An Integrated Transcriptomic, Proteomic, and Metabolomic Approach to Unravel the Molecular Mechanisms of Metal Stress Tolerance in Plants

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1 Introduction

Rapid rate of industrialization, urbanization, intensive agriculture, and mining has been causing widespread contamination of our soil and aquifer resources with heavy metals. This is a problem of paramount importance since metal pollution can lead to dangerous consequences by entering into the food chain (Rascio and Navari-Izzo 2011). Some of the metals may be required for plants as micronutrients (Cu, Fe, Mg, Mn, Ni, Zn) for normal growth and development, but many (Ag, As, Cd, Co, Cr, Hg, Pb, Sb, Se) may not be essential for plant growth or may have unknown functions in the plant systems (Rascio and Navari-Izzo 2011). But, if heavy metals are present in excess amount, they may hamper the metabolic pathways and disrupt the functions of enzymes (Hossain et al. 2012a, b, Ali et al. 2013). Enhanced levels

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of heavy metals generate reactive oxygen species (ROS) or non-free radical species such as singlet oxygen and hydrogen peroxide and also cytotoxic compounds such as methylglyoxal which cause oxidative stress (Hossain et al. 2012a, b, Sytar et al. 2013). Heavy metal stress also leads to programmed cell death due to membrane and DNA damage, ion leakage, and redox imbalance (Rascio and Navari-Izzo 2011). But, over a period of time, plants have acquired diverse mechanisms to cope with adverse effects of heavy metal stress. They may avoid metal stress to preclude the onset of stress either by excluding or preventing the entry (Viehweger 2014). Dalvi and Bhalerao (2013) reported that plants prevent the entry of metals by immobilizing them with mycorrhizal association and complexing them by organic acids secreted from roots. If metals enter into the plant systems, plants use other tolerance mechanisms for getting rid of them. These strategies include metal sequestration into vacuoles (Patra et al. 2004) and metal binding to cell walls, biosynthesis of phytochelatins, and metallothioneins (John et al. 2009). Apart from metal sequestration mechanisms, plants synthesize osmolytes such as proline and activate antioxidant defense mechanisms (Manara 2012). Thus, plants adapt a wide range of strategies to overcome heavy metal toxicity. But, the intricate molecular mechanisms that are associated with such strategies are not fully known. Advances in the study of transcriptome, using next-generation sequencing methods, matrix-assisted laser desorption ionization (MALDI) time-of-flight, time-of-flight (MALDI-TOF-TOF) for the study of proteome are assisting us to dissect out different pathways and complex network of genes and proteins. This chapter describes an account of the biosynthesis of two important ligand peptides, phytochelatins, and metallothioneins that are used by plants, their functions in metal detoxification, and how transcriptomic, proteomic, and metabolomic data are helping us to unravel some of the molecular mechanisms associated with heavy metal toxicity. Some directions are also provided at the end for forming a future framework of research.

2   Heavy Metal-Induced Nutritional and Water-Deficit Stresses

2.1   Nutritional Stress in Metal-Exposed Plants

Heavy metal stress causes transient deficiencies of nutrients in plants due to spatial and temporal variations in plant nutrient demands and supplies. The movement of mineral elements into the plant system may in turn depend upon several factors like diffusion of elements, transport of bulk soil solution, etc. (Marschner 1995). While most of the nutrients (e.g., N, P, K, S, Mg) are transported through phloem, calcium and boron are usually supplied through the xylem to the growing parts (Nazar et al. 2012). Thus, both xylem and phloem should function efficiently under nutrient deficiency caused by metal stress. Heavy metals like cadmium compete with Ca, Mg, and Fe in transport process across membrane via ZIP and NRAMP family members

or Ca channels and transporters (Llamas et al. 2000, Perfus-Barbeoch et al. 2002). Further, Gothberg et al. (2004) pointed out that the competition between nutrients and toxic metals for binding sites at the membrane or cell wall level influences the entry and distribution of these metals. Sun and Shen (2007) showed that the reduction decrease of Mn, Fe, Mg, S, and P levels in cabbage plants sensitive to Cd stress is the reason for decreased growth. In *Aeluropus littoralis* (Rezvani et al. 2012), heavy metals caused an increase in macronutrient and decease in micronutrient concentrations. Yoshihara et al. (2006) reported Cd-induced iron deficiency responsive genes like *HvIDS*2pro::*GUS*, *NtFRO*1, and *NtIRT*1 in tobacco roots. Work carried out by Wu et al. (2012) shows that high Fe content in shoots under Cd stress alleviates Cd toxicity in *Arabidopsis*. Thus, nutrient deficiency in plants appears to be widespread under heavy metal stress. As pointed out by Guerinot (2000), comprehensive understanding of the functions of the ZIP and other family transporters is pivotal for enhancing mineral nutrient content and developing crop plants that accumulate or exclude toxic metals.

2.2   Water Stress in Metal-Exposed Plants

Water and inorganic nutrients absorbed through the roots support the plant growth and development. Roots are directly exposed to higher heavy metal concentrations from where they are exported to shoots and leaves (Burkhead et al. 2009, Feleafel and Mirdad 2013). Due to the presence of soluble salts including heavy metal salts, water relations would be severely affected in plants. However, Vaculik et al. (2012) reported changes in root anatomy and morphology of *Salix caprea* under Zn and Cd stress. Besides this, decreased root elongation, increased root dieback, and reduced root hair surface were also noticed in other plants (Sharma 2012, Gallego et al. 2012, Anjum et al. 2013, Feleafel and Mirdad 2013). If heavy metals are present in excess amount, they influence the flow of water by lowering the transpiration rate (Barcelo and Poschenrieder 1990). Maggio and Joly (1995), and Carvajal et al. (1996) reported reduction in pressure-induced water flux from tomato and wheat, respectively, in response to Hg. While roots of *Brassica juncea* displayed an increase in the vacuole size (Han et al. 2004), in *Lutea luteus*, Pb-induced vacuolization was recorded with high values of relative water content (Rucinska-Sobkowiak et al. 2013). In roots of *Oryza sativa* and *Pisum sativum*, extensive tissue damage was noticed in the presence of Cd and Ni (Gabbrielli et al. 1999, Lux et al. 2011, Llamas et al. 2008). Stomata are closed due to the interaction of heavy metals with guard cells and reduced osmotic potentials (Rucinska-Sobkowiak 2016). They also pointed out that ABA-induced signals play a vital role in stomatal movement. It appears that metals decelerate short-distance water transfer both in symplast and apoplast. Such a situation certainly reduces the flow of water into the vascular system and impairs the supplies to the shoot and leaves. Likewise, long-distance transport of water is hindered due to decreased hydraulic conductivity in the roots, stems, and leaves. Also, such metal-induced perturbations in water relations modulate

aquaporin gene expressions in plants resulting in water loss reductions (Rucinska-Sobkowiak 2016). However, further research is needed to unravel the cross talk between signaling elements and their role in the transduction of metal-induced signals from below-the-ground parts to the aboveground parts. Such studies help to evolve plants with better resilience to metal-induced water-deficit conditions.

3 Role of Thiol- and Non-thiol Compounds and Metal(loid) Chelation

Certain ions, though essential for plant growth and development, can cause toxicity when present in excess. However, plants have evolved multiple strategies to maintain metal homeostasis, alongside the mitigation of heavy metal stress. When plants are exposed to higher metal concentrations, either they can exclude or efflux out the metals into the xylem (xylem loading) or accumulate and sequester into vacuoles (Robinson et al. 1994; Montargès-Pelletier et al. 2008). Lasat (2002) and others (Seth et al. 2008, Cuypers et al. 2009) pointed out that exclusion of metal ligands from root symplasm into xylem vessels or sequestration into the vacuoles leads to metal homeostasis and metal-induced oxidative stress. Thiol compounds such as glutathione (GSH), phytochelatins (PCs), and metallothioneins (MTs) contain sulfhydryl (–SH) groups and bind with high affinity to many metals (Seth et al. 2012). GSH is a tripeptide (γ-Glu-Cys-Gly) and a major –SH compound and acts as a precursor for the synthesis of phytochelatins (Clemens 2006, Srivalli and Khanna-Chopra 2008). MTs are sulfur (S)-containing cysteine (Cys)-rich, gene-encoded polypeptides and bind to metals in plants (Verbruggen et al. 2009). Though PCs partake in metal detoxification, their role during normal physiology of plants in the absence of metal stress is largely unknown. In addition to thiol compounds, in a number of plants, non-thiols like organic acids (citrate, malate, oxalate, malonate, aconitate, tartarate) and amino acids such as proline, histidine, cysteine, arginine, glutamate, and nicotianamine either alone or in combination with thiol compounds, have been shown to contribute to metal chelation (Hall 2002). Thus, it appears that plants utilize a wide array of mechanisms/resources for metal sequestration/detoxification.

3.1 Phytochelatins and Their Induction

PCs are a family of cysteine-rich polypeptides with general structure (γ-Glu-Cys) n-X, in which X represents Gly, γ-Ala, Ser, Gln, or Glu and n = 2–11 depending on the organism, though the most common forms have 2–4 peptides (Gekeler et al. 1989). PCs were discovered first in *Saccharomyces pombe* after exposing them to cadmium which produced PC_2 and PC_3 (Kondo et al. 1985). They have been named

as cadystin A and B, but later the name PC was adopted (Wu et al. 2014). They are mostly induced by Cd when compared to Zn (Thangavel et al. 2007) and Hg (Sobrino-Plata et al. 2009). Interestingly, more GSH has been found in Hg-exposed *Hordeum vulgare* aerial parts than in roots. However, longer-chain PCs (such as PC3, PC4, and PC5) were abundant in roots in comparison with aerial parts (Dago et al. 2014). Morelli and Scarano (2001) discovered that Pb and Cd can cause the synthesis of PC2 and PC4, respectively, in *Phaeodactylum tricornutum*. Though the –SH group of the Cys residues helps PCs to bind to the metals, the PC-metal complexes are transported into the vacuoles via ABC transporters or a group of solute transporters for detoxification (Verbruggen et al. 2009, Solanki and Dhankhar 2011).

3.2 Biosynthesis of PCs and Variations (Homo-PCs, Hydroxymethyl-PCs, and Iso-PCs)

Biosynthesis of phytochelatins can be plant or metal specific. Zenk (1996) reported that Hg, Cd, As, Ag, and Fe are strong inducers, Pb and Zn are weak inducers, and Cu and Ni are moderate inducers. It appears that Pb is a strong inducer of PCs in comparison with other metals in many plant species (Dago et al. 2014). They also reported more longer-chain PCs such as PC3, PC4, and PC5 in *Hordeum vulgare* roots than in leaves and shoots. In plants such as *Phaeodactylum tricornutum* (Morelli and Scarano 2001), exposure to Pb and Cd causes the production of PC2 and PC4, respectively. However, how Cd can stimulate PC synthesis or its activation is not currently known. Song et al. (2014) are of the opinion that essential metals like Zn, Cu, and Mn are transported into vacuoles in the form of PC2-metal complexes through ATP-binding cassette (ABC) transporters. Grill et al. (1989) pointed out that the biosynthesis of PCs is triggered by exposure of cells to metals like Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Ag^{2+}, and others. Glutamate and cysteine are the precursors for the biosynthesis of γ-glutamylcysteine, and the reaction is catalyzed by γ-glutamylcysteine (EC) synthetase (γ-ECS) in plants. Glycine molecule is then added to this dipeptide, and the enzyme associated with this biochemical conversion is ATP-dependent glutathione synthetase. γ-ECS is converted to γ-glutamylcysteine glycine (γ-Glu-Cys-Gly) which is then catalyzed by the enzyme phytochelatin synthase (PCS) to form PCs (Zenk 1996). However, variations occur in the formation of PCs. In leguminous plants (Phaseoleae tribe), phytochelatins contain β-alanine instead of glycine and are analogous to homo-GSH; therefore, they are named as homo-phytochelatins (Grill et al. 1986). If homoglutathione occurs in some plants, and in such cases, β-alanine-specific ATP-dependent enzyme homoglutathione synthase utilizes γ-Glu-Cys to produce γ-Glu-Cys-β-Ala (homo-phytochelatins as in the case of Leguminosae family). On the other hand, in members of the family Poaceae, biosynthesis of γ-Glu-Cys-Ser [(Glu-Cys)n-Ser] is common (Klapheck et al. 1992). Klapheck et al. (1992) found that hydroxymethyl-GSH(γ-Glu-Cys-Ser), which upon exposure to metals,

produces homologous peptides [(Glu-Cys)n-Ser] and later named as hydroxymethyl-PCs. However, *Zea mays* is an exception to this, where γ-Glu-Cys-Glu has been noticed (Meuwly et al. 1993, 1995). Thus, C-terminal-modified PC peptides have been noticed with different residues (other than glycine) and termed as iso-phytochelatins. In addition to PCs, iso-PCs, peptides without C-terminal amino acids represented as (g-Glu-Cys)n and are called as desGly-PCs. Such desGly-PCs were noticed in *Saccharomyces pombe* (Mehra and Winge 1988) as well as in maize (Bernhard and Kagi 1987). The catalytic moiety of phytochelatin synthase (PCS) contains cysteine (Cys) residue. Wang et al. (2009) pointed out that though the C-terminal domain is not highly conserved, in all species, the termini contain multiple Cys residues that bind Cd ions with high affinity. Irrespective of their names, the major function of PCs is metal detoxification in plants. However, why such a diverse group of PCs is synthesized in varied taxa is not exactly known.

3.3   *Mode of Action of PCs*

It is necessary for plants to chelate metals so as to prevent the disruption of vital metabolic activities by heavy metals in the cytosol. Once metals enter the cytosol, they activate the phytochelatin synthase (PCS) enzyme, which is otherwise inactive. PCS then produce PC molecules with variable length utilizing GSH. The metal-binding peptides are then biosynthesized in the cytosol and form a metal-PC complex as detailed above. It has been pointed out later by Toppi and Gabbrielli (1999) that PCs complex with metal ions such as Cd^{2+} through the thiolic group (-SH) of cysteine and the PC-metal complexes are accumulated in the vacuole through the activity of ABC transporter family members or a group organic solute transporters (Solanki and Dhankhar 2011). DalCorso et al. (2008) found out the metal-PC complexes are effectively sequestered into vacuoles, and the heavy metals are also transported over a long distance through the xylem and phloem vessels in higher plants (Mendoza-Cozatl et al. 2008). Since both metal-tolerant and metal-sensitive plants produce PCs, it is believed widely that PCs alone are not responsible for hyperaccumulation of Zn, Ni, or Pb (Shen et al. 1997, Leopold et al. 1999). Rauser (1999) pointed out that metal ions after combining with PCs are transported to the vacuoles and form a complex with sulfides and organic acids. Formation of PC-As (III) complex in rice leaves was shown to reduce the translocation of arsenic (As) from leaves to the grains (Duan et al. 2011). Phytochelatin synthase genes have been isolated from higher plants and overexpressed in many plants. Heterologous overexpression of *Triticum aestivum TaPCS1* in rice increased the sensitivity to the metal cadmium(Cd) and accumulation in shoots but not in roots (Wang et al. 2012). However, overexpression of *Ceratophyllum demersum PCS1* (*CdPCS1*) in *Nicotiana tabacum* or *Arabidopsis thaliana* (Shukla et al. 2013) increased the synthesis of PCs and accumulation of Cd and As. Thus, PCs detoxify the heavy metals in plants, besides transporting them (Salt and Rauser 1995). However, this does not fully

explain how precisely the heavy metal specificity or species specificity of hyperaccumulation takes place in certain plants as has also been pointed out by Baker et al. (2000) and Hossain et al. (2012a, b).

3.4 The Superfamily of Metallothioneins: Classification and Structure of Metallothioneins

Metallothioneins (MTs) are intracellular, gene-encoded, low molecular weight (2–7 kDa), cysteine (Cys)-rich (20–30%) polypeptides that act as metal chelators. MTs are distributed very widely in microbes (prokaryotes), fungi, and animal and plant kingdoms. Based on their sequence similarity and phylogenetic relationship, MTs have been classified into 15 families. Depending on the number of Cys regions and their distribution, they are classified again into type 1–type 4 subfamilies (Cobbett and Goldsbrough 2002, Peroza et al. 2009, Hassinen et al. 2011). MT has α and β structural domains at the C- and N-termini which are composed of Cys clusters and has the ability to bind to four metal ions very tightly with the help of mercaptide bonds (Cobbett and Goldsbrough 2002, Ruttkay-Nedecky et al. 2013), but at the same time, metal ions are easily exchanged for other proteins (Hassinen et al. 2011). MT was first reported as a cadmium-binding protein in the cortex of horse kidney by Margoshes and Valle (1957). Casterline and Barnett (1982) were the first to discover MTs in roots of soybean. However, plant MTs differ from that of animal MTs in the amino acid sequence, charge, homocysteine number, and distribution pattern (Mir et al. 2004). Type 1 MTs are characterized by the arrangement of Cys residues in the order of Cys-amino acid-Cys in N- and C-termini. On the other hand, type 2 MTs have Cys-Cys, Cys-amino acid-amino acid-Cys and Cys-amino acid-Cys in the N-terminus, and Cys-amino acid-Cys in the C-terminus. Sequence alignment of the *MT* cDNAs with that of genomic sequences showed that *MT* genes have 1–3 introns, the size ranging from 71 to 275 bp. Like other gene sequences, *MT* gene promoters also contained TATA and CAAT boxes (8–635 bp) upstream of the start codon along with other regulatory elements like MYC, ABRE-like, and MYB-binding sites (Samson and Gedamu 1997). This indicates that they are associated with abiotic stress tolerance in plants, maybe by scavenging reactive oxygen species (ROS).

3.5 Expression of MT Genes

The work of Cobbett and Goldsbrough (2002) and Hassinen et al. (2011) reveals that MT genes display a very distinct spatial and temporal expression patterns in plants. MTs are certainly constitutively expressed, but they are also stimulated by many endogenous and exogenous agents. This signifies that there is a functional

divergence for MT subfamilies and is also necessary for plant survival and development. Guo et al. (2003) studied for the first time the expression of MT gene family (including senescence) in *Arabidopsis* and their responses to Cu treatment. MTs are expressed under varied abiotic stress conditions like drought, salinity, high temperature, cold, light, wounding, and senescence (reviewed in Anjum et al. 2015). Also, metals such as Cu, Cd, Pb, and Zn strongly induce plant MT gene expressions (Mehes-Smith et al. 2013). Gene expressions in plants differed depending upon metal accumulators and non-accumulators. In *Arabidopsis thaliana*, *MT1a* and *MT1b* are highly expressed in roots when exposed to Cd, Cu, and Zn (Maestri et al. 2010). But in a metal accumulator *Thlaspi caerulescens*, high levels of MT1 transcripts were noticed in leaves when compared with leaves under the influence of Cu. Likewise, type 3 MT of *Thlaspi caerulescens* showed higher Cu binding capacity compared to *Arabidopsis*. Roosens et al. (2004) on the other hand found higher expression of *MT3* genes during leaf aging and *MT4* in seeds of *A. thaliana*. Gautam et al. (2012) reported many MT genes for detoxification in rice. They noticed 11 class I MT genes in rice genome that are differentially expressed during growth. Both *MT1* and *MT2* were expressed when rice was exposed to As (V) (Nath et al. 2014). Such a tissue-specific expression of MTs reveals that they play a crucial role during plant development besides metal detoxification. In *T. caerulescens*, modifications in protein sequences of TcMT1 and TcMT3 were observed in their Cys domains. Later, Roosens et al. (2005) and Gautam et al. (2012) noted a close association between the number of Cys residues and metal tolerance. They noted that the lower the number of Cys residues, the lower the metal tolerance when *TcMT1* was overexpressed in yeast. Thus, the distribution and organization in Cys residues may have bearing on metal chelation in plants and metal homeostasis.

3.6   Metal(loid) Specificity and Chelation

MTs bind not only to toxic metals such as Cd but also to other transition metals that are essential for plant nutrition like Cu^+ and Zn^{2+} (Freisinger 2011). The binding of heavy metals to MTs is crucial for detoxification of heavy metals. At the same time, transport of Zn and Cu ions helps the maintenance of metal homeostasis. It appears that like in mammalian cells, plant type 1 MT protein (from *Triticum durum* and *Fucus vesiculosus*) assumes a dumbbell shape and the protein can bind very tightly to six Cd^{2+} along with four S^{2-} molecules (Zimeri et al. 2005). Besides, MT protein also binds to four Zn^{2+} ions. Jin et al. (2006) found that *MT* genes are affected by metal and oxidative stresses as well as by salt, drought, and temperature implying that they also play a role in abiotic stress. Usman et al. (2015) showed that *MT4* gene from *Hordeum vulgare* binds to zinc in the aleuronic layer of the grains and act in putative storage. However, structural information and understanding of metal-binding mechanisms in plant MTs are scanty.

3.7 Functions of MTs

In animals, MTs play a role in zinc and copper homeostasis and protection against cadmium toxicity (Vasak and Hasler 2000). In humans, while cells that contain high levels of MTs are resistant to the heavy metal cadmium, lines that synthesize low MTs are sensitive to it (Karin et al. 1983, Enger et al. 1986). Loebus et al. (2013) reported that metallothionein proteins detoxify heavy metals like cadmium and mercury in plants. Poplars were found to accumulate 800 mg of Cd and Zn per kilogram of plant weight (Laureysens et al. 2004). When willows were used in short rotation for removing metals, pollution was reduced to safer levels (Witters et al. 2009). Zhou et al. (2014) noticed homeostasis of metals such as zinc and copper under the influence of MTs. Lv et al. (2013) found out that MTs have functions like ROS scavenging (Akashi et al. 2004), and Higashimoto et al. (2009) reported protection against DNA damage in animals, plants, and microorganisms. Thus, the four different types of MTs have unique and overlapping functions in metal homeostasis, metal detoxification, and antioxidative defense. It appears that there is a functional variation for MTs, but understanding the structure-function relationship and their metal-binding attributes is highly vital for making use of MTs in genetic engineering techniques aimed at metal detoxification/environmental cleanup.

4 Heavy Metal Stress Tolerance at Molecular Level: Omics Approach

Molecular strategies are being employed to understand mechanisms of heavy metal stress tolerance using OMIC tools (Fig. 1). Plants growing in metal-contaminated sites/areas develop tolerance strategies as well as mechanisms to regulate the process of metal uptake, translocation, and accumulation. These strategies involve the role of transcriptome, proteome, and metabolome (reviewed by Singh et al. 2015). In the following section, we discussed the details how transcriptome, proteome, and metabolome regulate the process of heavy metal stress tolerance in plants.
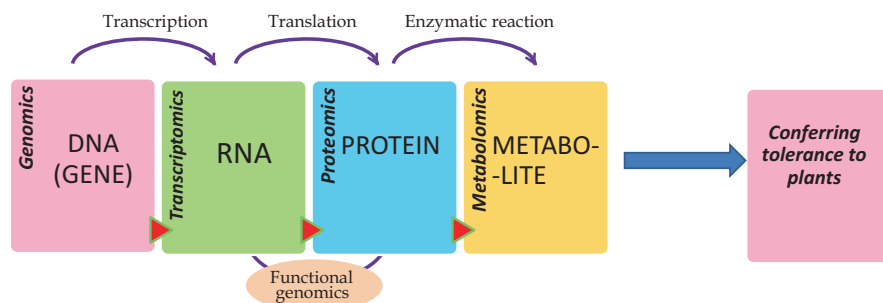

Fig. 1 An overview of omic tools involved in conferring tolerance to plants under heavy metal stress

4.1 Transcriptomics

RNA sequencing is the most important tool for developing transcriptome profile for characterizing and analyzing functionality of gene(s) (Wang et al. 2015). With the help of transcriptome analyses, the role of genes related to metal tolerance has been identified in several plant species like *Arabidopsis*, ramie, radish, and *Viola yedoensis* (Herbette et al. 2006, Xu et al. 2015, Liu et al. 2015a, Gao et al. 2015). Stressors trigger several genes and proteins to provide the plant survival mechanisms under such adverse conditions. The set of genes are either regulatory or functional, where the regulatory gene encodes transcription factor(s) while the functional gene encodes enhanced production of several metabolites like amino acids, alcohol, sugars, etc. The regulatory genes encoding transcription factors are thought to be the center of gene cluster and belong to the multigene family. It has been suggested that one transcription factor could regulate many genes, and their action involves the binding of transcription factor to the *cis*-acting elements of the target genes (Wray et al. 2003, Nakashima et al. 2009) and its further interaction with protein domain that oligomerize the transcription factors with other regulatory substances (Wray et al. 2003, Shiu et al. 2005). Several comparative transcriptome analyses have been performed with two cultivars of *Brassica rapa* under Cd stress, in order to identify the reason for heavy metal tolerance capability, and it was found that several plasma membrane-localized and tonoplast-localized transporters were overexpressed that aid the plant to survive under Cd stress (Yu et al. 2017). In another study by Liu et al. (2015a, b), regulatory role of several genes was analyzed by exploring the stress-responsive miRNA, and it was suggested that several miRNA-encoding transcription families were differentially expressed and these transcription factors were associated with the uptake and homeostasis of Cr. Transcription families like AREB/ABF, MYB, AP2/EREBP, WRKY, bHLH, bZIP, MYC, HSF, ARF, AtSR, CPP, SBP, MADS, TUB, C2C2-YABBY, C2C2-CO-like, C2C2-Gata, E2F-DP, ABI3VP1, CCAAT-HAP3, CCAAT-HAP5, C2H2, C3H, C2C2-Dof, DREB1/CBF, NAC, HB, ARID, EMF1, CCAAT-HAP2, and CCAATDR1 are known to regulate important processes under stress conditions (Shiu et al. 2005, Shameer et al. 2009). Some studies in relation with transcriptome analyses under heavy metal stress have been detailed in Table 1.

4.2 Proteomics

It basically includes the complete changes in protein profile at cellular, tissue, and organ levels. Techniques being deployed for analyzing the metal-responsive proteins involve conventional two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS). Although transcriptome analyses are helping a lot to understand the response of plants, not all the changes taking place in transcriptome are reflected in the proteome (Hossain and Komatsu 2012). For example, in a study

Table 1 Regulation at transcriptome level in heavy metal-stressed plants

Plant	Metal	Transcription factors involved	Plants response	Reference
Brassica rapa	Cd	ZIPs, P_{1B}-type ATPase, and MTPs	Enhanced expression of transcription factors associated with plasma membrane (i.e., *ZIP2*, *ZIP3*, *IRT1*, *HMA2,* and *HMA4*) and tonoplast (i.e., *CAX4*, *HMA3*, *MRP7*, *MTP3,* and *COPT5*)	Yu et al. (2017)
Phytolacca americana	Cd	ZRT/IRT, ABC transporters, expansin (EXP), nicotianamine synthase (NAS), natural resistance-associated macrophage protein (NRAMP), metal-nicotianamine transporter (YSL)	The expression of transcription factors associated with absorption, transportation, and accumulation of heavy metals	Chen et al. (2017)
Oryza sativa	Cd	GST, MT, and DREB (drought-responsive element-binding protein) 1E, DREB/C-repeat binding factor (CBF), NRAMP1 (natural resistance-associated macrophage protein), HLH DNA-binding domain containing transcription factor (Os04g0301500)	Expression of Cd-responsive metal transporter gene was enhanced that helped the seedlings to survive under Cd stress	Oono et al. (2016)
Sedum alfredii	Cd	ARF4 (auxin response factor 4) and AAP3 (amino acid permease 3)	Enhanced expression of two hub genes, ARF4 (auxin response factor 4) and AAP3 (amino acid permease 3), played significant role in regulating Cd stress	Han et al. (2016)
Raphanus sativus	Cr	SPLs, MYBs, ERFs, and bZIPs	Enhancement in transcriptional processes like SPLs, MYBs, ERFs, and bZIPs. Expression of these transcription factors aids the plant under Cr stress	Liu et al. (2015a)
Oryza sativa	Cd	DREB/CBF, bZIP, NAC, AP2/ERFs (AP37, AP59), C2H2 zinc finger (ZFP252), TIFY (TIFY11), and MYB (Myb4)	Expression of transcripts related to metal transport which was found to be linked with tolerance under Cd stress	Oono et al. (2014)

(continued)

Table 1 (continued)

Plant	Metal	Transcription factors involved	Plants response	Reference
Arabidopsis thaliana	As	WRKY6	Coordinated action of transcription factors associated with uptake and transport of As as well as expression of some factors involved in signaling under stress condition	Castrillo et al. (2013)
Oryza sativa	Al	ASR5	Expression of transcription factors associated with Al tolerance was found to be enhanced thereby conferring resistance to the plant under stress condition	Arenhart et al. (2013)
Sedum alfredii	Cd	MYB, bHLH, bZIP	Upregulation in the expression of these transcription factors helped the plants to cope up with the damage induced by Cd and also helped in hyperaccumulating the metal	Gao et al. (2013)
Oryza sativa	As	ATP-binding cassette (ABC), phosphate transporter (PT), metal transporters (MT), ABC subfamily G (ABCG), DEGs, cation exchanger (CAX), cation diffusion facilitator (CDF), zinc iron permease (ZIP), copper transporter (CTR), and natural resistance-associated macrophage protein (NRAMP)	The expression of these transcription factors suggests toward their regulatory role under As stress. Apart from this, some transcriptional regulation took place at the level of hormonal signaling and lipid metabolism, and their further regulation was carried by miRNA	Yu et al. (2012)
Arabidopsis thaliana	Cu, Cd	WRKY22, WRKY25, and WRKY29	Overexpression of these transcription factors was found to be involved in enhancing stress responses by regulating MAPK and oxylipin signaling	Opdenakker et al. (2012)
Oryza sativa	–	ZIP39	Endoplasmic reticulum (ER) stress-responsive genes were found to be overexpressed and that helped the plants in regulating stress response	Takahashi et al. (2012)
Arabidopsis thaliana	Cd	bHLH38 and bHLH39	Expression of bHLH38 and bHLH39 enhanced Cd tolerance in plants as well as improved the homeostasis of Fe	Wu et al. (2012)

on *Arabidopsis*, abundance in proteins of Zn and Mg transporters was noticed; however, at transcriptome level, the factors were not found to be abundant (Elbaz et al. 2006). These differences in transcriptome and proteome expression could be resultant of changes at posttranscriptional and translational level (DalCorso et al. 2013). Therefore, it is mandatory to analyze the changes taking place at proteome levels and identify the target proteins under heavy metal detoxification process. Proteins directly participate in the detoxification process and expression profile of several antioxidants for detoxification of ROS and molecular chaperons for re-establishing the functionality of proteins, which were found to be enhanced (Zhao et al. 2011, Sharmin et al. 2012, Wang et al. 2012). Semane et al. (2010) reported that large subunit of RuBisCO-binding protein, proteins related with oxygen evolution, PS I and II were found to enhance under heavy metal stress. In a proteome study by Kieffer et al. (2008), Cd was found to affect the expression of proteins of carbon metabolism and oxidative stress in poplar plants. Moreover, accumulation of heat shock proteins, chaperons, foldases, and pathogenesis-related (PR) proteins was differentially regulated in roots and shoots (Kieffer et al. 2009). Another proteome analysis in arsenic-stressed *Anabaena* species showed abundance in phytochelatins that conferred tolerance (Pandey et al. 2012). Similar to this, Bona et al. (2011) showed that arsenic hyperaccumulator *Pteris vittata* enhances accumulation of phytochelatins as well as glutathione. Some studies in relation with proteome analyses under heavy metal stress have been shown in Table 2.

4.3 Metabolomics

Metabolome includes the role of metabolites like ascorbate, glutathione, phenols, proline, amino acids, etc. in conferring tolerance to heavy metal-stressed plants, and identification and quantification of these metabolites are referred to as metabolomics. Metabolites are important growth-regulating component of cells that are not only involved in regulating normal growth and development process but also aid the plant in combating the stress generated due to abiotic factors, especially heavy metals. The role of several amino acids like histidine and proline has been investigated, and studies showed that these are involved in chelating metal ions. Proline is reported to accumulate under metal stress (Yusuf et al. 2012) and maintain the redox status of cell by enhancing the synthesis of glutathione (Siripornadulsil et al. 2002). Similar to proline, histidine has also been reported to play an important role under heavy metal stress (Sharma and Dietz 2009). Chia et al. (2015) showed that Cd stress enhances the proline as well as histidine accumulation in *Chlorella vulgaris*, and accumulation of these metabolites helps in chelating the metal ions. Apart from amino acids, organic acids are also involved in conferring metal tolerance in the cellular system. Among the organic acids, malate, citrate, and others have been reported to play a crucial role in chelating the metal ions (Rauser 1999). Xu et al. (2012) showed that treatment with citrate enhanced the Cd accumulation in leaves rather than roots that might be linked to its sequestrating mechanism. In a recent

Table 2 Regulation at proteome level in heavy metal-stressed plants

Plant	Metal	Method employed	Plants response	Reference
Glycine max	Cd	2-DE, nano-liquid chromatography, tandem mass spectrometry	Abundance in glutamine synthetase ascorbate peroxidase, catalase, and superoxide dismutase that aids the seedlings to survive damages under cadmium stress. Increase in the molecular chaperone expressions for refolding of proteins and for their stabilization	Hossain et al. (2012a, b)
Glycine max	Cd	IPG, 2-DE, nanoLC-MS/MS	Cd-chelating proteins and amino acids were found to enhance; moreover, proteins for biosynthesis of lignin were also upregulated	Ahsan et al. (2012)
Lycopersicon esculentum	Cd	IPG, 2-DE, MALDI-TOF-MS, LIFT TOF-TOF	Cd stress enhanced the protein amount twofold higher, and out of these polypeptides some were identified for glycolytic pathway, TCA cycle, and respirations; moreover, some corresponded to proteins involved in detoxification	Rodriguez-Celma et al. (2010)
Oryza sativa	Cd	IPG, 2-DE, MALDI-TOF MS	Differential regulation of proteins like NADH-ubiquinone oxidoreductase, putative vacuolar proton-ATPase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, enolase, formyltetrahydrofolate synthetase, hypothetical protein OsJ_009523, glutathione reductase, alpha-1,4-glucan-protein synthase, guanine nucleotide-binding protein subunit beta-like protein, endo-1,3-beta-glucanase, L-ascorbate peroxidase 1	Lee et al. (2010)

Table 2 (continued)

Plant	Metal	Method employed	Plants response	Reference
Hordeum vulgare	Cd	iTRAQ labeling, MALDI-TOF/ TOF MS	Enhancement in inorganic pyrophosphatase and a gamma-tonoplast intrinsic protein (gamma-TIP) ratio of a CAX1a and natural resistance-associated macrophage protein (NRAMP), an ABC transporter homolog to AtMRP3	Schneider et al. (2009)
Ectocarpus siliculosus	Cu	IPG, 2-DE, MALDI-TOFMS	Proteins of PS II (proteins basically involved in OEC stabilization, glycolysis, and pentose phosphate), HSP70, and vBPO for protein stabilization and detoxification of ROS were enhanced	Ritter et al. (2010)
Oryza sativa	Cu	IPG, 2-DE, MALDI-TOF MS	Putative cytochrome and metallothionein proteins were upregulated	Zhang et al. (2009)
Lupinus albus	B	IPG, 2-DE, LC-MS/MS	Important proteins involved in glycolysis, TCA cycle and oxidation-reduction, and cell division were found to decline under deficiency of B	Alves et al. (2011)
Chlamydomonas reinhardtii	As	2DE, MALDI-TOF-MS, LC-MS/MS	Proteins were differentially modulated under As stress	Walliwalagedara et al. (2012)
Anabaena sp.	As	IPG, 2-DE, MALDI-TOF, and LC-MS	Proteins involved in maintaining glycolysis (FBA II, PGK, TK, FBPase, ATP synthase, Prx, Trx, oxidoreductase), pentose phosphate pathway, and calvin cycle were upregulated to overcome the As stress	Pandey et al. (2012)
Oryza sativa	As	IPG, 2-DE, MALDI-TOF MS, ESI-MS/MS	Proteins involved in metabolism and energy-generating processes were found to enhance under As stress, and some important proteins like that are involved in synthesis of RuBisCO, ribonucleoproteins were found to decline	Ahsan et al. (2010)

Table 2 (continued)

Plant	Metal	Method employed	Plants response	Reference
Miscanthus sinensis	Cr	2DE, MALDI-TOF, MALDI-TOF-TOF	Proteins like that of nitrate reductase, adenine phosphoribosyltransferase, formate, and IMPase were found to accumulate, as these are considered as Cr-responsive proteins	Sharmin et al. (2012)
Zea mays	Cr	2DE, MALDI-TOF-MS-MS	Differential expression in proteins leads to Cr toxicity	Wang et al. (2013)
Pseudokirchneriella subcapitata	Cr	IPG, 2-DE, LC-ESI-MS/MS	Target proteins like RuBisCO activase, RuBisCO, and light-harvesting protein complex that are involved in photosynthetic processes and amino acid metabolism were affected by Cr stress	Vannini et al. (2009)
Suaeda salsa	Hg	2-DE, MALDI-TOF, TOF-MS	Proteins associated with photosynthesis, stress responses, energy metabolism, signaling, etc. were altered	Liu et al. (2013)
Oryza sativa	Hg	2DE, ESI-MS/MS	Differential expression of proteins involved in homeostasis of redox status and hormones, molecular chaperon activity, transcriptional processes and regulation	Chen et al. (2012)
Arabidopsis thaliana	Zn	iTRAQ	Proteins related with FRO2, a ferric-chelate reductase, IRT1, an iron and zinc transporter, and V-ATPase were enhanced to aid the plant under Zn stress.	Fukao et al. (2011)
Populus sp.	Zn	2DE-MS	Proteins associated with photosynthesis, S assimilation, metabolism of sugar, chaperons and antioxidants proteins like APX, DHAR, GST were differentially regulated	Lingua et al. (2012)

(continued)

Table 2 (continued)

Plant	Metal	Method employed	Plants response	Reference
Brassica juncea	Ni	2DE, MALDI-TOF-MS	Proteins related with S metabolism, photosynthesis and ATP generation, antioxidants were enhanced to aid the plant under Zn stress	Wang et al. (2012)
Hordeum vulgare	Mn	2DIEF/SDS-PAGE, 2D blue native BN/SDS-PAGE	Proteins related to universal stress protein (Usp) family, i.e., inorganic pyrophosphatase, a probenazole-inducible protein (PBZ1), a chloroplast translational elongation factor (Tu), and ribosomal protein were differentially regulated	Führs et al. (2010)

study, the role of malic acid (a dicarboxylic acid) was investigated under Cr stress and found that this organic acid upregulates the activity of antioxidants, viz., super-oxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione peroxidase (GPX), and the enzymes of the gly-oxalase system as well as accumulation of metabolites, viz., ascorbate and glutathi-one, with a reduction in the accumulation of ROS (Mahmud et al. 2017). Plants deploy the synthesis of low molecular weight chelators, an important strategy for metal detoxification in cells, as these chelators scavenge heavy metal and dump them into the vacuole thereby reducing these metals from cytosol. Among the important chelators, two important heavy metal-binding proteins have been recog-nized, viz., PCs and MTs as mentioned above (Cobbett and Goldsbrough 2002). Biosynthesis and the functions of these two proteins are discussed in detail in the above sections. With the help of proteomic as well as metabolomics study, it has been suggested that phytochelatins are involved in detoxification of metal ions. Some studies in relation with metabolome analysis under heavy metal stress have been given in Table 3.

5   Conclusions and the Way Forward

Heavy metals accumulate in soils because of intense human activities. The basic aim of studying the heavy metal pollution/contamination and hyperaccumulator species is to unravel the physiological and molecular mechanisms underlying metal stress tolerance. Thus, one can explore the possibility of using such plants ulti-mately to sequester/remove metals from the sites of contamination/metal-rich areas.

Table 3 Regulation at metabolome level in heavy metal-stressed plants

Metabolite/antioxidants	Plant	Metal	Method employed	Plants response	Reference
Sugars, amino acids, organic acids, inorganic acids	*Raphanus sativus*	Cd, Pb	GC-MS	Sugar, amino acids, and organic acids were altered under Pb or Cd stress	Wang et al. (2015)
Glutathione (GSH)	*Arabidopsis thaliana*	Cd and Hg	Biochemical estimation	GSH was found to enhance metal tolerance by maintaining the redox status of the cell for proper functioning	Sobrino-Plata et al. (2014)
Antioxidative enzymes	*Eichhorniacrassipes*	Pb	Biochemical estimation	The activity of stress-related antioxidants like SOD, CAT, and APX. was found to enhance under Pb stress	Malar et al. (2014)
Ascorbate peroxidase, glutathione reductase and glutathione,	*Brassica juncea*	Ni and Zn	Biochemical estimation	Reduction in oxidative stress was noticed due to increased activity of the APX, GR, and GSH content	Khan and Khan (2014)
ascorbate, glutathione, polyamines	*Vigna radiata*	Cd	Biochemical estimation	Enhanced activity of antioxidants, i.e., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione S-transferase (GST), and glutathione peroxidase (GPX), and content of ascorbate, glutathione, phytochelatins and polyamines were noticed, and this increase helped the plant to survive under Cd stress	Nahar et al. (2016)
Proline and catalase	*Chlorella*	Pb and Cd	Biochemical estimation	Both the metabolites were found to enhance under the Pb and Cd stress	Subashchandrabose et al. (2017)
Proline	*Lactuca sativa*	Al	Biochemical estimation	Proline content was found to enhance under Al stress	Silva and Matos (2016)
SOD, POD, CAT, and APX	*Eichhorniacrassipes*	Pb	Biochemical estimation	APX and POD activity were found to enhance under the stress condition, while SOD and CAT activity were differentially regulated	Malar et al. (2014)

Metabolite/antioxidants	Plant	Metal	Method employed	Plants response	Reference
Proline, CAT, GSH, and POD	*Vicia faba*	Cd, Cu, Ni, Pb and Zn	Biochemical estimation	Enhancement in GPX activity and proline content was noticed	Nadgórska-Socha et al. (2013)
Vitamin E (α-tocopherol), ascorbate, and glutathione	*Arabidopsis thaliana*	Cd and Cu	Biochemical estimation	Differential expression of metabolites was noticed as vitamin E was found to increase, while ascorbate and glutathione content declined	Collin et al. (2008)
Amino acids (proline, histidine, and glutamine)	*Chlorella vulgaris*	Cd	Biochemical estimation	Enhanced accumulation of amino acids was noticed that helped in chelating metal ions within the cells	Chia et al. (2015)
Amino acid (proline and histidine)	*Solanum nigrum* and *Solanum torvum*	Cd	LC-MS/MS Q-TRAP	Enhanced biosynthesis as well as accumulation of amino acids, viz., proline and histidine, which suggests toward their involvement in improving Cd tolerance of the test seedlings	Xu et al. (2012)
Organic acid (citric acid)	*Solanum nigrum* and *Solanum torvum*	Cd	LC-MS/MS Q-TRAP	Biosynthesis of organic acid, i.e., citric acid, was enhanced in the seedlings treated with Cd that helped the plant to combat with the stress	Xu et al. (2012)
Organic acid (maleic acid)	*Brassica juncea*	Cr	Biochemical estimation	Exogenous supplementation improved the antioxidant as well as metabolite status within the cell thereby conferring tolerance to the cell	Mahmud et al. (2017)

In spite of an avalanche of information that has accumulated over the years with regard to heavy metal tolerance in plants, many aspects of this area still await explanation. Till date, we could not generate a plant that can withstand multiple heavy metals and useful for phytoremediation/phytomining strategies. It appears that the heavy metal-accumulating species get protection from the herbivores and other pathogens, but this needs to be further validated fully. Therefore, there is a need to generate plants either by breeding or using genetic engineering/genome editing tools immediately. Extensive research is needed under field conditions which can increase our understanding of heavy metal tolerance in plants/hyperaccumulators under various sites of metal contamination. Such an understanding would provide clues for removing the metals effectively from the contaminated sites, preventing their entry into food chain, and saving human and animal lives. The fact that metal accumulators may also serve as ideal genetic resources to develop plants with increased essential metal accumulation like Fe, Zn, and selenium and food crop biofortification for improving the health of the general public is a silver lining in this area of research.

Conflict of Interest Authors declare that they do not have any conflict of interests regarding the publication of this paper.

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