changes in ultrastructures of membranes have been reported in several studies (Azzarello et al., 2012; Basile et al., 2012; Esposito et al., 2012; He et al., 2012; Sánchez-Pardo et al., 2012; Ali et al., 2013a,b). Moreover, heavy metals have been reported to affect another key physiological process, i.e., nitrogen metabolism, which is involved in plant function, from metabolism to allocation of resources, thereby regulating plant growth and development. Heavy metals have been found to enhance protease activity (Chaffei et al., 2003), and thus, reducing the activity of enzymes involved in nitrate (Nitrate reductase; NR and Nitrite reductase; NiR) and ammonia (Glutamine synthetase; GS, Glutamine oxoglutarate aminotransferase; GOGAT and Glutamate dehydrogenase; GDH) assimilation. The heavy metal Cd has been reported to affect nitrogen metabolism by inhibiting nitrate uptake and transportation, nitrate reductase, and GS activity (Hernández et al., 1997; Lea and Mifflin, 2004), thereby affecting primary N assimilation processes.

Heavy metal-mediated alteration in hormonal balance correlates with their toxicities in plants (Petö et al., 2011; Wilkinson et al., 2012). For instance, in *Brassica juncea*, As causes toxicity by changing the levels of the auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA) and altering the expression of about 69 microRNAs (Srivastava et al., 2013). However, exogenous supply of IAA improves the growth of *B. juncea* under As stress, suggesting an implication of the regulation of the hormone level in the management of As stress.

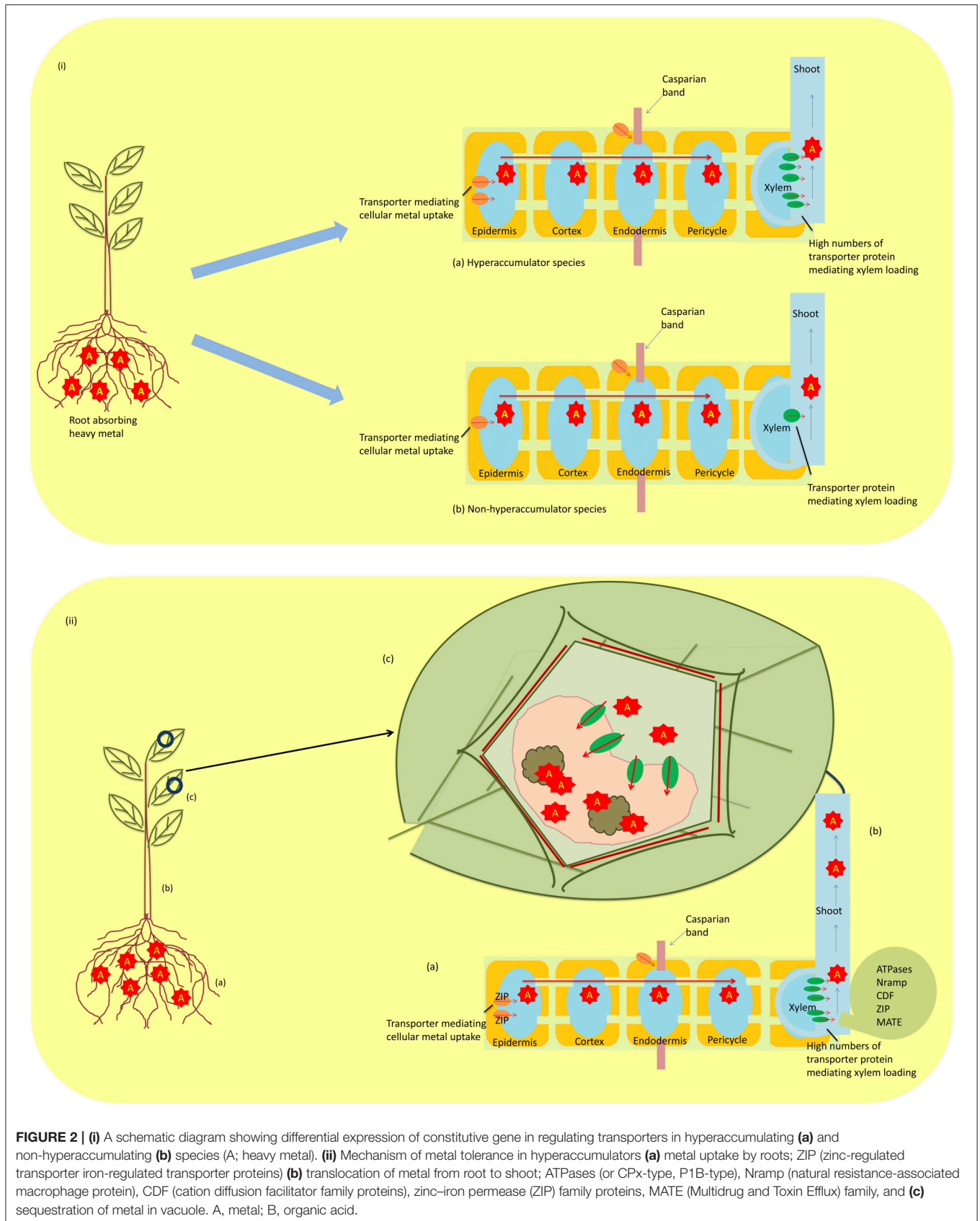
Metallophytes under Heavy Metal Stress

Metallophytes, also known as hyperaccumulators, have the ability to uptake large amounts of heavy metals from the soil, and this property makes them unique to be utilized in technologies

such as biogeochemical and biogeobotanical prospecting and phytoremediation. The absorbed heavy metals from the soil by these hyperaccumulators are not retained in the roots but are translocated to the shoots and accumulated in the aboveground organs at concentrations 100–1000-fold higher than the observed in non-hyperaccumulating species (Figures 2ia,b). However, this high concentration does not pose any toxic effect on plants (Rascio, 1997; Reeves, 2006; Prasad et al., 2010). With significant advances in our understanding of the mechanisms adopted by hyperaccumulators, there has been implication of three hallmarks that distinguish them from non-hyperaccumulators. These are (i) greater capability of heavy metal uptake, (ii) root-to-shoot translocation of heavy metal, and (iii) detoxification and sequestration of heavy metal (Figures 2iia–c). Studies on *Thlaspi caerulescens* and *A. halleri*, model plants for studying heavy metal tolerance strategies, have been done (Milner and Kochian, 2008; Singh et al., 2009; Frérot et al., 2010; Krämer, 2010). The studies have revealed that hyperaccumulation is not due to the presence of a novel gene, but it arises only from differential expression of genes that are common to hyperaccumulators and non-hyperaccumulators (Verbruggen et al., 2009). Hyperaccumulation of heavy metal includes three complex phenomena discussed below:

Heavy Metal Uptake

Hyperaccumulators have an extraordinary ability to absorb heavy metals from the soil under varying concentration of heavy metals (Ma et al., 2001; Yang et al., 2002). Although heavy metals are taken up by hyperaccumulators, their uptake is affected by several factors such as pH, water content, organic substances, etc. Moreover, heavy metal uptake requires a suitable transporting system to enter the plant (Figure 2i). Several researchers have reported that pH affects (i) proton secretion by roots that further acidify rhizosphere, thus enhancing metal dissolution, and (ii) the growth of metal-accumulating plant species (Bernal et al., 1994; Peng et al., 2005; Kuriakose and Prasad, 2008). Apart from pH, organic substances released from the roots affect growth in hyperaccumulating plants. Krishnamurti et al. (1997) have reported that organic acids released influence Cd solubility by forming Cd complexes. Therefore, pH and organic substances released from the rhizosphere of a hyperaccumulator mobilize heavy metal and enhance absorption (Krishnamurti et al., 1997; Peng et al., 2005). High uptake of heavy metal has also been associated with enhanced root proliferation (Whiting et al., 2000). Furthermore, constitutive overexpression of genes also attributes to enhanced heavy metal uptake. To pinpoint the genes involved in overexpression, several comparative studies have been performed in hyperaccumulating *Arabidopsis halleri* and *Thlaspi caerulescens* with that of congener non-hyperaccumulating species. Studies on *T. caerulescens* and *A. halleri* have revealed that increased Zn uptake is due to overexpression of genes belonging to the ZIP (Zinc-regulated transporter Iron-regulated transporter proteins) family encoding plasma membrane located transporters (Assunção et al., 2001): ZTN1 and ZTN2 in *T. caerulescens* and ZIP6 and ZIP9 in *A. halleri*. The decreased uptake of Cd under increasing Zn concentration was noticed in both genera,



and it clearly demonstrated that expression of ZIP genes is Zn regulated (Assunção et al., 2010) and Cd influx is mainly due to Zn transporters having strong preference for Zn over Cd (Weber et al., 2006). Evidence exists that As being a chemical analog of phosphate enters the plant cell *via* phosphate transporters (Meharg and Hartley-Whitaker, 2002; Kanoun-Boulé et al., 2009). Similarly, a study on the As hyperaccumulator *Pteris vittata* and non-hyperaccumulator *Pteris tremula* has shown that plasma membranes of root cells of *P. vittata* had high density of phosphate/arsenate transporters than *P. tremula* (Caille et al., 2005), possibly due to constitutive gene overexpression. In addition, a study on the Se hyperaccumulators *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae) revealed that there was a higher Se/S ratio in the shoots of these species in comparison to the non-hyperaccumulator sister species, and this observation also supported the fact that an enhanced Se uptake was through sulfate transporters (Galeas et al., 2007).

Root-to-Shoot Translocation of Heavy Metals

Unlike non-hyperaccumulator plants, hyperaccumulators do not retain the heavy metal absorbed from roots but translocate them into shoots *via* xylem and several classes of proteins are involved in this translocation. The proteins involved are heavy metal-transporting ATPases (or CPx-type, P1B-type), natural resistance-associated macrophage proteins (Nramp), cation diffusion facilitator (CDF) family proteins, zinc-iron permease (ZIP) family proteins, and MATE (Multidrug And Toxin Efflux) protein family. The CPx-type ATPases are involved in transporting toxic metals like Cu, Zn, Cd, and Pb using ATP across cell membranes (Williams et al., 2000). The P1B-type ATPases also have the similar role of transporting heavy metal, but they also regulate metal homeostasis as well as tolerance (Axelsen and Palmgren, 1998). These heavy metal ATPases (HMAs) overexpressed in roots and shoots of hyperaccumulators suggest their upregulation in hyperaccumulators in comparison to non-hyperaccumulators (Papoyan and Kochian, 2004). Nramp is another class of protein family that has been found to be involved in transporting heavy metal ions, and genes coding for these proteins are termed as *Nramp* genes. Studies on rice revealed three homologs of this protein, namely OsNramp1, OsNramp2, and OsNramp3, and these proteins expressed in different tissues of rice transport distinct but related ions (Belouchi et al., 1997). Another class of proteins (CDF) have been found to be involved in transporting Zn, Co, and Cd, and regulate effluxing of cation out of the cytoplasmic compartment, and therefore, they are termed as “cation efflux transporters” (Mäser et al., 2001). A related Zn transporter (ZNT1) from *T. caerulescens* was reported by Pence et al. (2000), which belongs to a superfamily known as ZIP gene family, and was found to be expressed at high levels in roots and shoots. Another Zn transporter ZAT1 was also reported in *Arabidopsis* that was highly expressed in root tissues (van der Zaal et al., 1999). The transporter protein MATE is also involved in heavy metal translocation; FDR3, a protein of this family, was found to be expressed in roots of *T. caerulescens* and *A. halleri*, and the gene encoding this protein FDR3 plays a role in translocation of heavy

metal (Talke et al., 2006; van de Mortel et al., 2006; Krämer et al., 2007). Therefore, the abovementioned studies provide strong evidence that multiple transporter proteins are involved in the translocation of heavy metal.

Detoxification/Sequestration of Heavy Metal

After translocating, hyperaccumulators sequester and then detoxify the heavy metal, a process that allows them to survive under metal-contaminated areas without suffering from any toxic effect (Figure 2ii). The process of detoxification/sequestration occurs in the vacuole of plants (Vögeli-Lange and Wagner, 1990; Kanoun-Boulé et al., 2009; Singh et al., 2011a) and several transporter families are involved in this process, namely ABC, CDF, HMA, and NRAMP transporters. The ABC transporters are involved in transporting heavy metal into the vacuole and mainly two subfamilies (MRP and PDR) are active. The HMT1, first vacuolar ABC transporter reported in *Schizosaccharomyces pombe*, localized in the tonoplast aids in transporting PC–Cd (phytochelatin–cadmium) complexes formed in the cytosol (Ortiz et al., 1992, 1995; Kuriakose and Prasad, 2008). Later on, a functional homolog of HMT1 has been reported in *Caenorhabditis elegans* and *Drosophila* (Vatamaniuk et al., 2005; Sooksa-Nguan et al., 2009); however, no such homolog was studied in plants. Studies in *A. thaliana* have revealed two transporters AtMRP1 and AtMRP2 in transporting PC–Cd complexes into the vacuole (Lu et al., 1997, 1998), and these transporters confer the metal tolerance. The CDF transporter family, also named “metal tolerance protein (MTP),” is also involved in transporting metal cations such as Zn²⁺, Cd²⁺, Co²⁺, Ni²⁺, or Mn²⁺ from the cytosol to the vacuole (Krämer et al., 2007; Montanini et al., 2007). They have been categorized into two of four distinct groups of which groups I and III are the most important (Blaudez et al., 2003). Comparative studies in *A. halleri* and *T. caerulescens* with those of non-hyperaccumulators have shown higher expression of MTP1 (group III), MTP8 (group I), and MTP11 (group I) (Becher et al., 2004; Talke et al., 2006; van de Mortel et al., 2006). Similarly, AhMTP1 protein also showed a constitutive higher expression in leaves of *A. halleri* under exogenous supply of Zn (Dräger et al., 2004). The MTP11 and MTP8 were found to be close homologs of ShMTP8 (formerly ShMTP1) and confirmed Mn tolerance in *A. thaliana* (Delhaize et al., 2003), thus suggesting a role of these proteins in metal tolerance. Likewise, other transporter proteins such as HMA and NRAMP are also involved in transporting the metal from the cytosol to the vacuole. However, HMAs are thought to be involved in detoxification mechanisms due to their overexpression, as reported in *A. thaliana* (Morel et al., 2009).

Apart from the role of transporter proteins, organic acids are also involved in detoxification mechanisms, as they help in entrapping the metal ion and chelating them. For instance, citrate binds with Ni in leaves of *Thlaspi goesingense*, enabling formation of metal–organic acid complex for chelation (Krämer et al., 2000). Similarly, malate binds with Zn in *A. halleri* and Cd in *T. caerulescens* (Salt et al., 1999; Sarret et al., 2002). The role of amino acids in hyperaccumulator has been found to be important due to the formation of stable complexes with bivalent cations (Callahan et al., 2006), thus helping largely in sequestering

metal cations. For example, histidine (His) is involved in Ni hyperaccumulation, and a high concentration of His has been reported in the roots of Ni hyperaccumulators (Assunção et al., 2003). The mechanism of heavy metal detoxification in hyperaccumulators also relies on the overexpression of genes related with antioxidant activity such as reduced glutathione (GSH), cysteine and *O*-acetylserine (Anjum et al., 2014). Studies have revealed that upstream signaling of salicylic acid results in increased serine acetyltransferase (SAT) activity and higher GSH level (Freeman et al., 2005a). Similarly, overexpression of *NgSAT* in *Noccaea goesingense* resulted in enhanced levels of GSH that resulted in Ni, Co, Zn, and to a small extent Cd tolerance (Freeman et al., 2004; Freeman and Salt, 2007).

HEAVY METAL AND REACTIVE OXYGEN SPECIES (ROS) PRODUCTION

Another negative consequence of heavy metal accumulation is the generation of ROS. In plants, ROS accumulation depends upon the balance between ROS production and ROS scavenging (Mittler et al., 2004), which in turn also depends on growth conditions such as temperature, light intensity, presence of heavy metal, etc. For instance, the presence of excess heavy metals results into a limitation of CO₂ fixation in the chloroplasts, which coupled with an over reduction of the photosynthetic electron transport chain serves as a major site of ROS production (Mittler et al., 2004). Over reduction of the electron transport chains in the mitochondria is also a major site of ROS generation (Davidson and Schiestl, 2001; Keunen et al., 2011). Møller et al. (2007) reported that 1–5% of O₂ consumed by the isolated mitochondria converts into ROS. ROS hydrogen peroxide (H₂O₂) is produced in the peroxisomes after glycolate is oxidized to glyoxylic acid during photorespiration (Mittler et al., 2004). Therefore, ROS such as singlet oxygen (¹O₂), superoxide anion (O₂^{•-}), H₂O₂, and hydroxyl radicals (•OH) are produced in these organelles because of spin inversion and one-two and three-electron transfer reactions to O₂, respectively, during functioning of the electron transport chains (Sharma and Dietz, 2009). The redox active heavy metals such as Cu, Cd Fe, and Zn can induce ROS formation directly by participating in Haber–Weiss and Fenton reactions or indirectly by inhibiting the functioning of enzymes in the cellular antioxidant defense network (Schützendübel and Polle, 2002; Halliwell, 2006; Keunen et al., 2011).

ROS are unstable, highly reactive, and thus, promptly react with other macromolecules to generate more free radicals because unpaired electrons tend to pair and give rise to two stable electron bonds (Foyer and Halliwell, 1976). Being extremely reactive in nature, ROS can interact with macromolecules such as DNA, pigments, proteins, lipids, and other essential cellular molecules depending on the properties like chemical reactivity, redox potential, half-life, and mobility within the cellular system, ultimately leading to a series of destructive processes collectively termed as “oxidative stress” (Mittler, 2002; Sharma and Dietz, 2009; Hossain et al., 2012a,b). Among ROS, •OH is the most reactive, highly damaging, and short-lived

(1 ns), and can oxidize macromolecules within a diffusion distance. Therefore, ROS might induce reversible as well as irreversible modifications in lipids, proteins, and nucleic acids; however, most of these ROS effects are damaging and irreversible.

Conversely, ROS also act as signaling molecules involved in the regulation of many key physiological processes such as root hair growth, stomatal movement, cell growth, and cell differentiation when finely tuned and regulated by an antioxidative defense system (Foreman et al., 2003; Kwak et al., 2006; Tsukagoshi et al., 2010). It has been shown in several studies that ROS generated by NADPH oxidases during stress are channeled by the plant to serve as a stress signal to activate acclimation and defense mechanisms, which in turn counteract oxidative stress (Mittler et al., 2004; Davletova et al., 2005; Miller et al., 2008, 2010). Therefore, the fate of ROS (i.e., whether it will act as signaling molecule or damaging one) in the cellular system depends upon the output of many complex processes that involve in antioxidative system, signaling cascades, redox alterations, etc. When the generation of ROS exceeds that of the scavenging potential of antioxidants, oxidative stress occurs (Figure 3).

Plants possess a specific mechanism to keep the routinely formed ROS at physiological limit, preventing them from exceeding toxic threshold levels, thereby playing an important role in the acclimation process against an imposed stress (Mittler et al., 2004). This mechanism is known as the plant antioxidant defense system that regulates ROS levels in the cellular system at a particular time (Figure 3). An antioxidant system comprises two types of components: enzymatic and non-enzymatic. The enzymatic antioxidants include superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione peroxidase (GPX; EC 1.11.1.9), and glutathione-S-transferase (GST; EC 2.5.1.18), whereas non-enzymatic antioxidants include water-soluble compounds such as ascorbate, glutathione, proline, and α-tocopherol (Apel and Hirt, 2004; Sharma and Dietz, 2009; Hossain et al., 2011, 2012a,b). Although ascorbate and glutathione both function as cofactors of enzymatic antioxidants, both can also directly quench ROS and regulate the gene expression associated with biotic and abiotic stress responses (Hossain et al., 2012a,b). The importance of antioxidants is based on the facts that their increased and/or decreased levels are generally related to an enhanced or declined stress tolerance of stressed plants. Since the evolution of O₂, antioxidants play an important role in sustaining ROS concentration at an appropriate level that can promote plant development and reinforce resistance to stressors by modulating the expression of a set of genes and redox signaling pathways (Neill et al., 2002). Keeping into consideration the importance of antioxidants in managing ROS levels and oxidative stress, the responses of transgenic plants and/or organisms overexpressing antioxidant and/or its biosynthetic pathway gene(s) against heavy metal stress are listed in Table 1.

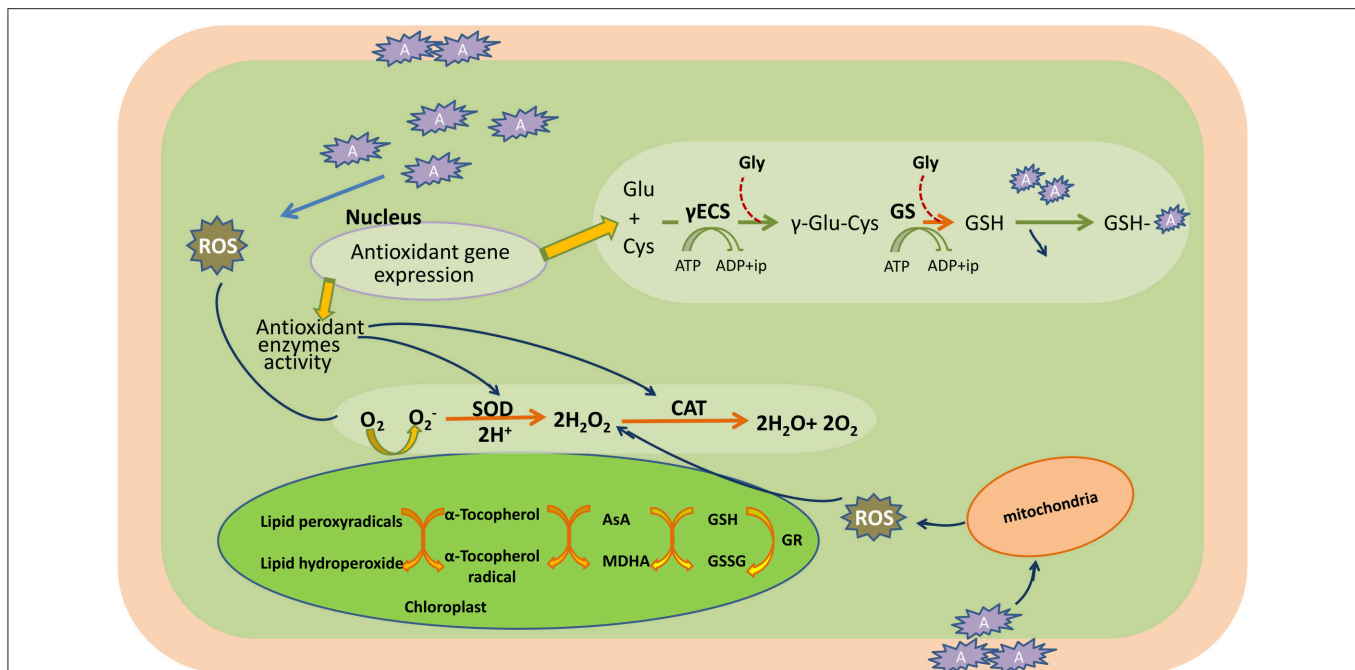


FIGURE 3 | Heavy metal induced-oxidative stress, tolerance, and detoxification mechanisms in the plant cell. AsA, ascorbic acid; CAT, catalase; Cys, cysteine; c-ECS, c-glutamylcysteinyl synthetase; Glu, glutamine; Gly, glycine; GR, glutathione reductase; GS, glutathione synthetase; GSH, glutathione (reduced); GSSG, oxidized glutathione; H_2O_2 , hydrogen peroxide; MDHA, monodehydroascorbate; O_2 , oxygen molecule; O_2^- , superoxide radicals; ROS, reactive oxygen species; SOD, superoxide dismutase; A, heavy metal.

Plant Antioxidant Defense System

The term “antioxidant” refers to a class of compounds that protect cells from damage caused by exposure to certain highly reactive species like ROS. The network and coordination of antioxidants are solely responsible for removing, neutralizing, and scavenging ROS. SOD is an enzyme involved in dismutating superoxide radicals generated by oxidation of molecular oxygen into H_2O_2 and O_2 in all the cellular compartments (Fridovich, 1989).

H_2O_2 produced by the action of SOD is quite dangerous as it can diffuse through the membrane very easily and damage other cellular components, and thus, metabolites (ascorbate and glutathione) and enzymes (monodehydroascorbate reductase; MDHAR, dehydroascorbate reductase; DHAR and glutathione reductase; GR) are implicated in scavenging of H_2O_2 (Foyer et al., 1997). Three types of SODs have been reported in plants on the basis of the metal containing (1) the chloroplastic or cytosolic Cu–Zn SOD; the cytosolic Cu–Zn SOD is referred to as Cu–Zn SOD I, whereas the chloroplastic one is referred to as Cu–Zn SOD II; (2) Mitochondrial Mn SOD, and (3) the chloroplastic Fe SOD. APX is regarded as a housekeeping protein in the cytosol and chloroplast, and is involved in scavenging of H_2O_2 . The substrate for this enzyme is ascorbate and the product, which is a radical, is reduced to dehydroascorbate by an enzyme MDHAR in the presence of an electron donor NADPH (Asada, 1992, 1996). CAT is an important oxidoreductase enzyme that catalyzes decomposition of H_2O_2 into H_2O and O_2 , and it is found in most plants and is localized in the peroxisome. CAT

is a key enzyme involved in detoxifying peroxides generated during photorespiration (Morita et al., 1994; Lin and Kao, 2000). Although APX and CAT serve the same function of detoxifying, different affinities (on the basis of Km values) of APX and CAT depict the role of APX in modulating H_2O_2 for signaling and CAT in detoxifying excess H_2O_2 during stress (Mittler, 2002).

The above mentioned enzymatic components play a relevant role in mitigating heavy metal stress. Several studies have revealed that treatment of heavy metal enhances ROS formation, and thus, substantial increase in the activities of SOD, CAT, and APX was observed (Bharwana et al., 2013; Bashri and Prasad, 2015). A study by Wang et al. (2004) revealed a considerable increase in the activities of POD, APX, and SOD under Cu stress in *B. juncea* seedlings. Similarly, Bharwana et al. (2013) showed that under Pb treatment, there was an appreciable rise in SOD, guaiacol peroxidase, APX and CAT activities, and their activities were further enhanced with the rising concentration of Pb from 50 to 100 μM . Similar to this, Singh et al. (2013) reported increased activity of SOD and CAT under As exposure (5 and 50 μM). These results suggest that cooperative action of antioxidants is required for a detoxification mechanism under heavy metal stress.

“OMICS” TOOLS

Metabolomics

Metabolomics refers to the identification and quantification of all low-molecular weight metabolites required by the organisms

TABLE 1 | Summary of transgenic plants over-expressing gene(s) of enzymatic and non-enzymatic antioxidants and their performance against heavy metal stress.

Antioxidant and/or its biosynthetic pathway gene(s)	Source	Target transgenic	Response of transgenic plants and/or organisms	References
CAT3	<i>Brassica juncea</i>	<i>Nicotiana tabacum</i>	Cd stress tolerance, better seedling growth, and longer roots	Gichner et al., 2004
CAT	<i>Brassica juncea</i>	<i>Nicotiana tabacum</i>	Zn and Cd stress tolerance, 2.0-fold higher CAT activity than wild type, lower H ₂ O ₂ level, and cell death	Guan et al., 2009
CAT1 and CAT2	<i>Brassica oleracea</i>	<i>Arabidopsis</i>	Low level of H ₂ O ₂ and enhanced stress tolerance	Chiang et al., 2013
Cu/ZnSOD and/or CAT	<i>Zea mays</i>	<i>Brassica campestris</i>	Less reduction in photosynthetic activity than wild type under SO ₂ stress	Tseng et al., 2007
MnSOD	<i>Triticum aestivum</i>	<i>Brassica napus</i>	SOD activity was 1.5–2.5-fold greater than wild type and enhanced Al tolerance	Basu et al., 2001
Cu/ZnSOD and APX	–	<i>Festuca arundinacea</i>	Increased tolerance against Cu, Cd, and As due to depressed oxidative stress	Lee et al., 2007
cpGR/cpGR	Bacterial	<i>Brassica juncea</i>	cpGR transgenic showed lower Cd accumulation and 50 times higher GR activity than wild type plants	Pilon-Smits et al., 2000
GR	<i>Brassica rapa</i>	<i>Escherichia coli</i>	Increased tolerance against H ₂ O ₂ induced by Cd, Zn, and Al due to an enhanced GR activity	Kim et al., 2009
DHAR/GR/GST	<i>Escherichia coli</i>	<i>Nicotiana tabacum</i>	Overexpression enhanced metal tolerance due to maintained redox couples such as ascorbate and glutathione	Le Martret et al., 2011
DHAR	<i>Oryza sativa</i>	<i>Escherichia coli</i>	DHAR-overexpressing <i>E. coli</i> strain was more tolerant to oxidant and metal-mediated stress conditions than the control <i>E. coli</i> strain	Shin et al., 2008
MDHAR/DHAR	<i>Arabidopsis</i>	<i>Nicotiana tabacum</i>	DHAR but not MDHAR enhanced Al tolerance by maintaining ascorbate level	Yin et al., 2010
GST	<i>Trichoderma virens</i>	<i>Nicotiana tabacum</i>	Enhanced Cd tolerance simultaneously no Cd accumulation, increased activity of SOD, CAT, GST, APX, and GPX than wild type	Dixit et al., 2011
Sulfite oxidase (SO)	<i>Zea mays</i>	<i>Nicotiana tabacum</i>	Increased tolerance against S due to enhanced CAT-mediated H ₂ O ₂ scavenging	Xia et al., 2012
TcPCS1	<i>Thlaspi caerulescens</i>	<i>Saccharomyces cerevisiae</i> and <i>Nicotiana tabacum</i>	Increased tolerance to Cd due to the decreased lipid peroxidation and enhanced activities of SOD, POD, and CAT	Liu et al., 2011
Serine acetyltransferase	<i>Thlaspi goesingense</i>	<i>Escherichia coli</i>	Imparts Ni and Co tolerance due to involvement of glutathione	Freeman et al., 2005a
gshII	<i>Escherichia coli</i>	<i>Brassica juncea</i>	Transgenic plants had higher level of glutathione, phytochelatin, and thiols and thus showed enhanced Cd tolerance	Liang Zhu et al., 1999
AsPCS1/GSH1	<i>Allium sativum</i> / <i>Saccharomyces cerevisiae</i>	<i>Arabidopsis</i>	Elevated production of phytochelatin and glutathione that imparts Cd and As tolerance	Guo et al., 2008a
APS1	<i>Arabidopsis</i>	<i>Brassica juncea</i>	Increased Se tolerance due to its rapid reduction	Pilon-Smits et al., 1999
MTH1745	<i>Methanothermobacter thermoautotrophicum</i>	<i>Oryza sativa</i>	Increased Hg tolerance, higher photosynthesis, SOD and POD activity, and lower superoxide radicals, H ₂ O ₂ , and lipid peroxidation than wild type	Chen et al., 2012b
PCs	<i>Arabidopsis</i>	<i>Nicotiana tabacum</i>	Enhanced Cd tolerance and hampers root-to-shoot Cd transport	Pomponi et al., 2006
PCS1	<i>Arabidopsis</i>	<i>Arabidopsis</i>	Enhanced As tolerance but increased Cd hypersensitivity	Li et al., 2004
PCS	<i>Anabaena</i> sp. PCC 7120	<i>Escherichia coli</i>	Enhanced tolerance against multiple stresses such as Cd and Cu by increasing phytochelatin production	Chaurasia et al., 2008
MT1	<i>Mus musculus</i>	<i>Nicotiana tabacum</i>	Enhanced Hg accumulation and tolerance	Ruiz et al., 2011
MT1	<i>Paxillus involutus</i>	<i>Hebeloma cylindrosporum</i>	Increased Cu and Cd tolerance	Bellion et al., 2007
Δ1-pyrroline-5-carboxylate synthetase	<i>Vigna aconitifolia</i>	<i>Chlamydomonas reinhardtii</i>	Transgenic grows rapidly in toxic Cd concentration (100 μM), and bind four-fold more Cd than wild-type cells. Proline likely acts as an antioxidant in Cd-stressed cells and thus increases Cd tolerance	Siripornadulsil et al., 2002
Alkyl hydroperoxide reductase	<i>Anabaena</i> sp. PCC 7120	<i>Escherichia coli</i>	Enhanced tolerance against Cu and Cd by enhancing scavenging of H ₂ O ₂ and reactive sulfur species	Mishra et al., 2009b

during developmental stages (Arbona et al., 2013), and some metabolites have been reported to be involved under heavy metal stress tolerance strategies. In the following section, we discuss the role of metabolomics under heavy metal stress.

Amino Acids and Amines

Amino acids and their derivatives have been reported to chelate metal ions, thus conferring metal tolerance to plants. Amino acids, particularly proline and histidine, have been found to chelate metal ions in cells as well as in the xylem sap (Rai, 2002; Sharma and Dietz, 2006). Proline has been reported to accumulate under heavy metal stress (Talanova et al., 2000; Yusuf et al., 2012a). A study on microalgae has demonstrated an increased level of proline under Cd stress (Siripornadulsil et al., 2002). The mechanism of action of increased levels of proline is not sequestration, but it reduces the formation of free radicals and also maintains reducing environment by enhancing the level of GSH (Siripornadulsil et al., 2002). Histidine, another important amino acid, has been found to play an important role under heavy metal stress. Krämer et al. (1996) reported increased histidine levels in the xylem sap of *Alyssum lesbiacum* (Ni hyperaccumulator) under Ni stress. Similarly, Kerkeb and Krämer (2003) reported simultaneous uptake of Ni and Histidine in *B. juncea*. Changes in the histidine content have functional significance in metal stress tolerance (Sharma and Dietz, 2006). NA (aminocarboxylate), an amino acid derivative synthesized by condensation process of three S-adenosyl-L-methionine, has been also reported to chelate metal ions. They have been found to be involved in the movement of mineral nutrients (Stephan and Scholz, 1993). The physiological role of NA has been confirmed by studying the tomato mutant lacking NA synthase, an enzyme involved in catalyzing formation of NA, which showed accumulation of Fe and Cu (Scholz et al., 1985; Herbiak et al., 1996). Apart from its chelating action, NA has been reported to be precursor of phytosiderophore mugineic acid involved in binding metals such as Zn, Cu, and Fe (Treeby et al., 1989).

Organic Acids

Organic acids such as malate, citrate, and oxalate have been reported to transport metals through xylem and are involved in sequestering ions in vacuole (Rauser, 1999). Citrate, synthesized from citrate synthase, has been shown to have high affinity for Fe, Ni, and Cd, but it is majorly involved in chelating Fe (Cataldo et al., 1988). Malate has been reported to chelate Zn and is mainly involved in chelating cytosolic ions (Mathys, 1977).

Glutathione and α -Tocopherol

Glutathione (GSH) is a water-soluble tripeptide thiol having low molecular weight (c-Glu-Cys-Gly) and plays a role in the cellular defense against the toxic actions of heavy metals (Meister and Anderson, 1983). Glutathione reductase (GR) readily converts an oxidized glutathione (GSSG) form to reduced form of GSH. GR contains a conserved disulfide bridge that breaks off under metal stress (Creissen et al., 1992; Lee et al., 1998) and plays an important role in defense by reducing GSSG, thus allowing a high GSH/GSSG ratio to be maintained. Studies on *Luffa* seedlings showed an increasing trend in GR activity with an increasing

concentration of As (Singh et al., 2015). GR-catalyzed reduction of glutathione disulfide (GSSG) to glutathione (GSH) is NADPH dependent, and to maintain the proper ratio of GSH/GSSG, GSH biosynthesis must be initiated with rapid reduction in GSSG by GR (Kumar et al., 2012).

Alpha-tocopherol is the most active form of vitamin E and is synthesized in the plastids of higher plants. It is found to be involved in scavenging ROS and lipid peroxides (Munne-Bosch, 2005) by quenching $^1\text{O}_2$ in the chloroplast and thus, prevents cell membrane from damage under stress. Several studies have reported changes in the levels of α -tocopherol under heavy metal stress (Collin et al., 2008; Yusuf et al., 2010; Kumar et al., 2012; Lushchak and Semchuk, 2012). A study by Collin et al. (2008) reported an increased concentration of α -tocopherol in *Arabidopsis* under Cd treatment, and the authors suggested that there is an upregulation of genes related to its biosynthesis (Figure 3).

Phenols

Synthesis of phenolic compounds under heavy metal stress is due to their high tendency to chelate metals, which is due to the presence of hydroxyl and carboxyl groups that bind to metal ions particularly iron and copper (Jun et al., 2003). Winkel-Shirley (2002) reported induction of phenolic compounds in maize under aluminum exposure. Similarly, Díaz et al. (2001) reported accumulation of phenols in leaves of *P. vulgaris* when exposed to Cu stress. This increase in phenolics is correlated with increased activity of enzymes involved in biosynthesis of phenols under heavy metal stress. Phenols have been reported to be directly involved in chelating Fe ions and thus, suppressing Fenton's reaction, which is the important source of ROS production. Stimulation of CHS (Chalcone synthase) and PAL (phenylalanine ammonia-lyase) activities has been reported in several plants exposed to Cu, Cd, Al, Pb, and Ni (Babu et al., 2003; Sobkowiak and Deckert, 2006; Kováčik and Klejdus, 2008; Kováčik et al., 2009; Pawlak-Sprada et al., 2011). Lavid et al. (2001) reported that tea plants rich in tannin are tolerant to Mn and prevent from Mn toxicity by directly chelating the Mn.

Ionome and Ionomics

Ionome includes the role of mineral nutrients, namely nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulfur (S) and magnesium (Mg) and trace metals namely iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo), cobalt (Co), and zinc (Zn) in alleviating heavy metal toxicity. Although all the mineral nutrients and trace elements are essential for growth and development processes of plants, concentration greater than the required level becomes toxic to the plants. Apart from posing toxicity at higher concentration, nutrients under safe limit play important role in alleviating toxicity induced by heavy metals.

Nitrogen is the most essential nutrient as it is the major constituent of proteins, nucleic acids, vitamins, and hormones. It has the potentiality of alleviating heavy metal toxicity, as it enhances the photosynthetic capacity by increasing chlorophyll synthesis, often synthesizes N-containing metabolites like proline, GSH, etc. and by enhancing the activity of antioxidant enzymes (Sharma and Dietz, 2006; Lin et al., 2011). In a study

by Pankovic et al. (2000), it has been shown that supplementing 7.5 mM (optimal level) of N to sunflower reduced the inhibitory effect of Cd on photosynthesis by enhancing Rubisco activity and by increasing protein content. In another study by Zhu et al. (2011), it has been shown that supplementing N fertilizer in the form of 16 mM $(\text{NH}_4)_2\text{SO}_4$, alleviated Cd-induced toxicity in *Sedum*. The alleviating potential not only depends on the supplemented level of N but also on the source of N. For instance, when N was applied in the form of NH_4^+ -N, it reduced the Cd concentration in leaves of rice plants that was found to be below 100 mg kg^{-1} (Jalloh et al., 2009), but when supplemented as NO_3^- -N, it increased the Cd concentration, which suggests antagonistic behavior of NH_4^+ - while synergistic of NO_3^- toward Cd. Another mineral nutrient, phosphorus (P) is the major constituent of cell membrane and nucleic acid, and majorly required for phosphorylation reaction. It has also been reported in alleviating metal-induced toxicity either by diluting the metal or by decreasing the mobility of the metal by forming metal-phosphate complex (Sarwar et al., 2010). In addition, P can also increase GSH content and prevent membrane damage, thereby conferring tolerance to plants (Wang et al., 2009).

Potassium (K) ion is required by the plant to maintain anion-cation balance in cells and plays important regulatory role in protein synthesis and enzyme activation. By improving nutritional status of K, condition of oxidative stress in plants can be minimized (Shen et al., 2000). Supplementation of K at 60 mg kg^{-1} alleviated the toxicity induced by Cd at 25 mg kg^{-1} by increasing the content of AsA and GSH. Similar to nitrogen, K source may also play an important role in alleviating toxicity. A study by Zhao et al. (2004) clearly demonstrated that application of KNO_3 , K_2SO_4 , and KCl at the rate of 55, 110, and 166 mg.K.kg^{-1} , respectively, to the soil has differential effect on Cd (concentration 15 mg Kg^{-1}) accumulation. When KCl and K_2SO_4 were applied in increasing concentration from 0 to 55 mg kg^{-1} , there was 60–90% increase in Cd accumulation in shoots, whereas similar increasing concentrations of KNO_3 increased the Cd content very marginally, suggesting its protective action against Cd stress.

Sulfur (S), another mineral nutrient, serves as an important constituent of several coenzymes, vitamins, and ferredoxin. Wangeline et al. (2004) reported that Cd toxicity could be alleviated by the upregulation of S-assimilation pathway, thus suggesting toward alleviating role of S under heavy metal toxicity. Studies on *Triticum aestivum* (Khan et al., 2007), *B. juncea* (Wangeline et al., 2004), and *Arabidopsis* (Howarth et al., 2003) have shown increased ATP-sulfurylase (ATPS) and serine acetyl transferase (SAT) activities under Cd stress, and thereby conferring tolerance to these plants. As ATPS activity helps in maintaining GSH level required for regulating Ascorbate (AsA)-GSH cycle (Khan et al., 2009), it has been reported that S at 40 mg.S.Kg^{-1} enhanced the AsA-GSH cycle, thereby reducing Cd-induced toxicity in mustard (Anjum et al., 2008). Thus, indicating toward the possibility that S supplementation to soil system might enhance the formation of S-containing defense compounds such as GSH and phytochelatin. Study by Astolfi et al. (2004) has shown that Cd ($100 \mu\text{M}$) exposure enhanced the ATPS, O-acetyl serine (OAS) thiol lyase activity, which is

related to the production of phytochelatin that play the most effective detoxifying mechanism in plants (Zhang et al., 2010). Apart from enhancing the formation of phytochelatin, S also regulates ethylene signaling and thereby helping under heavy metal stress (Masood et al., 2012). Calcium (Ca) is majorly involved in activating the enzymes and also plays an important role in regulating metabolic activities. Due to chemical similarity as well as due to same channels and intracellular Ca-binding sites (Lauer Júnior et al., 2008) of Ca^{+2} and Cd^{+2} , Cd present in external medium, replaces the Ca, and thereby affects the growth of plant. However, Ca has been shown to decrease the heavy metal-induced toxicity (Suzuki, 2005; Farzadfar et al., 2013). It has been reported that 30 mM Ca reduced the Cd content from 46.7 to $17.4 \mu\text{g}$ in *Arabidopsis* seedlings (Suzuki, 2005). Similar to this, Zhenyan et al. (2005) reported enhanced Cd (concentration 0.5 mM) tolerance in *Lactuca sativa* when supplied with 4 mM CaCl_2 , which was due to enhanced expression of phytochelatin synthase. Ca reduces heavy metal-induced toxicity by reducing their uptake, influencing physiological processes, or activating expressions of other defense compounds.

Magnesium (Mg), an important constituent for chlorophyll biosynthesis, plays an essential role under heavy metal toxicity. Abul Kashem and Kawai (2007) reported that Cd ($0.25 \mu\text{M}$) - induced toxicity in Japanese mustard spinach was alleviated by Mg at 10 mM, and Cd accumulation was reduced by 40%. Mg-induced alleviation is not due to inhibition in uptake but due to enhanced antioxidant enzymes (Chou et al., 2011). Moreover, Mg-induced alleviation has been also correlated with expression of some genes *OsIRT1*, *OsZIP1*, and *OsZIP3* of rice.

Trace elements are required in lesser amount for the biological system, which include iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo), cobalt (Co), and zinc (Zn), and their high levels could be toxic. The essentialities of these trace metals are due to their active participation in the redox reactions as well as because of their roles as enzyme cofactors (Sanita di Toppi and Gabbrielli, 1999). However, apart from their roles in biological system, they have been reported to play a crucial role in alleviating metal toxicity. Several trace elements have direct as well as indirect effects on heavy metal availability and toxicity (Sarwar et al., 2010). Direct effects include lowered solubility of heavy metals in the soil (Hart et al., 2005; Shi et al., 2005; Matusik et al., 2008), competition between heavy metals and trace elements for the same membrane transporters (Baszynski et al., 1980; Qiu et al., 2005), and heavy metal sequestration in the vacuoles (Salt and Rauser, 1995; Zaccheo et al., 2006). Indirect effects include dilution of heavy metal concentration by increasing plant biomass (dilution effect) and alleviation of heavy metal stress by increasing antioxidant defense system (Hassan et al., 2005; Suzuki, 2005; Jalloh et al., 2009). Zn, being an important group of metal transporter family, has been suggested to prevent damage caused by Cd toxicity. As reported in the case of *Thalpsi violacea*, plants supplied with 2 mgL^{-1} Cd showed 48.5 mg Kg^{-1} Zn accumulation than that of control (16.8 mgKg^{-1}), whereas when the plant was supplemented with 5 mgL^{-1} Cd, Zn accumulation decreased upto 12.8 mg Kg^{-1} , suggesting Cd/Zn antagonism (Street et al., 2010). Furthermore, Zn also enhances the activities of antioxidant enzymes and

competes with Cd to bind with the membrane protein in order to protect plant (Wu and Zhang, 2002). Other trace metal Fe, under Cd stress, showed reduced Cd uptake and translocation, thus increasing plant growth. Study by Qureshi et al. (2010) revealed that exogenous application of 40 μM Fe reduces the condition of oxidative stress by stabilizing the thylakoid complex under Cd stress. It was also reported that at Fe concentrations of 1.89 mg L^{-1} (moderate) and 16.8 mg L^{-1} (high), under low level of Cd (0.1 mM), plant height showed increment (Nada et al., 2007).

Similarly, studies have also revealed the protective roles of trace elements in ameliorating toxic effects of heavy metals by protecting photosynthetic tissue and increasing antioxidant capacity (Zornoza et al., 2010; Tkalec et al., 2014). Pal'ove-Balang et al. (2006) have shown that Mn-mediated amelioration of Cd toxicity was associated with a decreased Cd uptake. Apart from the beneficial role played by trace metals, there are some beneficial nutrients like selenium (Se), and silicon (Si) that also play a major role under heavy metal stress. Earlier, selenium (Se) was considered as toxic element but later on Schwarz and Foltz (1957) confirmed it to be an essential one. Studies on lettuce grown under Pb and Cd toxicity supplemented with Se showed a decrease in heavy metal accumulation as well as enhanced uptake of essential nutrients (He et al., 2004). Belokobylsky et al. (2004) and Feng and Wei (2012) have found that Se level up to 5 mg L^{-1} has beneficial effects on *Spirulina platensis* and *P. vittata*, respectively. Filek et al. (2008) have shown that exogenous application of Se alleviates toxic effects of Cd by enhancing the activities of antioxidant enzymes such as SOD, CAT, GPX, and APX. Several reports have revealed that appropriate dose of Se can protect plants against damage by heavy metals such as Hg, Pb, Cd, Cr, and Sb (Khattak et al., 1991; Shanker et al., 1996; Belokobylsky et al., 2004; He et al., 2004; Feng et al., 2011). Role of Si under heavy metal stress is also well established (Singh et al., 2011b; Dragišić Maksimović et al., 2012; Tripathi et al., 2012). Study by Song et al. (2009) has shown that supplementation of Si under Cd stress decreased an uptake and root to shoot translocation of Cd as well as enhanced the activities of enzymes of the defense machinery in *B. chinensis*. Similarly, study by Bharwana et al. (2013) revealed that Si application reduces Pb uptake and enhances the activities of antioxidants viz., SOD, GPX, APX, and CAT.

The measurement of elemental composition and their changes as a response to some stimuli in living organisms comes under the study of ionomics. Alteration in ionome could be direct or indirect. Direct one includes the changes in nutrient level in soil or due to impairment of ion transporter, whereas indirect changes might be due to changes in cell wall structure (Salt et al., 2008). Heavy metals due to their interaction with nutrient elements affect the uptake and distribution of these elements and may result in deficiency of minerals thus affecting the growth. Sarwar et al. (2010) suggested that Cd affects the permeability of plasma membrane and thus interferes with the nutrient uptake. However, there exists both antagonistic as well as synergistic interaction between heavy metals and micronutrient uptake, which could be due to differences in plant species and nutrient concentration. Likewise, a study by Cataldo et al. (1983) reported

antagonistic interaction between Cd and Fe, Zn, Cu, and Mn in soybean plants, whereas Nan et al. (2002) reported synergistic interaction between Cd and Zn in wheat and corn. In a study by Yang et al. (1998), decreased accumulation of Fe, Mn, and Cu in ryegrass, maize, cabbage, and white clover was observed after Cd exposure, whereas there was increased P accumulation. Similarly, Cui et al. (2008) reported decrease in Fe and Zn uptake in rice after Cd treatment in hydroponic system. A study by Safarzadeh et al. (2013) determined the effect of different doses (0, 45, and 90 mg kg soil) of Cd on uptake of Fe, Zn, Cu, and Mn in seven rice cultivar and reported decrease in Zn, Fe, Mn, and Cu uptake. Not only the uptake decreased but also there was decrease in the translocation of these minerals as Cu and Fe contents found to be greater in roots than in shoots that indicate toward impairment of ions transporters.

Similar to Cd, As has also been reported to influence nutrient uptake and their distribution in plants. Meharg and Hartley-Whitaker (2002) reported As-induced decrease in P uptake is due to chemical similarity between P and arsenate and due to which arsenate enters the plant via the phosphate transport systems. However, the concentration of As also plays an important role in P uptake. Burló et al. (1999) reported higher uptake of P at lower level of As in tomato plants. Similarly, Carbonell et al. (1998) reported increased P uptake in tomato plant when exposed to low level of As. As not only influences P uptake but also affects the uptake of other nutrients like N, P, K, Ca, etc. A study by Carbonell-Barrachina et al. (1997) observed increased concentration of N, P, K, Ca, and Mg in *P. vulgaris* L. plants when exposed to arsenite. Similarly, Carbonell-Barrachina et al. (1994) reported decreased uptake of K, Ca, and Mg (macronutrients), B, Cu, Mn, and Zn (micronutrients) in *Lycopersicon esculentum* Mill. The effect of As concentration on nutrient level of hyperaccumulator *P. vittata* L. had also been studied by Tu and Ma (2005), and the authors reported that both micro- and macronutrients were in the range of normal concentration as in non-hyperaccumulators. However, there was enhancement in P and K contents in the fronds of *P. vittata* L. at lower level of As. They reported molar ratio of P/As to be 1.0 in fronds of *P. vittata* L., which is the threshold value for normal growth of plants.

Heavy metal ions such as Cu^{+2} , Zn^{+2} , Mn^{+2} , and Fe^{+2} are essential for plant metabolism but when they are present in excess amount become highly toxic. For instance, Zn and Mn when present in excess impairs growth and compete with Fe. Excess Fe in the plant system participates in the fenton reaction, thereby creating a condition of oxidative stress (Williams and Pittman, 2010; Shanmugam et al., 2011). In order to avoid toxicity induced by mineral elements and trace elements, these are chelated by low molecular weight compounds and sequestered in vacuoles or excluded to extracellular spaces by transporters situated in the tonoplast or plasma membrane, which plays central role in maintaining metal homeostasis under safe limit. These transporters belong to (1) $\text{P}_{1\text{B}}$ -ATPase or CPX-type ATPase, (2) Cation Diffusion Facilitator (CDF) also known as Metal Tolerance Proteins (MTPs), (3) Natural Resistance-Associated Macrophage Proteins (NRAMPs), and (4) ZRT-IRT-like Protein (ZIP) transporters.

P_{1B}-ATPases (also Known as Heavy Metal ATPases: HMAs)

P_{1B}-ATPases (also known as Heavy Metal ATPases: HMAs) are found in a wide range of organisms ranging from prokaryotes to eukaryotes including yeasts, insect, mammals, and plants. Being energized by ATP hydrolysis, they translocate heavy metals (Zn, Co, Cu, Cd, and Pb) out of cytoplasm (to plasma membrane and into vacuole) and thus play important roles in their transport, compartmentalization, and detoxification (Williams et al., 2000; Grennan, 2009). HMA members (HMA2, HMA3, and HMA4) export Zn and Cd. For instance, HMA4 protein that plays a role in nutrition and transport of Zn from root to shoot also protects plants from Cd *via* its efflux (Mills et al., 2005). Hussain et al. (2004) demonstrated that though HMA2 and HMA4 are essential for Zn homeostasis in *Arabidopsis*, double mutants of HMA2 and HMA4 (*hma2* and *hma4*) exhibited increased sensitivity of plant to Cd, suggesting that they may also play a role in Cd detoxification. Similarly, loss of function in HMA2 and HMA4 has been shown to increase Cd sensitivity in *Arabidopsis* under phytochelatin deficient, *cad1-3* as well as CAD1 backgrounds (Wong and Cobbett, 2009). A recent study on 349 wild varieties of *A. thaliana* with combined approach of genome-wide association mapping, linkage mapping, and transgenic complementation revealed that HMA3 is solely responsible for variation in amount of Cd accumulated. Varieties with high Cd accumulation indicate toward reduced HMA3 function (Chao et al., 2012). Similarly, Song et al. (2014) found expression of C-type ATP-binding cassette (ABC) transporter (OsABCC) family in *Oryza sativa* and reported its involvement in detoxifying and reducing As accumulation in grains. They reported higher expression of these transporters under higher level of As.

Cation Diffusion Facilitators (CDFs) or Metal Tolerance Proteins (MTPs)

Cation Diffusion Facilitators (CDFs), also known as Metal Tolerance Proteins (MTPs) family, were first reported by Nies and Silver (1995) and found in diverse group of organisms such as bacteria, fungi, animals, and plants. Members of this family are involved in cellular heavy metals homeostasis with principal selectivity for Zn²⁺, Mn²⁺, and Fe²⁺ (Podar et al., 2012). Despite their specificities for Zn²⁺, Mn²⁺, and Fe²⁺, many CDFs may also transport other heavy metals such as Co²⁺, Ni²⁺, and Cd²⁺ (Ricachenevsky et al., 2013). CDFs transporters are involved in heavy metals efflux from the cytoplasm either to the extracellular space or into the organelles (Haney et al., 2005; Peiter et al., 2007; Ricachenevsky et al., 2013). Twelve MTP genes have been recognized so far in *A. thaliana* and 10 in *O. sativa* (Gustin et al., 2011). In *A. thaliana*, the first CDF gene was characterized as the Zinc Transporter 1 gene (ZAT1) and later renamed as METAL TOLERANCE PROTEIN 1 (*AtMTP1*) (van der Zaal et al., 1999; Delhaize et al., 2003). The *AtMTP1* gene is expressed constitutively in roots as well as in shoots, and when overexpressed in *Arabidopsis*, it enhances Zn tolerance (van der Zaal et al., 1999). However, RNA interference (RNAi)-mediated silencing (Desbrosses-Fonrouge et al., 2005) or T-DNA insertion mutation (Kobae et al., 2004) of this gene increases Zn sensitivity, indicating its important role in regulation of Zn homeostasis.

In *A. halleri*, a Zn hyperaccumulator plant, *AhMTP1* gene is believed to have a role in Zn hypertolerance (Shahzad et al., 2010). Unlike *AtMTP1* gene, *AtMTP3* is expressed predominantly in roots and reported to be engaged in maintenance of Zn homeostasis by excluding Zn under Zn oversupply (Arrivault et al., 2006). Another member of MTPs family, *AtMTP11*, has been reported to transport as well as provide Mn tolerance (Delhaize et al., 2007; Peiter et al., 2007). In rice, an ortholog of MTPs, *OsMTP1*, has been characterized and supposed to be located on chromosome 5 and highly expressed in mature leaves and stem (Lan et al., 2012; Yuan et al., 2012).

Menguer et al. (2013) demonstrated that *OsMTP1* gene localized on tonoplast, and when heterologously expressed in the yeast-mutant *zrc1* and *cot1*, complemented Zn hypersensitivity. Besides, its expression also alleviated Co sensitivity, rescued Fe hypersensitivity of the *ccc1* mutant, and restored growth of the Cd-hypersensitive mutant *ycf1*, indicating potential role of this gene in possible biotechnological applications, such as bio fortification and phytoremediation.

Natural Resistance-Associated Macrophage Proteins (NRAMPs) Transporters

Nramp is a highly conserved family of integral membrane proteins that are conserved in different species and located in the plasma membrane of root apical cells (Simões et al., 2012). They are involved in proton-coupled active transport of various heavy metals (Fe²⁺, Zn²⁺, Mn²⁺, Co²⁺, Cd²⁺, Cu²⁺, Ni²⁺, and Pb²⁺) in wide range of organisms including bacteria, fungi, animals, and plants (Hall and Williams, 2003; Cailliatte et al., 2009). However, the physiological role of NRAMP was primarily related with Fe and to a lesser extent for Mn transport (Cailliatte et al., 2009). AtNRAMP1 and 6, forms the first group, and AtNRAMP2–5 constitute the second group (Mäser et al., 2001). Of these, *AtNRAMP1*, 3, 4, and 6 have been shown to encode functional plant heavy metal transporters (Krämer et al., 2007; Cailliatte et al., 2009). Yeast mutants defective in heavy metals uptake have been utilized to investigate transport specificities of plant Nramps. Study revealed that *AtNRAMP1* can complement the *fet3fet4* yeast mutant that is defective in both low and high-affinity Fe transporters, whereas overexpression of *AtNRAMP1* in *Arabidopsis* increases plant resistance to toxic Fe concentrations (Curie et al., 2000). Furthermore, AtNRAMP3 and AtNRAMP4 have been shown to mediate the remobilization of Fe from the vacuolar store and are essential for seed germination under low Fe conditions (Thomine et al., 2003; Lanquar et al., 2005), indicating a role of Nramps in Fe homeostasis.

Since the roles of NRAMPs family proteins were previously related with Fe uptake and transport in biological systems, however, increasing numbers of studies indicated that members of this family have wide range of specificities for pumping cations inside and/or outside the cell. Cailliatte et al. (2010) demonstrated that NRAMP1, localized on plasma membrane, restores the capacity of the *iron-regulated transporter1* (a ZIP family metal transporter) mutant to take up Fe and Co, indicating that NRAMP1 has a broad selectivity for heavy metals *in-vivo*. An *AtNRAMP4* homolog, *TjNRAMP4*, was cloned from the Ni hyperaccumulator *Thlaspi japonicum*, and its expression

increased Ni²⁺ sensitivity of wild-type yeast due to elevated Ni accumulation, indicating that this protein might transport Ni into the cytoplasm (Mizuno et al., 2005). Besides regulating uptake and distribution of essential heavy metals, Nramps have also been found to be involved in the transport of non-essential heavy metals. In *Arabidopsis*, *AtNRAMP3* disruption increases Cd²⁺ resistance, whereas overexpression of this gene confers increased Cd²⁺ sensitivity, indicating that it plays a role in Cd²⁺ transport and sensitivity in plant (Thomine et al., 2000; Mäser et al., 2001).

A *O. sativa* *Nrat1* (*OsNrat1*) gene, a Nramp aluminum transporter and localized at all cells of root tips, when expressed in yeast transports only Al³⁺ but not the Mn, Fe, and Cd, indicating that this transporter gene specifically transports only Al (Xia et al., 2011). Furthermore, in knockout of *Nrat1*, Al sensitivity increased, whereas in wild type, its expression is up-regulated by Al in root that is believed to be required for a prior step of final Al detoxification through sequestration of Al into vacuoles. Study of Cailliatte et al. (2009) demonstrated that *Arabidopsis* transgenic plants overexpressing *AtNRAMP6* gene were hypersensitive to Cd, although plant Cd content remained unchanged, thereby indicating that modification in expression pattern of *AtNRAMP6* affects distribution and availability of Cd within the cell. However, Sano et al. (2012) have shown that *Nicotiana tabacum* NRAMP1 gene (*NtNRAMP1*), a plasma membrane transporter, when overexpressed in tobacco BY-2 cells increases resistance of the cells to both Fe and Cd, and suggested that *NtNRAMP1* moderates Fe-uptake and prevents toxicity resulting from excess Fe or Cd application. Tiwari et al. (2014) also demonstrated that *OsNRAMP1*, localized on plasma membrane of endodermis and pericycle cell, when expressed in *Arabidopsis* provides tolerance against As and Cd with their enhanced accumulation in root and shoot, and proposed that modification in this gene may be helpful in reducing the risk of food chain contamination by these toxic heavy metals. These studies clearly indicate that NRAMP genes are able to encode multi-specific heavy metals transporters. In recent years, a new Nramp5 belonging to rice (*OsNramp5*) has been characterized, which is responsible for accumulation of Mn in rice and has been reported to encode proteins localized on plasma membrane, thus suggesting that Nramp5 is a major transporter responsible for transport of Mn and Cd (Sasaki et al., 2012). To gain deep insights into the roles of NRAMP genes transporter in heavy metals uptake and homeostasis in plants, a more systematic characterization of the different members of the NRAMP family is further required.

ZRT, IRT-Like Proteins (ZIP) Transporters

Members of the ZIP family named on the first member identified ZRT IRT-like Protein in *Arabidopsis*, expressed in roots of iron deficient plants and found to be capable of transporting various heavy metals such as Fe, Zn, Mn, Cd and Ni within cellular systems (Mäser et al., 2001). The key feature of the ZIP family is that these proteins can transport heavy metals from the extracellular space or from organelles lumen into the cytoplasm. In *Arabidopsis*, 15 genes viz. *ZIP1-12*, *IRT1*, *IRT2*, and *IRT3* of the ZIP family are reported (Milner et al., 2013). Among these

members, *AtIRT1*, *AtIRT2*, and *AtIRT3* transporters are well characterized, with *AtIRT1* being the most studied (Eide et al., 1996; Lin et al., 2009; Vert et al., 2009) for their involvement in regulation of Zn and Fe homeostasis in plants. Rest of the ZIP family members has been studied for their membrane localization and heavy metals they transport into or outside of a specific organelle (Milner et al., 2013). In a model legume *Medicago truncatula*, six ZIP family transporters *MtZIP1*, *MtZIP3*, *MtZIP4*, *MtZIP5*, *MtZIP6*, and *MtZIP7* have been tested for their ability to complement yeast heavy metals uptake mutants, and each family member was able to rescue the growth of Zn, Mn, and Fe uptake mutants, indicating their function in heavy metals transport (López-Millán et al., 2004).

Apart from the abovementioned transporters, recently another transporter arsenate reductase (ACR) has been characterized in yeast *Saccharomyces cerevisiae*, a model system for As resistance. It was reported that a 4.2-kb region conferred arsenite (AsIII) resistance in *S. cerevisiae*; they found three ACR genes, namely *ACR1*, *ACR2*, and *ACR3* (Bobrowicz et al., 1997). These authors also reported that *ACR1* regulates *ACR2* and *ACR3* by transcriptional factor and any loss in *ACR1* function yeast conferred arsenite and arsenate hypersensitivities (Bobrowicz et al., 1997; Ghosh et al., 1999). Later on, Landrieu et al. (2004a,b) reported that *ACR2* represents arsenate reductase that showed homology to yeast *ASCR2* (*ScACR2*). Similarly, Ellis et al. (2006) reported other transporter *PvACR2* from *P. vittata* and *OsACR2.1* and *OsACR2.3* from *O. sativa* (Duan et al., 2007). Earlier, *ACR2* (called as *CDC25*) was thought to be involved in As metabolism in *A. thaliana*. Recent studies on *A. thaliana* have revealed the involvement of new arsenate reductase (ACR), namely *HAC1* (Chao et al., 2014) or *ATQ1* (Sánchez-Bermejo et al., 2014). Chao et al. (2014) reported that loss of function of *HAC1* in *A. thaliana* resulted in decreased As accumulation in roots, and thus, there was diminished As efflux to external medium. Another transporter, *OsABCC1* localized in phloem cells of *O. sativa*, has been reported to be involved in sequestration of As to vacuole (Song et al., 2014). However, in anaerobic paddy fields, As (mainly Arsenite) uptake is regulated by transporters of Si, namely *Lsi1* (low silicon 1; influx transporter) and *Lsi2* (low silicon 2; efflux transporters) (Ma et al., 2008). Apart from these transporters, there are some other transporters as well that transfer arsenate and arsenite. Likewise, a transporter from *P. vittata*, *PvACR3* has been reported to compartmentalize As into the vacuoles and loss in its function results in As hypersensitivity (Indriolo et al., 2010).

Transcriptomics

Investigations on the basic mechanisms of heavy metal tolerance and adaptation are the area of great scientific interest and an intensive research. Various stressors induce an expression of a set of genes in plants (Nakashima et al., 2009).

At molecular level, the regulation of gene expression is very important for the biological processes, which determines the fate of plant development as well as tolerance to heavy metal stress. Stressors trigger large number of genes and several proteins in order to link the signaling pathways that confer stress tolerance (Umezawa et al., 2006; Valliyodan and Nguyen,

2006; Manavalan et al., 2009; Tran et al., 2010). These genes are classified into two groups: the regulatory genes and the functional genes (Tran et al., 2010). The genes of regulatory group encode various transcription factors (TFs), which can regulate various stress-responsive genes cooperatively and/or separately and thus, constitute a gene network. However, the genes of functional group encode metabolic compounds such as amines, alcohols, and sugars, which play a crucial role in heavy metal stress tolerance. The TFs, which are reported to be master regulators, control an expression of gene clusters and usually members of multigene families. Studies reveal that a single TF can control the expression of many target genes *via* specific binding of the TF to the cis-acting element in the promoters of its target genes (Wray et al., 2003; Nakashima et al., 2009). Most of the TFs contain a DNA-binding domain that interacts with cis-regulatory elements in the promoters of its target genes and *via* a protein-protein interaction domain that helps in oligomerization of TFs with other regulators (Wray et al., 2003; Shiu et al., 2005). This type of transcriptional regulatory system is referred as “regulon” (Nakashima et al., 2009). Various TFs families such as AREB/ABF, MYB, AP2/EREBP, WRKY, bHLH, bZIP, MYC, HSE, DREB1/CBF, NAC, HB, ARID, EMF1, CCAAT-HAP2, CCAAT-DR1, CCAAT-HAP3, CCAAT-HAP5, C2H2, C3H, C2C2-Dof, C2C2-YABBY, C2C2-CO-like, C2C2-Gata, E2F-DP, ABI3VP1, ARE, AtSR, CPP, E2F-DP, SBP, MADS, TUB, etc. are known to influence stress response in plants (Singh et al., 2002; Shiu et al., 2005; Shameer et al., 2009). LeDuc et al. (2006), in a transcriptome analysis on plants, reported that plants treated with heavy metals could induce transcription factors that regulate corresponding transcriptional processes.

Liang et al. (2013) reported first FER regulatory gene involved in Fe uptake in tomato, and the functional analog of FER is FER-like Deficiency Induced Transcription Factor (FIT) that has been conferred to play an important role under Fe deficiency in *Arabidopsis* (Yuan et al., 2005). In addition to this, there are several other subgroups of bHLH family *viz.*, AtbHLH38, AtbHLH39, AtbHLH100, and AtbHLH101 that have been shown to be upregulated under Fe deficiency in roots and leaves of *Arabidopsis* (Wang et al., 2007; Yuan et al., 2008). Later, several researchers proposed that AtbHLH38 or AtbHLH39 interacts with FIT and forms heterodimers and directly activates transcription factors for ferric chelate reductase and ferrous transporters, which are the two major genes regulating Fe uptake under deficient condition (Varotto et al., 2002; Vert et al., 2002; Yuan et al., 2008). In *Arabidopsis*, IRT1 has been reported to be the most essential ferrous transporter. Beside transporting Fe, it can also transport Zn, Mn, Co, Ni, and Cd, and thus, these metals get accumulated under Fe deficiency (Vert et al., 2002; Schaaf et al., 2006). A recent study by Wu et al. (2012) in *Arabidopsis* revealed that expression of FIT with AtbHLH38 or AtbHLH39 further activates expression of several other transporters *viz.*, HMA3, (MTP3), Iron Regulated Transporter2 (IRT2) that play regulatory role in maintaining Fe content under Cd exposure.

Transcriptome analysis in *A. thaliana* and *B. juncea* exposed to Cd stress has revealed the induction of basic region leucine zipper (bZIP) and zinc finger transcription factors (Ramos et al., 2007). ERF1 and ERF5, two transcription factors belonging

to AP2/ERF superfamily (characterized by AP2/ERF domain; Nakano et al., 2006), have been reported to be induced when *A. thaliana* was exposed to Cd (Herbette et al., 2006). Similar induction of TFs has been reported in *A. halleri* under Cd stress (Weber et al., 2006). Differential expression of ERF factors under Cd indicates toward their responses to various levels of Cd stress. A study by Nakashima and Yamaguchi-Shinozaki (2006) reported down-regulation of dehydration-responsive element-binding protein (DREB) transcription factor (involved in cold and osmotic stress responsive genes) in roots of *A. thaliana* under heavy metal treatment and suggested it could be acclimation response and DREB might have helped in normalizing osmotic potential, so that flow of heavy metal-contaminated water could be reduced, thus helping plants to avoid toxic effects of heavy metal. Therefore, acquiring a deep knowledge of the interrelated mechanisms, which regulate the expression of these genes, is a crucial issue in plant biology and necessary to generate genetically improved crop plants for extreme environments like heavy metal stress (Umezawa et al., 2006; Valliyodan and Nguyen, 2006; Nakashima et al., 2009). Summary of an involvement of TFs in conferring heavy metal and other abiotic stresses tolerance is given in **Table 2**.

Mitogen-Activated Protein Kinase MAPK Cascade

MAPK cascade are activated in response by plants when exposed to heavy metal stresses. This cascade has its significance in activation of signal transduction pathway used in hormone synthesis (Jonak et al., 2002). This cascade involves three kinases *viz.*, MAPK kinase kinase (MAPKKK), the MAPK kinase (MAPKK), and the MAPK, which are activated by phosphorylation process. The finally formed phosphorylated MAPK cascade phosphorylates substrates in cells including transcription factors in nucleus. Therefore, MAPK regulates the transduction of information downstream. Jonak et al. (2004) have shown four isoforms of MAPK that are activated under Cu or Cd stress in *Medicago sativa*. All these pathways finally lead to regulation of transcription factors that in turn activate genes for activation of metal transporters, biosynthesis of chelating compounds, and other defending compounds.

Proteomics

Proteomics is a well-established technique in the post-genomic era (Liu et al., 2013). Proteomics deals with the study of large-scale expression of proteins in an organism encoded by its genome (Anderson and Anderson, 1998). Proteomics not only serves as a powerful tool for describing complete protein changes in any organisms but it can also be used to compare variation in protein profiles at organ, tissue, cell and organelle levels under various stress conditions including heavy metal stress (Ahsan et al., 2009). Although genomic analysis has enhanced our understanding regarding plants' response to heavy metal toxicity, transcriptomic changes in the genome are not always reflected at protein level (Gygi et al., 1999; Hossain and Komatsu, 2013). For instance, putative Zn and Mg transporter protein MHX was more abundant in *Arabidopsis* even though its corresponding transcript level was not different (Elbaz et al., 2006). This suggests that transcription of any gene is not a guaranty

TABLE 2 | Summary of transcription factors (TFs) whose overexpression in plants confers heavy metal stress tolerance.

Name of TF	Family of TF	Studied plant	Plant response	References
WRKY6	WRKY	<i>Arabidopsis thaliana</i>	Plant exhibits dual WRKY-dependent signaling mechanism that modulates As ^v uptake and transposon expression and provides a coordinated strategy for As ^v tolerance and transposon gene silencing	Castrillo et al., 2013
WRKY22, WRKY25, and WRKY29	WRKY	<i>Arabidopsis thaliana</i>	TFs induced by Cu and Cd involve in stress response <i>via</i> MAPK and oxylipin signaling	Opdenakker et al., 2012
WRKY45	WRKY	<i>Arabidopsis</i> spp.	Involved in Zn and Fe stress response and homeostasis	van de Mortel et al., 2006
ART1	C2H2	<i>Oryza sativa</i>	Constitutively expressed in roots and regulates genes related to Al tolerance and thus increases Al tolerance	Yamaji et al., 2009
ASR5	–	<i>Oryza sativa</i>	Overexpression enhanced Al tolerance. Authors suggested that this protein is localized in nucleus and acts as a transcription factor to regulate the expression of different genes that collectively protect rice cells from Al-induced stress	Arenhart et al., 2013
ZIP39	bZIP	<i>Oryza sativa</i>	Overexpression regulates endoplasmic reticulum (ER) stress-responsive genes and thus regulates ER stress response	Takahashi et al., 2012
HsfA4a	HSF	<i>Oryza sativa</i>	Expression of this TF increases Cd tolerance by inducing up-regulation of MT gene expression	Shim et al., 2009
Hsfs	HSF	<i>Arabidopsis</i> spp.	TF up-regulated by Cd stress and plays a role in Cd stress tolerance	Herbette et al., 2006; Weber et al., 2006
CaPF1	AP2/EREBP	<i>Pinus Virginiana Mill.</i>	Overexpression of TF enhanced production on of APX, GR, and SOD which confer tolerance against oxidative stress induced by Cd, Cu, and Zn	Tang et al., 2005
OXS2	C2-H2 ZF	<i>Arabidopsis thaliana</i>	Enhanced Cd tolerance	Blanvillain et al., 2011
ACEI	–	<i>Saccharomyces cerevisiae</i>	TF binds metal-regulatory elements (MREs) upstream promoter of target gene for induction of MT which plays a role in Cu homeostasis	Fürst et al., 1988
ACE1	–	<i>Arabidopsis thaliana</i>	Overexpression protects plant against Cu stress by inducing activity of SOD and POD, and suppressing inhibition in chlorophyll biosynthesis	Xu et al., 2009
ACE1	–	<i>Saccharomyces cerevisiae</i>	TF binds MREs upstream promoter of target gene for induction of MT which plays a role in Cu homeostasis	Dixon et al., 1996
ACP1	AP2/EREBP	<i>Physcomitrella patens</i>	Expression of this gene enhances metal responding genes which confer tolerance against Cd and Cu	Cho et al., 2007
OSISAP1	Zinc-finger protein	<i>Nicotiana tabacum</i>	Overexpression enhances tolerance against various abiotic stresses including heavy metal like Cu, Cd, Mn, Ca, Zn, and Li	Mukhopadhyay et al., 2004
STOP1	C2-H2 ZF	<i>Arabidopsis thaliana</i>	Expression protects plants from Al toxicity by proton pump regulation	Iuchi et al., 2007
bHLH38 and bHLH39	bHLH	<i>Arabidopsis thaliana</i>	Overexpression enhanced Cd tolerance by increased Cd sequestration in roots and also improved Fe homeostasis in shoots	Wu et al., 2012
bHLH100	bHLH	<i>Arabidopsis</i> spp.	Involved in Zn and Fe stress response and homeostasis	van de Mortel et al., 2006
–	MYB, bHLH, bZIP	<i>Sedum alfredii</i>	These TFs families were up-regulated by Cd and involved in Cd hyperaccumulation and tolerance	Gao et al., 2013
PYE	bHLH	<i>Arabidopsis thaliana</i>	Expression is implicated in regulating plant growth response against Fe deficiency	Long et al., 2010

that gene would be translated into a functional protein. This may occur due to the potential impact of post-transcriptional and translational modifications, protein folding, stability and localization, protein–protein interactions, which are considered important determinants of a protein function (Dalcorso et al., 2013b). Therefore, depth analyses of proteomics offer a new platform for identifying target proteins, which take part in heavy metal detoxification, and in studying complex biological processes and interactions among the possible pathways that involve a network of proteins (Ahsan et al., 2009).

Furthermore, it is known that proteins directly take part in plant stress responses, and plant adaptations to heavy metal stress

are always accompanied with deep proteomic changes. Therefore, technique of proteomics can be exploited for deciphering the possible relationships between proteins abundance and plant stress adaptation. It can contribute to better understanding of physiological mechanisms under heavy metal stress such as perception of stress and further signaling cascade that leads to changes in the expression of huge numbers of genes at transcriptional level and in metabolite profile, which could be used for an acquisition of an enhanced plant tolerance under heavy metal toxicity (Kosová et al., 2011). Studies have revealed that an abundance of defense proteins was increased for scavenging of ROS, and molecular chaperones play a role

in re-establishing the conformation of a functional protein that contributes in helping heavy metal stressed plants to maintain the redox homeostasis (Zhao et al., 2011; Sharmin et al., 2012; Wang et al., 2012). Under heavy metal stress, modulations of various metabolic pathways occur such as photosynthesis, respiration, nitrogen metabolism, sulfur metabolism, etc. particularly in photosynthesis and mitochondrial respiration that help stressed plants to produce more reducing power such as NADPH, NADH, and FADH₂ and assimilatory power ATP to compensate high energy demand of heavy metal-challenged plants (Hossain and Komatsu, 2013). For example, an increased abundance of RUBISCO large sub unit (LSU)-binding proteins, oxygen-evolving enhancer protein 1 and 2, NAD(P)H-dependent oxidoreductase, and photosystem I and II-related proteins is an adaptive feature to withstand heavy metal stress (Semane et al., 2010). The cellular mechanism of stress sensing and further transduction of signals into the cell appear to be the first reactions in the plant cell against heavy metal. Furthermore, an intracellular communication of stress signals plays a fundamental role in signal transduction pathways under stress, which ultimately activate defense-related genes and thus signaling cascades (Hossain et al., 2012c). Therefore, to decipher an underlying molecular mechanism of alterations in the protein signature of a plant cell in order to withstand stress, a deep study on the cellular as well as organelle proteomics would be of great importance in developing heavy metal-tolerant crops. Alterations in protein profile under heavy metal stress, which could be utilized for developing heavy metal-tolerant plants, are given in **Table 3**.

Apart from inducing synthesis of amino acids (proline and histidine), amines, organic acids, and plant antioxidant α -tocopherol and glutathione, some nitrogen containing metabolites like some peptides (phytochelatins, metallothioneins, and ferritins) have been reported to play an important role under heavy metal stress. In the following section, we will discuss about the roles of peptides in heavy metal tolerance.

PEPTIDES

Phytochelatins (PCs) have been the best-characterized chelators in plant systems. PCs belong to a family of metal-binding protein having general structure (c-Glu-Cys)*n*Gly (*n* = 2–11) (Cobbett and Goldsbrough, 2002). These are synthesized by the transpeptidation of the γ -Glu-Cys moiety of GSH, and the transpeptidation reactions are carried out by enzyme named phytochelatin synthase (PCS). It has been reported that PCS were activated under heavy metal exposure (Rauser, 1995; Cobbett, 2000), and similar PC synthase activity has been observed in several other crops (Klapheck et al., 1995; Chen et al., 1997; Mishra et al., 2009a). Loeffler et al. (1989) confirmed that metals induce PC synthesis, in *in-vivo* as well as in *in-vitro* cultures, and were continuously synthesized until activated metal ions chelated. Haag-Kerwer et al. (1999) reported induction of PCs in *B. juncea* after the accumulation of Cd in the cells, and thus plays important role in detoxifying heavy metals (Hirata et al., 2005). Due to the presence of thiol group, they have the capability of chelating metals and forming complexes (Cobbett, 2000),

which are then sequestered in the vacuole. From the preceding discussion, it is clear that chelation by PCs is not a simple process but involves a complex molecular mechanism, where firstly, the PCS gets activated by metal ion and biosynthesis of PCs takes place; secondly, formation of complexes and sequestration in vacuole; thirdly, more complexation with the sulfides or organic acids in the vacuole, and finally detoxified. Besides detoxifying heavy metals, PCs also play a major role in metal ion homeostasis and thus regulating the metal ion availability in plant cells (Guo et al., 2008b).

Like PCs, metallothioneins (MTs) are synthesized and activated under heavy metal toxicity. They belong to a family of low molecular weight protein having cysteine-rich metal binding peptide. Due to the presence of mercaptides, they have the ability of binding metal ions. Metal-binding activities of MTs have been expressed in *Escherichia coli* in presence of Cd, Zn, and Cu (Tommeij et al., 1991). In addition, Zhou and Goldsbrough (1994) reported restoration in Cu tolerance ability of MT-deficient yeast strains, when provided with the *Arabidopsis* MTs. Similar to this, Zhigang et al. (2006) conferred increased tolerance of *A. thaliana* to Cd and Cu, when ectopically substituted with *B. juncea* MT. Moreover, comparative study of mutant and wild-type *A. thaliana* has clearly revealed that MT mutant was hypersensitive to Cd and accumulated much lower amount of Cd than wild type, thus conferring role of MTs in both heavy metal tolerance as well as accumulation (Zimeri et al., 2005). In terms of transcript amount, expression of MT genes varies during different developmental stages of plant as well as under varying environmental condition (Rauser, 1999). Besides, chelating metal ions MTs can also catalyze antioxidant protection mechanism as well as plasma membrane repair (Hamer, 1986).

Ferritins are other multimeric proteins that could accumulate iron atom (Harrison and Arosio, 1996). However, animal ferritins have been reported to store other metals like Cu, Zn, Cd, etc., whereas plants ferritin could store only Fe. These are synthesized in plants when there is excess Fe in the surroundings and thus represents first-line defense against Fe-induced oxidative stress (Ravet et al., 2009). These are not only involved in storing or releasing Fe but also involved in scavenging free reactive iron (Ravet et al., 2009).

PLANT GROWTH HORMONES

In spite of five classical plant hormones, i.e., gibberellins (GAs), cytokinins (CKs), auxins, abscisic acid (ABA), and ethylene, jasmonate (JA), brassinosteroids (BR), and salicylic acid (SA) are also well known for regulating many physiological processes and heavy metal stress tolerance (Freeman et al., 2005b; Gangwar et al., 2010; Gangwar and Singh, 2011; Peleg and Blumwald, 2011; Choudhary et al., 2012a,b; Vriet et al., 2012). Furthermore, it is also expected that some more growth hormones are yet to be discovered in future. In laboratory as well as field studies, two strategies have been used for plant hormone-mediated increase in stress tolerance as well as crop yield. These strategies include exogenous application of plant hormones and genetic manipulation of their endogenous contents. Both approaches have given promising results for increasing crop yield and

TABLE 3 | Summary of heavy metal-induced changes in protein expressions and their potential uses in developing heavy metal tolerant plants.

Metal	Technique(s) used	Plant species	Alterations in protein(s) expression profile	Plant response	References
Cd	2DE, MALDI-TOF-MS, LC-ESI-QTOF-MS	<i>Arabidopsis thaliana</i>	~1100 Spots reported, 41 spots showed significant changes including phytochelatin, glutathione-S-transferases, ATP sulfurylase, glycine hydroxymethyl transferase, trehalose-6-phosphate phosphatase	Alterations in these proteins in plant roots help to withstand Cd stress via modulating S assimilation	Roth et al., 2006
	2DE, MALDI-TOF/TOF-MS	<i>Phytolacca americana</i>	32 Proteins are differentially expressed, 14 enhanced, and 11 reduced under Cd treatment. Major changes were in photosynthetic pathway, S and GSH metabolism, transcription, translation and chaperones, 2 cys-peroxidase and oxido-reductases proteins	These alterations play a key role in enhancing Cd hypertolerance in plant	Zhao et al., 2011
	2DE, MALDI-TOF/TOF-MS	<i>Arabis paniculata</i>	18 Proteins differentially expressed upon Cd treatment which were mainly related with photosynthetic pathway and antioxidant defense system such as ribulose-5-phosphate 3-epimerase (RPE), RuBisCO activase, Protein thylakoid formation 1 (THF1), Mn-SOD, APX, GST	Plant adopted alterations mainly in antioxidative/xenobiotic defense and hence exhibited increased Cd tolerance	Zheng et al., 2011
	2-D DIGE, MALDI-TOF/TOF	<i>Populus sp.</i>	A number of changes in the expression of proteins with various functions were identified; in particular a decreased abundance of oxidative stress regulating proteins, whereas pathogenesis-related proteins showed a drastic increase in abundance. Furthermore, a large number of proteins involved in carbon metabolism showed a decrease in abundance, while proteins involved in remobilizing carbon from other energy sources were up-regulated	Due to deep proteomic changes, plant experienced lesser negative impact of Cd on physiological parameters and hence plant showed Cd tolerance	Kieffer et al., 2008
	2DE, MALDI-TOF-MS	<i>Oryza sativa</i>	36 Proteins either up-and/or down-regulated by Cd treatment. Most of the proteins were related to oxidative stress and antioxidative system	Antioxidative system related proteins play a role in Cd tolerance	Lee et al., 2010
	2DE	<i>Thlaspi caerulescens</i>	48 Tentatively spots identified which represent core metabolic functions, e.g., photosynthesis, nitrogen assimilation, carbohydrate metabolism as well as putative signaling and regulatory functions	The possible roles of some of the proteins were related with metal accumulation and tolerance	Tuomainen et al., 2006
As	2DE, MALDI-TOF-MS	<i>Oryza sativa</i>	23 Proteins up-regulated related with defense proteins like S-adenosylmethionine synthetase (SAMS), GSTs, cysteine synthase (CS), GST-tau, and tyrosine-specific protein phosphatase proteins (TSPP), and an omega domain containing GST	SAMS, CS, GSTs, and GR presumably work synchronously and GSH plays a key role in protecting rice roots against As stress	Ahsan et al., 2008
	IPG, 2-DE, MALDI-TOFMS, ESI-MS/MS	<i>Oryza sativa</i>	12 Proteins differentially expressed related with energy production and metabolism. RuBisCO large subunit and chloroplast 29 kDa ribonucleoproteins were decreased	Reduction in photosynthetic machinery proteins was related with As toxicity	Ahsan et al., 2010
	2DE, MALDI-TOF-MS, LC-MS/MS	<i>Chlamydomonas reinhardtii</i>	15 Proteins overexpressed like oxygen-evolving enhancer protein, rubisco small subunit 1, chaperones, Fe-SOD, Mn-SOD, and heat shock like proteins	Organism exhibited time course acclimation against As stress by modulating protein signatures	Walliwagedara et al., 2012
Hg	2-DE, MALDI-TOF-TOF-MS	<i>Suaeda salsa</i>	43 Proteins with significant changes reported. They include proteins related to metabolic processes, photosynthesis, stress response, protein fate, energy metabolism, signaling pathways, and immunosuppression	Alterations in these proteins was linked with Hg toxicity	Liu et al., 2013
	2DE, ESI-MS/MS	<i>Oryza sativa</i>	25 Proteins differentially expressed by Hg involved in cellular functions including the redox and hormone homeostasis, chaperone activity, metabolism, and transcription regulation	Plant exhibited Hg toxicity due to alterations in these proteins	Chen et al., 2012a
	2DE	<i>Oryza sativa</i>	33 Proteins were highly reproducible. Most of the proteins showed homology to RuBisCO protein, and some to defense/stress-related proteins, like the pathogenesis related class 5 protein (OsPR5), the probenazole-inducible protein (referred to as the OsPR10), SOD, and the oxygen evolving protein	Severe fragmentation of ribulose-1,5-bisphosphate carboxylase/oxygenase and induction of stress-related proteins causes Hg toxicity	Hajduch et al., 2001

(Continued)

TABLE 3 | Continued

Metal	Technique(s) used	Plant species	Alterations in protein(s) expression profile	Plant response	References
Cr	2DE, MALDI-TOF, MALDI-TOF-TOF	<i>Miscanthus sinensis</i>	36 Proteins differentially expressed. The identified proteins included: heavy metal-inducible proteins such as carbohydrate and nitrogen metabolism, molecular chaperone proteins, and novel proteins such as inositol monophosphatase, nitrate reductase, adenine phosphoribosyl transferase, formate dehydrogenase, and a putative dihydrolipoamide dehydrogenase	<i>Miscanthus</i> plant experienced Cr toxicity due to altered vacuole Cr sequestration, nitrogen metabolism, and lipid peroxidation in roots	Sharmin et al., 2012
	2DE, MALDI-TOF-MS-MS	<i>Zea mays</i>	58 Proteins identified related with photosynthesis and chloroplast organization, the redox homeostasis and defense response, RNA processing, protein synthesis and folding, DNA damage response, mitochondrial oxidative phosphorylation, and miscellaneous with unknown function	Plant exhibited Cr toxicity due to the deep changes in proteomics	Wang et al., 2013
Cu	2DE	<i>Oryza sativa</i>	Changes RuBisCO, defense/stress-related proteins, like the pathogenesis related class 5 protein (OsPR5), the probenazole-inducible protein (referred to as the OsPR10), and SOD	Alterations in these protein resulting in Cu stress	Hajduch et al., 2001
	SDS-PAGE and 2DE	<i>Oryza sativa</i>	13 Proteins identified such as metallothionein-like protein, membrane-associated protein-like protein, putative wall-associated protein kinase, pathogenesis-related proteins, and the putative small GTP-binding protein Rab2 which were up regulated by Cu stress. Three proteins, a putative small cytochrome P450 (CYP90D2), a putative thioredoxin and a putative GTPase, were down regulated by Cu stress	Plant experienced Cu toxicity due to a decline in thioredoxin and CYP90D2 and thus engineering of this protein may enhance Cu tolerance	Zhang et al., 2009a,b
	2DE-MS	<i>Populus sp.</i>	450 Proteins were reproducibly separated, including metabolic processes proteins such as photosynthesis, S assimilation, sugar metabolism, chaperones, and defense related proteins such as GST, DHAR, APX	Plant adjusts its metabolism against Cu stress by changing protein expression. These proteomic temporal features should be taken into account for the future development of metal tolerant plants	Lingua et al., 2012
	IPG, 2-DE, MALDI-TOF-MS	<i>Ectocarpus siliculosus</i>	Up-regulation of photosynthesis (PSII Mn-stabilizing protein of OEC33), glycolysis, and pentose phosphate metabolism; higher accumulation of HSP70 and vBPO	Cu stress leads to up-regulation of certain proteins such as HSP70 and vBPO for proper protein folding and ROS detoxification, respectively	Ritter et al., 2010
	IPG, 2-DE, LC-MS/MS	<i>Cannabis sativa</i>	Induced aldo/keto reductase, PCs expression, suppression/no change in ROS scavenging enzymes	Cu induced aldo/keto reductase acts as a Cu chaperone reduce Cu ions to Cu(I), promote PCs-mediated vacuolar transport in order to reduce Cu toxicity	Bona et al., 2007
SDS-PAGE and 2DE	<i>Oryza sativa</i>	25 Protein spots were differentially expressed in Cu-treated samples. Among them, 18 protein spots were up-regulated, and 7 protein spots were down-regulated. Antioxidants proteins such as glyoxalase I, peroxiredoxin, aldose reductase, and DnaK-type molecular chaperone up-regulated. Moreover, down-regulation of key metabolic enzymes like alpha-amylase or enolase revealed also observed	Plant showed physiological alterations under Cu stress due to the change in metabolic pathway related proteins	Ahsan et al., 2007	
Zn	iTRAQ	<i>Arabidopsis thaliana</i>	521 Proteins identified. Among them, several were membrane proteins. IRT1, an iron and zinc transporter, and FRO2, a ferric-chelate reductase, increased greatly in response to excess Zn	Plant exhibits Zn stress in which V-ATPase activity might play a central role	Fukao et al., 2011
	2DE-MS	<i>Populus sp.</i>	450 Proteins were reproducibly separated, including metabolic processes proteins such as photosynthesis, S assimilation, sugar metabolism, chaperones, and defense related proteins such as GST, DHAR, APX	Plant adjusts its metabolism against Zn stress by changing protein expression	Lingua et al., 2012

(Continued)

TABLE 3 | Continued

Metal	Technique(s) used	Plant species	Alterations in protein(s) expression profile	Plant response	References
Ni	2DE, MALDI-TOF-MS	<i>Brassica juncea</i>	61 Proteins differentially expressed. The majority of proteins were found to be involved in S metabolism and protection against oxidative stress. The induced expression of photosynthesis and ATP generation-related proteins were also observed	An increased expression of defense proteins and those related with energy metabolism suggesting the Ni tolerance in plant is an energy-demanding process	Wang et al., 2012
	2-DE, LC-MS/MS	<i>Alyssum lesbiacum</i>	12 Proteins differentially expressed. They include proteins of S metabolism, antioxidants, heat shock	Modulation in S metabolic and defense related proteins enhanced Ni tolerance of plant	Ingle et al., 2005
Mn	IPG, 2-DE, Nano-LC-MS/MS, ESI MS/MS	<i>Vigna unguiculata</i>	8 Differentially expressed proteins identified involved in CO ₂ fixation, stabilization of the Mn cluster of the photosystem II, pathogenesis-response reactions, and protein degradation	Coordinated interplay of apoplastic and symplastic reactions help plant to withstand Mn toxicity	Führs et al., 2008
	2DIEF/SDS-PAGE, 2D Blue native BN/SDS-PAGE	<i>Hordeum vulgare</i>	A range of proteins differentially expressed in response to Mn. A putative inorganic pyrophosphatase, a probenazole-inducible protein (PBZ1), a protein belonging to a universal stress protein (Usp) family, a chloroplast translational elongation factor (Tu) and the 50S ribosomal protein L11	In young leaves toxicity resulted due to Mn-induced Mg and Fe deficiencies	Führs et al., 2010

enhancing stress tolerance in a variety of crop species (Vriet et al., 2012). Although SA and GAs both are cost effective and can easily be availed for their exogenous application in crop fields under stress conditions, high cost of synthetic BRs and the variability of the results have discouraged the use of exogenous BRs in agriculture and horticulture (Khrupach et al., 2000; Gomes, 2011). In this context, modulation of endogenous BRs levels by genetic engineering has emerged an efficient strategy for enhancing crop yield under normal as well as adverse growth conditions (Divi and Krishna, 2009). Herein, we have summarized recent advances made in enhancing heavy metal tolerance as well as achieving high yield with desired agronomic traits by using salicylic acid (SA), brassinosteroids (BRs), and gibberellins (GA).

Salicylic Acid (SA)

In recent years, SA has gained much scientific attention due to its function as an endogenous signaling molecule conveying local and systemic plant–pathogen defense responses. Besides this, it has been reported that SA also plays a role in plant response against abiotic stresses such as heavy metal toxicities, chilling, drought, osmotic stress, and heat. In this sense, SA appears to be an “effective therapeutic agent” for plants as in the case of mammals (Rivas-San Vicente and Plasencia, 2011). Salicylic acid is a phenolic compound biosynthesized in all the plant kingdoms through the phenylpropanoid pathway (Métraux, 2002).

Being well characterized and studied role of SA in pathogen resistance, an exogenous application of SA could also provide protection against several types of abiotic stresses such as heavy metals, high or low temperature, salinity, radiation, etc. (Horváth et al., 2007; Hayat et al., 2010). Since under stress condition, reduced plant growth could result from an altered hormonal status, and thus, an exogenous application of plant hormones like SA has been an attractive approach

to attenuate heavy metal stress. Studies carried out so far demonstrated that SA treatment to plants evoke acclimatization effect, which causes an enhanced tolerance toward heavy metal stress primarily due to the adjustment of metabolic processes such as enhanced antioxidative capacity. In one of the first works, it was demonstrated that SA may induce protective effects against Cu toxicity in tobacco and cucumber (Strobel and Kuc, 1995). Later, an increasing numbers of studies have demonstrated SA-mediated amelioration of toxicities produced by various heavy metals. Zhou et al. (2009) reported that 0.2 mM of SA ameliorates Hg toxicity in alfalfa by increasing activity of APX, POD, and NADPH oxidase, and amounts of ascorbate, glutathione, and proline, and decreased lipid peroxidation, and an increase in NADPH oxidase activity. It indicates a role of ROS signaling in such an amelioration process.

In maize plant, Cd declined the growth by inhibiting chlorophyll synthesis, ribulose 1,5-bisphosphate carboxylase and phosphoenolpyruvate carboxylase, and enhancing oxidative damage such as lipid peroxidation and electrolyte leakage, whereas SA pretreatment of seeds reversed these toxic effects (Krantev et al., 2008). In cucumber, an exogenous application of SA has also been reported to enhance Mn tolerance by modulating nutrients' statuses and antioxidant defense system (Shi and Zhu, 2008). Similarly, in pea seedlings, Cd toxicity caused decline in growth due to an inhibited photosynthetic process and enhanced oxidative damage, whereas SA pretreatment alleviated damaging consequences of Cd on growth and photosynthesis (Popova et al., 2009). Moreover, Guo et al. (2009) have demonstrated that SA pretreatment alleviated Cd toxicity in rice by enhancing antioxidant components such as SOD, CAT, POD, glutathione, and non-protein thiols, which in turn depressed oxidative damage induced by Cd. Conversely, Metwally et al. (2003) reported that SA down-regulates activities

of antioxidant enzymes such as CAT and APX under Cd stress and concluded that SA alleviates Cd toxicity not at the level of antioxidant defense system but by affecting other mechanisms of Cd detoxification. Contrary to this, SA at higher concentration may also cause tissue damage and cell death by inducing oxidative stress (Horváth et al., 2007). For instance, SA has been shown to potentiate generation of ROS in photosynthetic tissue under abiotic stresses and thus causes tissue damage (Borsani et al., 2001). Therefore, it can be concluded that the concentration of SA appears to be important in regulating stress responses. The SA-mediated alterations in genes that are involved in mediating stress tolerance are listed in **Table 4**.

It is known that SA also involves in the regulation of oxidative stress caused by various stress factors (Yang et al., 2004). An enhanced level of SA under heavy metal stress suggests a connection between the extent of plant tolerance to heavy metal, which is mediated by the SA signal and the redox balance (Metwally et al., 2003; Sharma and Dietz, 2009). In the SA signaling under heavy metal stress, several signaling molecules such as nitric oxide (NO), H₂O₂, Ca⁺², etc. and their interactions have been reported (Rodríguez-Serrano et al., 2009; Xu et al., 2014). Moreover, Cui et al. (2012b) have reported a cross-talk of haem oxygenase-1 and SA in alleviation of Cd stress in *M. sativa*. In spite of considerable progress in the understanding of

TABLE 4 | Summary of plant hormone-mediated alterations in genes and their relation with an increased heavy metal stress tolerance.

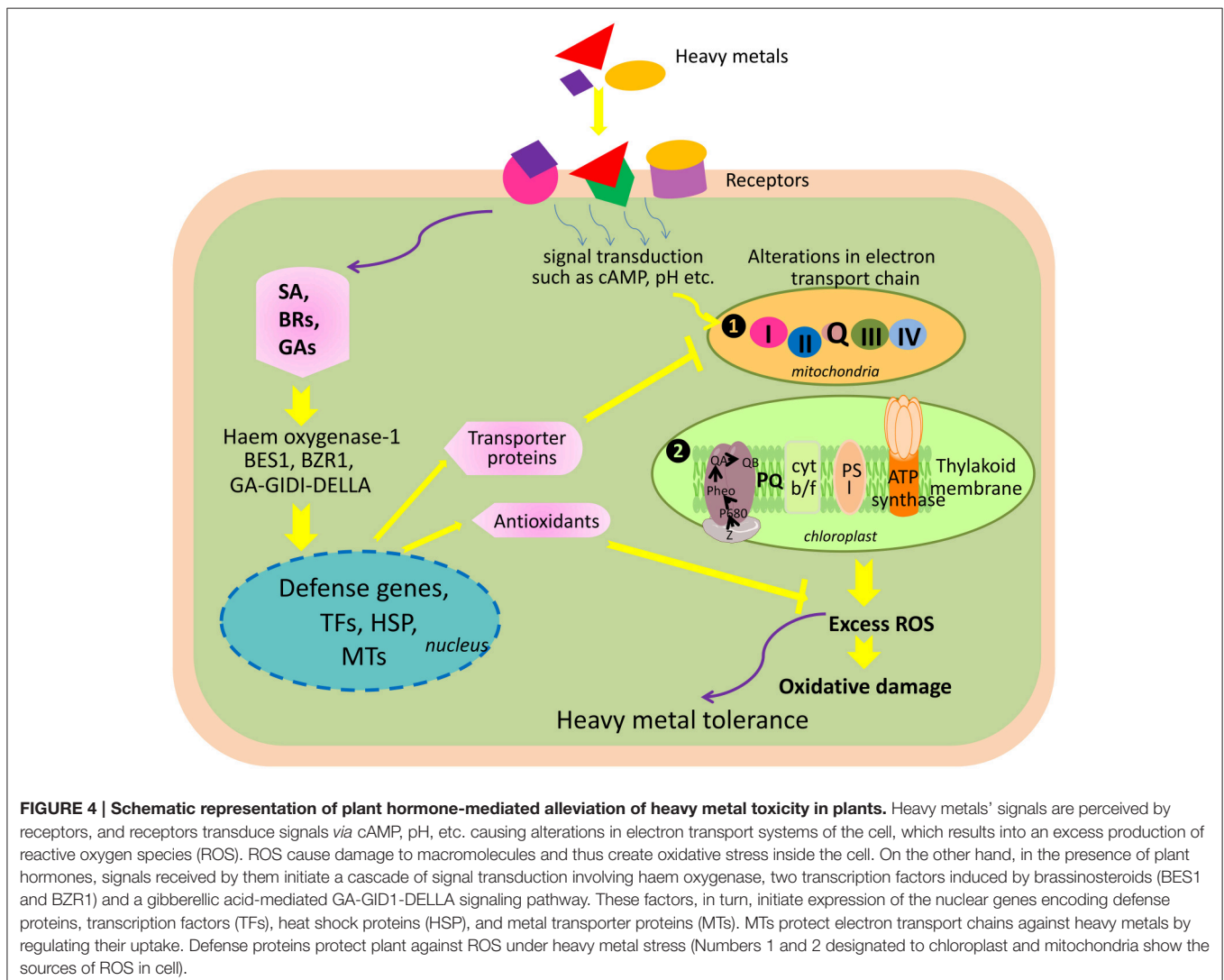
Plant hormone	Alteration in gene(s)	Studied plant	Response	References
Salicylic acid	Haem oxygenase-1 (<i>HO-1</i>)	<i>Medicago sativa</i>	Alleviation of Cd-triggered oxidative stress by re-establishing redox homeostasis	Cui et al., 2012b
	Serine acetyltransferase	<i>Thlaspi</i> spp.	Elevated level of glutathione and increased Ni tolerance	Freeman et al., 2005b
	Citrate synthase	<i>Cassia tora</i>	Enhanced Al tolerance through an efflux of citrate	Yang et al., 2003
	<i>SR3</i>	<i>Phaseolus vulgaris</i>	This gene up-regulated by SA and provides resistance against Hg, Cd, As, and Cu	Zhang et al., 2006
	<i>gsh1</i> , <i>gsh2</i> , or <i>gr1</i> and <i>gst</i>	<i>Arabidopsis thaliana</i>	SA did not influence expression of these genes except <i>gst</i> and thus did not affect Cu and Cd tolerance	Xiang and Oliver, 1998
	<i>MT1</i> and <i>MT2</i>	<i>Arabidopsis thaliana</i>	SA did not alter expression of these genes hence did not impart Cu tolerance	Murphy and Taiz, 1995
Brassinosteroids	Antioxidant defense related genes	<i>Raphanus sativus</i>	Increased resistance against Cr toxicity due to diminished production of ROS and an enhanced defense system	Choudhary et al., 2012b
	<i>Fe-SOD</i> , <i>CAT1</i> , <i>APX</i> , <i>GST1</i> , <i>GR</i> , <i>POD</i> , <i>GSH1</i> , <i>PAL</i> , <i>PPO</i> , <i>SKDH</i> , and <i>CAD</i>	<i>Solanum lycopersicum</i>	Alleviates Cd-induced inhibition on photosynthesis by up-regulating defense system and decreasing oxidative stress	Ahamed et al., 2013
	Genes encoding polyamines, IAA and ABA metabolic genes, and Cu homeostasis	<i>Raphanus sativus</i>	Lower ion leakage due to a maintenance of Cu homeostasis and hence an enhanced Cu tolerance	Choudhary et al., 2012a
	<i>HSP83</i> , <i>HAT2</i> , <i>GH3.9</i> , <i>SAL2</i> , <i>NIA1</i> , <i>GAS4</i> , <i>SAUR36</i> , <i>DWARF1</i> , <i>DWARF4</i> , and <i>BR6OX</i>	<i>Arabidopsis thaliana</i>	BR-exhibited synergistic effect with Cd and increased Cd sensitivity of plants	Villiers et al., 2012
	NADPH oxidase and <i>RBOH</i> , <i>MAPK1</i> , and <i>MAPK3</i>	<i>Cucumis sativus</i>	BR-mediated production of H ₂ O ₂ via NADPH oxidase increased stress tolerance in cooperation with kinases	Xia et al., 2009
	Induced NO production that up-regulates ABA biosynthetic gene <i>vp14</i>	<i>Zea mays</i>	BR-induced NO production that up-regulates ABA biosynthesis gene <i>vp14</i> and thus confers stress tolerance	Zhang et al., 2011
	Set of stress marker genes	<i>Brassica napus</i>	Increased tolerance against abiotic stresses such as drought and cold	Kagale et al., 2007
	<i>UBC32</i> , a stress-induced functional ubiquitin conjugation enzyme (E2)	<i>Arabidopsis thaliana</i>	Protects plants from abiotic stress through endoplasmic reticulum (ER)-associated protein degradation (ERAD) component and <i>UBC32</i> plays a crucial role in such protection	Cui et al., 2012a
Gibberellic acid	<i>IRT1</i>	<i>Arabidopsis thaliana</i>	GA-suppressed up-regulation of <i>IRT1</i> and enhanced accumulation of NO that enhanced Cd tolerance	Zhu et al., 2012
	<i>CAX2</i>	<i>Nicotiana tabacum</i>	GA did not influence expression of this gene and did not alter Mn and Cd tolerance	Hirschi et al., 2000
	GA-biosynthesis and redox genes	<i>Glycine max</i>	Increased Cu tolerance due to decreased oxidative damage and enhanced antioxidant defense system	Khan and Lee, 2013
	adenosine 5'-phosphosulfate reductase (APR)	<i>Arabidopsis thaliana</i>	GA plays a role in abiotic stress tolerance via regulating S assimilation pathway	Koprivova et al., 2008

SA signaling, molecular events, which are involved in the SA signaling in order to alleviate heavy metal stress, are still poorly known (Figure 4).

Brassinosteroids (BRs)

Brassinosteroids are group of hormones having ability of regulating ion uptake in plant cells and very effectively reducing the heavy metal accumulation in plants. BRs can also impart plant stress tolerance against variety of biotic and abiotic stresses such as heavy metal, salinity, drought, low and high temperatures, and pathogen attack (Bajguz and Hayat, 2009; Hao et al., 2013). An increasing numbers of studies have shown that an exogenous application of BRs is widely used in order to improve crop yield as well as stress tolerance in various plant species (Divi and Krishna, 2009; Peleg and Blumwald, 2011; Li et al., 2013). Cadmium, a heavy metal, very toxic even when present in trace amount, have been found to retard chlorophyll biosynthesis, activity of several enzymes, and inhibit light and dark reactions of photosynthesis by limiting the energy/reducing

sources (Vassilev and Yordanov, 1997). However, it has been reported that Cd-induced toxicity can be lowered with BR. For instance, Janecko et al. (2005) reported that Cd-induced inhibition in pigments content, cotyledon growth could be minimized with exogenous epibrassinolide (EPL: another BR). Hayat et al. (2007) have verified the role of HBL under Cd stress in *B. juncea*. In *Vigna radiata* L. Wilczek, Al stress caused a reduction in length, fresh and dry mass of root and shoot; activity of carbonic anhydrase; water use efficiency; relative water content; chlorophyll content; and the rate of photosynthesis, whereas addition of BR reversed these toxic effects and protected the plants *via* elevated level of proline in an association with an antioxidant defense system which at least in part was responsible for the amelioration of Al stress (Ali et al., 2008). In *B. juncea*, BR alleviates Cd toxicity through enhanced level of antioxidants (Hayat et al., 2007). The Cr, a known toxic metal, reduced the growth performance of *Raphanus sativus* L., whereas BR protects plants from adverse consequences of Cr toxicity by regulating antioxidant defense system (Sharma et al., 2011).



Micronutrients such as Cu and Ni are essential for growth and development, but in excess, they cause severe toxic effects. Cu, which has increasingly attained interest due to its use in fungicides, fertilizers, and pesticides, is also highly toxic to plants, but when seeds of *B. juncea* primed with epibrassinolide (a form of BR) were exposed to Cu stress, improvement in shoot emergence and biomass accumulation, along with reduced Cu uptake and accumulation, was noticed (Sharma and Bhardwaj, 2007). Similar protective responses of exogenous BR on *B. juncea* and *V. radiata* under Cu and Ni toxicities have been reported (Alam et al., 2007; Sharma et al., 2008; Fariduddin et al., 2009; Yusuf et al., 2012b). Besides higher plants, BR has also been found to be effective in alleviating heavy metals such as Cu, Pb, and Cd toxicities in algae, *Chlorella vulgaris*, through the regulation of antioxidant defense system (Bajguz, 2010). The BR-mediated alterations in the gene expressions and their roles in stress tolerance are listed in **Table 2**.

Gibberellic Acid (GA)

The gibberellins (GAs) are a large family of tetracyclic diterpenoid plant growth hormone associated with the plant growth and developmental processes (Matsuoka, 2003). To alleviate deleterious effects of stress, different types of plant hormones have been used that might complement decreased and/or imbalanced hormone level during exposure of stress. Of these, GA has been a focus of plant scientists (Hisamatsu et al., 2000; Iqbal et al., 2011; Zhu et al., 2012).

Several studies revealed that GA alleviates various abiotic stresses including heavy metal toxicity. In *A. thaliana*, GA (5 μ M) is reported to ameliorate Cd toxicity by reducing Cd uptake and lipid peroxidation (Zhu et al., 2012). Furthermore, authors demonstrated that GA reduces NO level which in turn down-regulates expression of *IRT1* gene, a Fe transporter (might be involved in Cd absorption) as indicated by no effect of GA in reduction of Cd uptake in an *IRT1* knockout mutant *irt1*. It is reported that an exogenous addition of GA reprograms the growth of soybean under stress conditions by enhancing the levels of daidzein and genistein contents, suggesting protective role of GA in mitigating adverse consequences of stressors (Hamayun et al., 2010). In wheat seedlings, Ni (50 mM) has been shown to decline growth, chlorophyll content, and carbonic anhydrase activity by enhancing oxidative stress, whereas an addition of GA ameliorates Ni-induced toxic effects (Siddiqui et al., 2011). Gangwar et al. (2011) have also reported that an exogenous addition of GA ameliorates toxic effects of Cr (50–250 μ M) on growth and ammonium assimilation of pea seedlings by regulating oxidative stress and an antioxidant system. In *B. napus* L., GA (50 μ M) has been shown to alleviate Cd (10–400 μ M)-induced negative impact on seed germination and growth by regulating oxidative stress and damage (Meng et al., 2009). It has been observed that Pb and Zn affect seed germination in *Cicer arietinum* cv. Aziziye-94 by altering hormonal balance, and an exogenous application of GA reverses the toxic effect of heavy metals (Atici et al., 2005). Furthermore, Sharaf et al. (2009) have also reported that GA mitigates detrimental effects of Cd and Pb on broad bean and lupin plants by regulating activities of proteases, CAT, and POD. These studies

clearly indicate that GA plays an important role in protecting plant metabolism against various stresses; however, this may occur *via* various routes suggesting complex GA signaling during plant acclimation against stresses. The GA-mediated alterations in genes and their relation with stress tolerance are summarized in **Table 4**.

CONCLUSION AND FUTURE PROSPECTS

Around 3.1 billion people from developing countries live in rural areas, and out of this population, ~2.5 billion people depend on agricultural practices for their livelihood, which contributes 30% to economic growth because of the gross domestic products obtained from agriculture (FAO, 2012). It is expected that world population will be about 10 billion by the middle of the twenty-first century, and we will witness serious food shortages (Smith et al., 2010; Naika et al., 2013). Furthermore, the situation will likely to be severe due to increased anthropogenic activities that have resulted into unwanted changes in the environment such as soil, air, and water pollution with various factors including heavy metal. These situations (pollution and population) are posing a continuously increasing burden on global crop productivity, and hence, there are demands for crop varieties that should be adaptive and resistant to various stresses.

In contrast to biotic stress, which is under the control of monogenic trait, abiotic stress tolerance is a genetically complex process that involves many components of signaling pathways, multigenic in nature, and thus, comparatively more difficult to control and engineer (Vinocur and Altman, 2005). Therefore, plant-engineering strategies for heavy metal tolerance depend on the expression of gene(s) whose product(s) are involved either in signaling and regulatory pathways or in the synthesis of functional and structural proteins and metabolites that confer heavy metal stress tolerance. Recently, several efforts are being made to improve heavy metal stress tolerance capacity through genetic engineering with several achievements; however, the genetically complex mechanisms of heavy metal stress tolerance and transfer of technology to field conditions make it difficult. Advances in various functional tools, resources, and “omics” have helped in the molecular characterization of the genes, metabolites, and proteins involved in heavy metal stress tolerance. Furthermore, genetic engineering of heavy metal-responsive genes (particularly TFs), metabolites, and proteins has shown surprising results but its full potential remains to be exploited. The design of future experiments that use a multidisciplinary approach with well-integrated “omics,” i.e., transcriptomics, metabolomics, proteomics, etc. ultimately required to significantly improve heavy metal tolerance as well as tolerance to other abiotic stresses in economically important crop plants.

ACKNOWLEDGMENTS

The University Grants Commission, Central Regional Office, Bhopal and New Delhi are greatly acknowledged for providing

financial support to Dr. VS (PI-UGC Minor research Project, File No. MS-27/201022/XII/13-14/CRO) and Dr. SP (PI-UGC Major research Project no: 41-460/2012(SR)) respectively. We are also very grateful to Department of Botany, University

of Allahabad for providing necessary lab facilities and also to University Grant Commission for providing financial support to PP and RS as research fellow to carry out this work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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