

Sudhakar Srivastava

Ashish K. Srivastava · Penna Suprasanna

*Editors*

# Plant-Metal Interactions

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# Preface

Metals have a widespread occurrence in the environment and are the basis of life on earth. The essential metals like zinc play several crucial roles in plants that their unavailability or deficiency leads to severe retardation of growth and development of the plant. However, at the same time, there are other metals like arsenic, cadmium, and chromium whose contamination has emerged as a problem of unprecedented scale in the last few decades. Due to the importance of metals in plants' life, the vast amount of research has been conducted on plant-metal interactions, which include morphological, anatomical, and physiological evaluation of plants' performance against deficiency or excess of a metal. The modern sophisticated techniques and instrumentation have led to rapid and thorough understating of responses of plants at gene, protein, and metabolite levels. This has resulted in enormous advancements in the field of "*plant-metal interactions*" from organ to tissue to cellular level responses with in-depth information about genes to proteins and metabolites. The present book includes a total of 13 chapters, dealing with various aspects of plant-metal interactions and present up-to-date knowledge on the subject.

The metal levels inside the plants are regulated via increased synthesis of a number of metal-complexing proteins, molecules, and peptides. In addition, the role of antioxidant enzymes is vital in the scavenging of toxic reactive oxygen species generated in response to toxic metals or in the presence of excess of essential metals. The holistic processes of metal homeostasis and detoxification are regulated through complex and intricate signaling and regulatory mechanisms. Chapter 1 of the book deals with transcriptomic, proteomic, and metabolomic approaches to delineate in-depth plant-metal interactions and gives an overview of metal-induced stress responses in plants and plants' mechanisms. Chapters 2, 3, and 4 deal with signaling-related aspects of metals in plants and with the roles of metal scavengers in plants and lucidly present the concepts and up-to-date knowledge on the topic. Furthermore, there are unique plants which are adapted to harsh conditions of metals and environment. Some plants can uptake and accumulate metals in exceptionally high amounts, while others can withstand excessive saline environments. Such hyperaccumulators and halophytic plants have been the keys to investigate tolerance mechanisms of plants by comparative evaluation with nonaccumulators and glycophytes,

respectively. Chapters 5 and 6 discuss the mechanisms of metal uptake and the accumulation and tolerance in hyperaccumulator and halophytic plants.

Arsenic contamination and toxicity are of great concern due to natural reasons behind the recent surge of arsenic in soil and groundwater in Southeast Asian countries. Arsenic toxicity to humans through rice is an important issue. Chapter 7 discusses the important aspects of arsenic uptake, transport, and detoxification in plants, while Chap. 8 discusses the metabolic modulations elicited in plants in response to arsenic stress. These chapters enhance our understanding of the arsenic problem to a great detail. The interactions of plants with another widely present contaminant metal, that is, chromium, are also discussed in fine detail in Chap. 9. Achieving sustainable and high crop yields is desirable, and therefore, understanding the impact of metals on crop production is crucial. Chapter 10 deals with the sources and effects of metals in crop productivity and discusses the impacts of both essential metals like copper, zinc, and nickel, and toxic metals such as cadmium, arsenic, chromium, lead, and mercury. In addition, Chap. 11 deals with the aspects of plant-microbe interactions in the context of metal tolerance of plants, while Chaps. 12 and 13 deal with phytostabilization and nanomaterials as a sustainable and feasible approach to deal with metal contamination issues.

The book therefore comprises a unique combination of chapters on various aspects and will provide the reader a comprehensive view of plant-metal interactions. The book covers metals whose contamination is widespread and which are of global environmental and public health concern like arsenic, cadmium, and chromium. This book would act as a guiding textbook for undergraduate and postgraduate students and as a mean to understand the latest research trends for doctoral students.

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# Contents

<b>An Integrated Transcriptomic, Proteomic, and Metabolomic Approach to Unravel the Molecular Mechanisms of Metal Stress Tolerance in Plants</b> . . . . .	1
Parul Parihar, Samiksha Singh, Rachana Singh, G. Rajasheker, P. Rathnagiri, Rakesh K. Srivastava, Vijay Pratap Singh, Penna Suprasanna, Sheo Mohan Prasad, and P. B. Kavi Kishor	
<b>Molecular Mechanism and Signaling Response of Heavy Metal Stress Tolerance in Plants</b> . . . . .	29
Rohit Joshi, Jeremy Dkhar, Sneh L. Singla-Pareek, and Ashwani Pareek	
<b>Heavy Metal Toxicity and Plant Productivity: Role of Metal Scavengers</b> . . . . .	49
P. L. Gratão, L. R. Alves, and L. W. Lima	
<b>Cadmium (Cd): An Emerging Regulatory Metal with Critical Role in Cell Signalling and Plant Morphogenesis</b> . . . . .	61
Renata Bączek-Kwinta	
<b>Heavy Metal Hyperaccumulator Plants: The Resource to Understand the Extreme Adaptations of Plants Towards Heavy Metals</b> . . . . .	79
Manoj Shrivastava, Ashish Khandelwal, and Sudhakar Srivastava	
<b>Halophytes and Heavy Metals: Interesting Partnerships</b> . . . . .	99
G. C. Nikalje, N. Saini, and Penna Suprasanna	
<b>Metabolome Modulation During Arsenic Stress in Plants</b> . . . . .	119
Preeti Tripathi and Rudra Deo Tripathi	
<b>Arsenic Transport, Metabolism, and Possible Mitigation Strategies in Plants</b> . . . . .	141
Kundan Kumar, Divya Gupta, Kareem A. Mosa, Kalidoss Ramamoorthy, and Pallavi Sharma	

<b>Plant-Chromium Interactions: From Toxicity to Remediation</b> . . . . .	169
Anoop Kumar Yadav, Pamela Jha, Neetin Desai, and Renitta Jobby	
<b>Metals, Crops and Agricultural Productivity:</b>	
<b>Impact of Metals on Crop Loss</b> . . . . .	191
Mitul Kotecha, Medhavi, Shivani Chaudhary, Naina Marwa, Farah Deeba, Vivek Pandey, and Vishal Prasad	
<b>Agroecotoxicological Aspect of Arsenic (As) and Cadmium (Cd) on Field Crops and its Mitigation: Current Status and Future Prospect</b> . . . . .	217
Debojyoti Moulick, Bhaben Chowardhara, and Sanjib Kumar Panda	
<b>Plant–Microbe–Metal (PMM) Interactions and Strategies for Remediating Metal Ions</b> . . . . .	247
Rahul Mahadev Shelake, Rajesh Ramdas Waghunde, and Jae-Yean Kim	
<b>Phytostabilization of Heavy Metals: Understanding of Principles and Practices</b> . . . . .	263
A. M. Shackira and Jos T. Puthur	
<b><i>In Planta</i> Synthesis of Nanomaterials for Environmental Remediation</b> . . . . .	283
Reshma V. Patil and Kiran D. Pawar	
<b>Index</b> . . . . .	309



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**Sudhakar Srivastava** is an Assistant Professor. He obtained his M.Sc. in Botany from the University of Lucknow in 2002 and his Ph.D. in Botany from the CSIR-National Botanical Research Institute (CSIR-NBRI) and University of Lucknow in 2008. After obtaining his Ph.D., he served as Scientific Officer in Bhabha Atomic Research Centre (BARC), Mumbai, from June 2009 to March 2014, and then moved to the Institute of Environment and Sustainable Development (IESD), Banaras Hindu University (BHU), in April 2014. He has more than 16 years of research experience in the field of plant-metal interactions with major interest on arsenic stress responses of plants. He has published 65 research articles and 20 review and letter articles in high-impact international and national journals. He has also published 20 book chapters in the books of international repute and few general articles in popular magazines and websites. He has also received several awards for his contributions, among which the notable ones are Young Scientist Award of the National Academy of Sciences, India (NASI), Allahabad, in 2011, Young Scientist Award from Uttar Pradesh Council of Science & Technology (UPCST) in 2013–2014, and Young Scientist Award from Science and Engineering Research Board (SERB) in 2015. He is an Associate Member of the National Academy of Agricultural Sciences (NAAS) and a Life Member of several societies as well as nongovernmental organizations. He is also an Associate Editor of reputed international journals, such as *Acta Physiologiae Plantarum*

and *Frontiers in Ecology and Evolution*. He is also an Expert Reviewer of several journals and project funding agencies.



**Ashish K. Srivastava** is presently working as Scientific Officer in Bhabha Atomic Research Centre, Mumbai. His research is focused on developing strategies for enhancing crop resilience toward different abiotic stresses. The stimulatory potential of thiourea (a reactive oxygen species scavenger) has been demonstrated for enhancing stress tolerance and crop productivity through lab and small-scale field experiments and by conducting multilocation field trials at salt- and drought-affected fields of Rajasthan. A small-scale technology has been developed for increasing yield and reducing arsenic accumulation from rice grains. Besides, thiourea has been used as a chemical probe for delineating redox regulatory components of stress tolerance. He has proposed a bifurcative model of thiourea action wherein redox homeostasis acts as “central” point to regulate gene expression through miRNA and hormones. To summarize, he is employing the combination of physiology, molecular biology, and biotechnology to understand crops behavior under stress and develop suitable strategy to minimize stress-induced yield losses in different crop plants. He has more than 40 research and review articles and book chapters to his credit. He has been awarded Young Scientist Award of the National Academy of Sciences, India (NASI), Allahabad, in 2018, Young Scientist Medal from the Indian National Science Academy (INSA) in 2014, and Young Scientist Award from the Department of Atomic Energy (DAE) in 2014. He has also received President International Fellowship Award from the Chinese Academy of Sciences, China, in 2016 and EMBO Short-Term Fellowship in 2011.



**Penna Suprasanna** is a Senior Scientist and Head of Plant Stress Physiology and Biotechnology Group, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, and is Professor, Homi Bhabha National Institute, Department of Atomic Energy, Mumbai. He made significant contributions to crop biotechnology research through radiation-induced mutagenesis, plant cell and tissue culture, genomics, and abiotic stress tolerance. His research on radiation-induced mutagenesis and in vitro selection in sugarcane yielded several agronomically superior mutants for sugar yield and stress tolerance. He has made intensive efforts to apply radiation mutagenesis techniques in vegetatively propagated plants through collaborative research projects with several national and international bodies (IAEA, Vienna). He is also serving as the DBT Expert/Nominee on Biosafety committees. He is the Recipient of the “Award of Scientific and Technical Excellence” by the Department of Atomic Energy, Government of India and is the Fellow of Maharashtra Academy of Sciences, Andhra Pradesh Academy of Sciences, Telangana Academy of Sciences, and Association of Biotechnology. He has published more than 250 research papers/articles in national and international journals/books, edited two Springer books, and has guided several doctoral students. His research is focused on molecular biology of abiotic stress tolerance and salt-stress adaptive mechanisms in plants. The research group led by him has successfully identified novel microRNAs and plant bioregulators besides validating the concept of redox regulation toward abiotic stress tolerance and crop productivity.

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



Parul Parihar, Samiksha Singh, Rachana Singh, G. Rajasheker, P. Rathnagiri, Rakesh K. Srivastava, Vijay Pratap Singh, Penna Suprasanna, Sheo Mohan Prasad, and P. B. Kavi Kishor

## 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 decrease in micronutrient concentrations. Yoshihara et al. (2006) reported Cd-induced iron deficiency responsive genes like *HvIDS2pro::GUS*, *NtFRO1*, and *NtIRT1* 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, Feleafeh 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, Feleafeh 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 ( $\gamma$ -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 *Phytochelatin and Their Induction*

PCs are a family of cysteine-rich polypeptides with general structure ( $\gamma$ -Glu-Cys) $_n$ -X, in which X represents Gly,  $\gamma$ -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<sub>2</sub> and PC<sub>3</sub> (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<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup>, Ag<sup>2+</sup>, and others. Glutamate and cysteine are the precursors for the biosynthesis of  $\gamma$ -glutamylcysteine, and the reaction is catalyzed by  $\gamma$ -glutamylcysteine (EC) synthetase ( $\gamma$ -ECS) in plants. Glycine molecule is then added to this dipeptide, and the enzyme associated with this biochemical conversion is ATP-dependent glutathione synthetase.  $\gamma$ -ECS is converted to  $\gamma$ -glutamylcysteine glycine ( $\gamma$ -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  $\beta$ -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,  $\beta$ -alanine-specific ATP-dependent enzyme homoglutathione synthase utilizes  $\gamma$ -Glu-Cys to produce  $\gamma$ -Glu-Cys- $\beta$ -Ala (homo-phytochelatins as in the case of Leguminosae family). On the other hand, in members of the family Poaceae, biosynthesis of  $\gamma$ -Glu-Cys-Ser [(Glu-Cys)<sub>n</sub>-Ser] is common (Klapheck et al. 1992). Klapheck et al. (1992) found that hydroxymethyl-GSH( $\gamma$ -Glu-Cys-Ser), which upon exposure to metals,

produces homologous peptides [(Glu-Cys)<sub>n</sub>-Ser] and later named as hydroxymethyl-PCs. However, *Zea mays* is an exception to this, where  $\gamma$ -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-phytochelatin. In addition to PCs, iso-PCs, peptides without C-terminal amino acids represented as (g-Glu-Cys)<sub>n</sub> 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 Gabbriellini (1999) that PCs complex with metal ions such as Cd<sup>2+</sup> 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  $\alpha$  and  $\beta$  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, Ruttkey-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

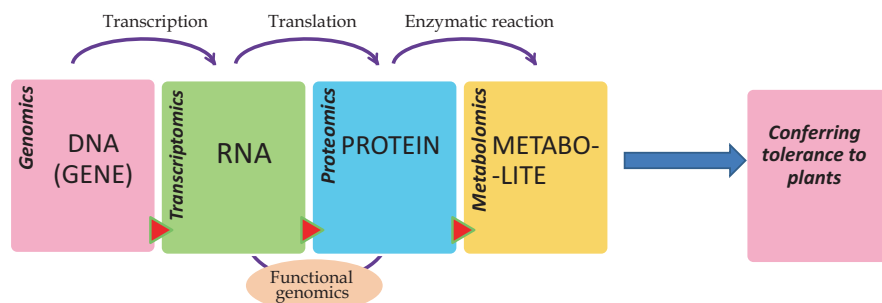
MTs bind not only to toxic metals such as Cd but also to other transition metals that are essential for plant nutrition like  $\text{Cu}^+$  and  $\text{Zn}^{2+}$  (Freisinger 2011). The binding of heavy metals to MTs is crucial for detoxification of heavy metals. At the same time, transport of Zn and 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  $\text{Cd}^{2+}$  along with four  $\text{S}^{2-}$  molecules (Zimeri et al. 2005). Besides, MT protein also binds to four  $\text{Zn}^{2+}$  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
<i>Brassica rapa</i>	Cd	ZIPs, P <sub>1B</sub> -type ATPase, and MTPs	Enhanced expression of transcription factors associated with plasma membrane (i.e., <i>ZIP2</i> , <i>ZIP3</i> , <i>IRT1</i> , <i>HMA2</i> , and <i>HMA4</i> ) and tonoplast (i.e., <i>CAX4</i> , <i>HMA3</i> , <i>MRP7</i> , <i>MTP3</i> , and <i>COPT5</i> )	Yu et al. (2017)
<i>Phytolacca americana</i>	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)
<i>Oryza sativa</i>	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)
<i>Sedum alfredii</i>	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)
<i>Raphanus sativus</i>	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)
<i>Oryza sativa</i>	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
<i>Arabidopsis thaliana</i>	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)
<i>Oryza sativa</i>	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)
<i>Sedum alfredii</i>	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)
<i>Oryza sativa</i>	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)
<i>Arabidopsis thaliana</i>	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	Opendakker et al. (2012)
<i>Oryza sativa</i>	–	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)
<i>Arabidopsis thaliana</i>	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 (Rausser 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 sequestering mechanism. In a recent

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

Plant	Metal	Method employed	Plants response	Reference
<i>Glycine max</i>	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)
<i>Glycine max</i>	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)
<i>Lycopersicon esculentum</i>	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)
<i>Oryza sativa</i>	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)

(continued)

**Table 2** (continued)

Plant	Metal	Method employed	Plants response	Reference
<i>Hordeum vulgare</i>	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)
<i>Ectocarpus siliculosus</i>	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)
<i>Oryza sativa</i>	Cu	IPG, 2-DE, MALDI-TOF MS	Putative cytochrome and metallothionein proteins were upregulated	Zhang et al. (2009)
<i>Lupinus albus</i>	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)
<i>Chlamydomonas reinhardtii</i>	As	2DE, MALDI-TOF-MS, LC-MS/MS	Proteins were differentially modulated under As stress	Walliwagedara et al. (2012)
<i>Anabaena</i> 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)
<i>Oryza sativa</i>	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)

(continued)