Chapter 11
Analysis of Signaling Pathways During Heavy Metal Toxicity: A Functional Genomics Perspective

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Abstract Abiotic stresses have major limiting factors for plant growth and crop productivity. Plants have different mechanisms to maintain the physiological concentrations of essential metal ions and to minimize exposure to non-essential heavy metals. Some mechanisms are ubiquitous because they are also required for general metal homeostasis, and they minimize the damage caused by high concentrations of heavy metals in plants by detoxification, thereby conferring tolerance to heavy metal stress. Metals in the cell are addressed using a range of storage and detoxification strategies, including metal transport, chelating, trafficking, and sequestration into the vacuole. A large number of genes encoding MAPK pathway components have a major role in cell proliferation and hormone action as well as in stress signaling. Germin-like protein genes were developed by various stresses including metal stress. Functional genomics (integrating genome sequencing, transcriptomics, proteomics, metabolomics, ionomics, and phenomics) allows large-scale gene function analysis with high-throughput technology and incorporates interaction of gene products at cellular and organism level.

Keywords Metal toxicity • Metal transport • Oxidative stress • Metal-binding protein • Signaling pathway

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G.K. Pandey (ed.), *Elucidation of Abiotic Stress Signaling in Plants*,
DOI 10.1007/978-1-4939-2540-7_11

11.1 Introduction

Agriculture faces various abiotic stresses, especially salinity and drought as major limiting factors for crop productivity. Cold, temperature, heavy metals, and UV radiation are also the abiotic factors affecting crop yields (Qin et al. 2011). Some of the essential micronutrients playing a key role in many metabolic mechanisms can be toxic when the content in tissues is higher than optimal. Abiotic stress is one of the primary causes of crop loss, causing average yield losses of more than 50 % for major crops. Plants can resist abiotic stresses through various distinct mechanisms. Both tolerance and susceptibility to abiotic stresses are very complex mechanisms. Plant traits that are associated with resistance processes are multigenic and thus difficult to control and engineer. Metals like copper, iron, nickel, and zinc are essential for the functioning of physiological and biochemical processes for normal growth and development of plants (Marschner 1995; Broadley et al. 2007). However, elements such as aluminum, cadmium, and lead are considered to be non-essential and generate toxic responses even at low exposure concentrations. An excess of toxic metals commonly has a negative impact on physiological and biochemical processes in organisms, resulting in major risks to the environment and for human health. Plants growing on metal-enriched soils suffer from decreased growth and performance, reducing crop yield. At the molecular level, oxidative stress is widely studied as a key sign of plant stress. This process is commonly described as an imbalance between reactive oxygen species (ROS) and antioxidants. ROS are not only generated from the normal metabolic activity of mitochondria and chloroplasts but also produced during both abiotic and biotic stresses (Neill et al. 2002; Nakata et al. 2002; Patnaik and Khurana, 2001). ROS can undergo further reactions, often catalyzed by metal ions as in the Fenton reaction, and so generate the much more reactive hydroxyl radical, which may be responsible for alterations of macromolecules and ultimately may contribute to cell death (Briat and Lebrun 1999). Sharma and Dietz (2009) reported the close relationship between metal toxicity, redox homeostasis, and antioxidant capacity in plants. Depending on the chemical properties of metals in biological systems, their toxicity is attributed to various mechanisms such as (1) interference with functional sites in proteins; (2) displacement of essential elements, thereby disturbing enzymatic functions; or (3) increased ROS production. High concentrations of heavy metals increase the ROS production in subcellular organelles such as peroxisomes, chloroplasts, and mitochondria, which together constitute the predominant sources of ROS production in plants. Keunen et al. (2011) reported that the mitochondria are key players in cellular redox homeostasis and signaling (Fig. 11.1).

Enzymatic degradation of superoxide is ensured by superoxide dismutases, while that of hydroperoxides is ensured by catalase, glutathione peroxidase, or ascorbate peroxidase (Chaudiere and Ferrari-Iliou 1999; Foyer and Noctor 2005). High peroxidase activity is also reported as a defensive mechanism, which may cause damage or disturb the normal functions of plant cells (Fang and Kao 2000).

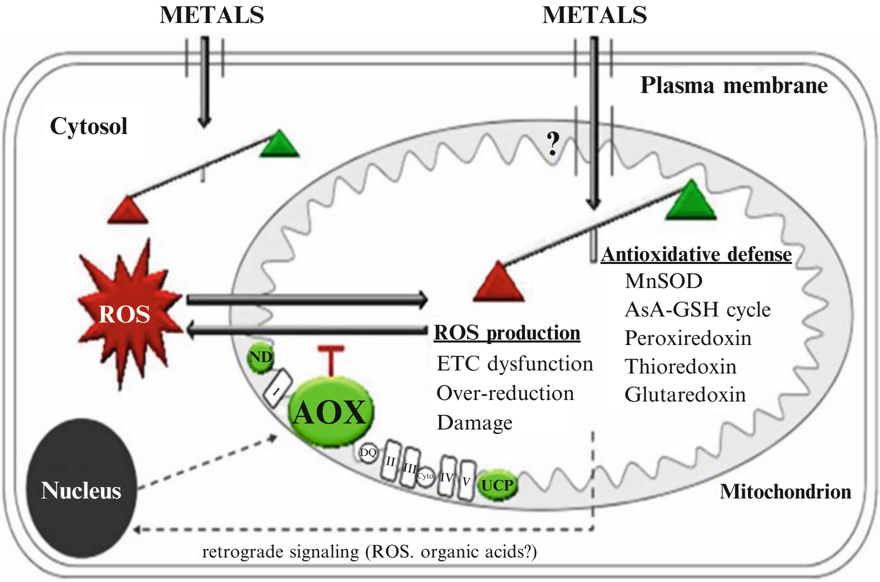

Fig. 11.1 Schematic overview of metal-induced responses in plant cells focusing on mitochondrial effects. Metal exposure has shown to cause mitochondrial electron transport chain dysfunction and over-reduction, thereby increasing mitochondrial ROS production (Keunen et al. 2011)

However, H_2O_2 has been shown to induce cell protection genes and has been shown to act as a diffusible compound, which mediates the regulation of gene expression (Desikan et al. 2001; Vanderauwera et al. 2005). Heavy metal absorption has affected seriously the quality and safety of harvested crop material; hence, an understanding of the biochemical detoxification strategies that plants can adopt against oxidative stress induced by metal ions in plants is key to manipulating metal tolerance in plants (Dixit et al. 2001). Danquah et al. (2014) reported that plants have specific mechanisms that allow them to rapidly perceive and respond to abiotic stresses in the environment. Among the evolutionarily conserved pathways, the ABA (abscisic acid) signaling has been identified as a central regulator pathway of abiotic stress response in plants, triggering major impact in gene expression and adaptive physiological responses. ABA induces protein kinases of the SnRK family to mediate a number of its responses. MAPK (mitogen-activated protein kinase) cascades have also been shown to be implicated in ABA signaling. Seo and Koshiba (2002) reported that except for the conversion of xanthoxin to ABA in the cytoplasm, all the steps for ABA synthesis occur in plastids. The early C5 precursor of ABA, isopentenyl pyrophosphate (IPP), is produced primarily in plastids via 1-deoxy-D-xylulose-5-phosphate (DXP) from pyruvate and glyceraldehyde-3-phosphate (Cutler and Krochko 1999; Nambara and Marion-Poll 2005; Seo and

Koshiba 2002; Wasilewska et al. 2008). This leads to the sequential production of farnesyl pyrophosphate, geranylgeranyl pyrophosphate (GGPP), phytoene, lycopene, and β-carotene. β-Carotene is converted to a xanthophyll, zeaxanthin, which is the first oxygenated carotenoid as reported by Seo and Koshiba (2002). Subsequent steps involve the synthesis of cis-isomers of violaxanthin and neoxanthin that are cleaved to form xanthoxin (the C15 precursor of ABA). Xanthoxin is presumed to migrate from the plastid to the cytosol (Nambara and Marion-Poll 2005) and converted to ABA through abscisic aldehyde, xanthoxic acid, or abscisic alcohol (Seo and Koshiba 2002). Cell-to-cell ABA transport was shown to be mediated by two plasma membrane-bound ATP-binding cassette (ABC) transporters and a family of low-affinity nitrate transporters (Kang et al. 2010; Kuromori et al. 2010; Kanno et al. 2012). Most ABC transporters are integral membrane proteins and act as ATP-driven transporters for a very wide range of substrates, including lipids, drugs, heavy metals, and auxin. Kuromori et al. (2010) isolated AtABCG25, which encodes a half-size ABC transporter protein and is responsible for ABA transport and responses in *Arabidopsis*. Kanno et al. (2012) isolated ABA-importing transporter 1 (AIT1) (which is also known as low-affinity nitrate transporter; NRT1.2) from a modified yeast-2-hybrid screen in which positive clones are capable of inducing interactions between the ABA receptor PYR/PYL/RCAR and PP2C protein phosphatase at low concentrations of ABA. AIT1 was preferentially localized to the plasma membrane of plant cells and was mainly expressed in vascular tissues in cotyledons, true leaves, hypocotyls, roots, and inflorescence stems.

11.2 Molecular Basis of Plant Responses to Heavy Metal Stress/Toxicity

Metals are required by plants in a wide range of concentrations. During the evolution of an angiosperm, the metal requirements are strongly steered by the demands of physiological processes in different organelles, cells, tissues, and whole plants. At the cellular level, the function of the cell and the presence of specific organelles determine the metal content. For example, in photosynthetic active cells, the metal demand of the chloroplasts varies from 15 % of the total cell; Zn in the carbonic anhydrase (Hewitt 1983) to nearly 50 % of the total cell, Cu in the plastocyanin (Hewitt 1983; Lolkema and Vooijs 1986). In mitochondria, however, Cu enzymes are already satisfied with 3–6 % of total cell of Cu (Peng et al. 2005) and Fe enzymes with 2–4 % of total cell of Fe (Hewitt 1983). The original compartmentalization concept (Ernst et al. 1974) has received much support during the past decade. At the cellular level, protection of physiologically active sites in the cell is achieved by a rapid cellular compartmentalization of the metal surplus, especially into the vacuole. The role of metal-binding metabolites in the cytosol is elaborated for Cd- and Cu-tolerant plants (Mengoni et al. 2003). The removal of surplus metals from the cytosol and their transport across the tonoplast are accelerated in metal-tolerant plants (Drager et al. 2004).

11.2.1 Heavy Metal-Induced Signaling in Plants

Metal-induced toxicity is very well reported. Several essential metals like iron, zinc, copper, and manganese participate in controlling the various metabolic and signaling pathways. One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress. The response to heavy metal stress involves a complicated signal transduction network that is activated by sensing the heavy metal and is characterized by the synthesis of stress proteins and signaling molecules and, finally, the transcriptional activation of specific metal-responsive genes to counteract the stress (Maksymiec 2007). The signal transduction pathways include the Ca–calmodulin system, hormones, ROS signaling, and the mitogen-activated protein kinase (MAPK) phosphorylation cascade, which converge by activating the stress-related genes. Different signaling pathways may be used to respond to the different heavy metals (DalCorso et al. 2010; Thiele 1992; Skórzynska-Polit et al. 1998). Ca^{2+} signaling features in responses to a number of abiotic stress factors, including temperature extremes, osmotic stress, oxidative stress, anoxia, and mechanical perturbation (Knight 1999). Excess heavy metals modify the stability of Ca^{2+} channels, thus increasing calcium flux into the cell. Intracellular Ca^{2+} is a secondary messenger, which interacts with calmodulin to provide the signal and ultimately to regulate downstream genes involved in heavy metal tolerance, metabolism, and transport (Yang and Poovaiah 2003). The Ca^{2+}–calmodulin system is also involved in the response to other heavy metal toxicity, such as Ni and Pb. Plant hormones are involved in many physiological and developmental processes and play a critical role in the adaptation to abiotic stress through the regulation of hormone synthesis in the presence of heavy metals (Peleg and Blumwald 2011). Heavy metals can produce ROS directly via the Fenton and Haber–Weiss reactions and indirectly by inhibiting antioxidant enzymes (Romero-Puertas et al. 2007). In particular, H_2O_2 acts as a signaling molecule in response to heavy metals and other stresses (Dat et al. 2000). Cuypers et al. (2000) reported a root-to-shoot signaling system that appears to be involved in copper-imposed oxidative stress as well as in the antioxidative defense response (Cuypers et al. 2002). Vitoria et al. (2001) reported the significant role in cadmium-induced increases in catalase, superoxide dismutase, and glutathione reductase activity in both leaves and roots of radish seedlings and also suggested that an oxidative stress signal is sent from roots to leaves. Verma and Dubey (2003) observed that the lipid peroxides in shoots were increased with elevated oxidative stress. The rationale for increased proline in a plant is linked with the ability of proline to quench singlet oxygen (Özturk and Demir 2002), which may arise as a by-product of lipoxygenase in the presence of Cu^{2+} (Arora et al. 2002). Proline can also react directly with the hydroxyl radicals that might result from metal-catalyzed Fenton chemistry, and therefore, increased proline would mitigate the damage from free radicals and leads to a more reduced cellular environment (Siripornadulsil et al. 2002).

Arasimowicz-Jelonek et al. (2012) reported that the roots of 3-day-old yellow lupine (*Lupinus luteus*) seedlings exposed to cadmium (89 mM $CdCl_2$) resulted in programmed cell death starting from 24 h of stress duration, which was evidenced by

TUNEL-positive reaction. Cd-induced programmed cell death was preceded by a relatively early burst of nitric oxide (NO) localized mainly in the root tips. These changes were accompanied by the NADPH oxidase-dependent superoxide anion (O) production. However, the concomitant high level of NO in 24 h of Cd exposure did not provoke an enhanced peroxynitrite formation. But the treatment with the NADPH oxidase inhibitor and NO scavenger significantly reduced superoxide anion and NO production, respectively, as well as diminished the pool of cells undergoing cell death.

11.2.2 Heavy Metal Ion Uptake and Its Translocation

In the biological system, cellular location and metal specificity of most of these transporters in plants are still unknown. The cell wall can play a key role in the immobilization of toxic heavy metal ions by providing pectic sites and histidyl groups, and extracellular carbohydrates such as callose and mucilage, and thus prevents heavy metal uptake into the cytosol. Metal availability and motility in the rhizosphere are influenced by root exudates and microorganisms (Wenzel et al. 2003). Higher plants possess highly effective systems for the acquisition of metal ions and other inorganic nutrients from the soil. One of the major roles of root exudates is to chelate metals and to prevent their uptake inside the cells. The binding of metal ions such as Cu and Zn in the apoplast also helps to control the metal content of root cells (Dietz 1996; Kobae et al. 2006). Cation-binding sites are also present on the root cell wall, and this allows metal exchange, thus influencing the availability of ions for uptake and diffusion into the apoplast (Allan and Jarrel 1989). Plants possess various form of plasma membrane transporters involved in metal uptake and homeostasis. Some of the transporters belong to the heavy metal P1B-ATPase, the NRAMP, and the CDF (Williams et al. 2000). Guerinot (2000) reported that ZIP family is one of the principal metal transporters involved in metal uptake. ZIP family of transporters have also been identified in many plant species and are involved in the translocation of divalent cations across the membranes. Certain ZIP proteins are induced in *A. thaliana* roots and shoots in response to Fe or Zn loading and thus appear to be part of a stress response. Most ZIP proteins are predicted to comprise eight transmembrane domains and have a similar topology, with the N- and C-termini exposed to the apoplast, and a variable cytoplasmic loop between transmembrane domains III and IV that contains a histidine-rich domain putatively involved in metal-binding site (Guerinot 2000). The first ZIP transporter to be characterized was *A. thaliana* IRT1. This was identified by functional complementation of the *S. cerevisiae efet3fet4* double mutant, which is impaired in iron transport (Eide et al. 1996). In *A. thaliana*, IRT1 is expressed in root cells and accumulates in response to iron deficiency, suggesting a role in Fe^{2+} uptake from the soil (Vert et al. 2002). Many metal transporters present at low ion selectivity (Korshunova et al. 1999). The plasma membrane plays an important role in plant response to heavy metals by reducing the uptake of metals into the cell or by active efflux pumping outside the cell. ABC transporters are also involved in metal ion efflux from the plasma membrane as reported by Kim et al. (2007).

11.2.3 Metal Sequestration in the Vacuole by Tonoplast Transporters

Plants respond to high intracellular concentrations of metal ions by using efflux pumps either to export the ions to the apoplast or to compartmentalize them within the cell. The main storage compartment for metal ions is the vacuole, which in plants accounts for up to 90 % of the cell volume (Vögeli-Lange and Wagner 1990). Several families of intracellular transporters involved in this process have been identified in plants and yeast, and they appear to be highly selective. Phytochelatins (PC) are the best-characterized heavy metal chelators in plants, especially in the context of Cd tolerance (Cobbett 2000). PCs are a family of metal-binding peptides with the general structure (c-Glu-Cys)nGly ($n=2$–11) (Cobbet and Goldsbrough 2002). The cysteine thiol groups allow PCs to chelate metals and form complexes with a molecular weight of 2.5–3.6 kDa (Cobbett 2000). PCs are synthesized in the cytosol and then transported as complexes to the vacuole. Their synthesis is rapidly activated in the presence of heavy metals such as Cd, Cu, Zn, Ag, Au, Hg, and Pb (Cobbett 2000). ABC transporters can transport xenobiotics and heavy metals into the vacuole and two subfamilies (MRP and PDR) particularly active in the sequestration of chelated heavy metals. Some of the CDF transporter family (also called MTPs in plants) are involved in the transport of metal ions from the cytoplasm to the vacuole (Krämer et al. 2007) and to the apoplast and endoplasmic reticulum (Peiter et al. 2007). CDF transporters have been characterized primarily in prokaryotes but are also found in many eukaryotes, where they transport divalent metal cations such as Zn, Cd, Co, Fe, Ni, and Mn (Montanini et al. 2007). The MAPK cascade in plants is a response to both biotic and abiotic stresses (He et al. 1999). It involved three kinases sequentially activated by phosphorylation: the MAPK kinase kinase (MAPKKK), the MAPK kinase (MAPKK), and the MAPK. At the end of this cascade of phosphorylation, MAPKs phosphorylate different substrates in various cellular compartments, including transcription factors in the nucleus. The MAPK cascade allows the transduction of the information to downstream targets. Four isoforms of MAPK were shown to be activated in alfalfa (*Medicago sativa*) seedlings exposed to Cu or Cd (Jonak et al. 1996), and a MAPK gene is also activated by Cd treatment in rice (Yeh et al. 2004). All these signaling pathways finally converge in the regulation of transcription factors that activate genes required for stress adaptation, particularly in the context of heavy metals.

11.3 Heavy Metal Toxicity-Induced Genes and Proteins and Its Identification

Heavy metal like aluminum (Al) inflicts a wide range of cellular injuries in plants that affect and reduced the root growth, nutrient and water uptake, and productivity. Duressa et al. (2010) reported that several genes might potentially have influence on soybean aluminum tolerance. Two transcription factors, cell wall metabolism enzymes and a cell proliferation gene, are interesting from the perspective of the physiological and molecular mechanisms of Al tolerance. The first transcription factor, *Cys2His2* zinc finger protein, coregulates molecular response to proton and aluminum toxicities, the major acid soil stress factors (Luchi et al. 2007). The second transcription activator, *ADR6*, is an auxin downregulated gene. Aluminum suppresses the biosynthesis of auxins and transport in root system, which might be one possible mechanism of aluminum-induced root growth inhibition (Kumari et al. 2008). They also stated that *ADR6* is triggered under Al stress acting in a parallel pathway to restore root growth under Al stress. Root cell wall rigidification by Al binding is one of the principal mechanisms of Al toxicity. Contreras-Porcia et al. (2011) reported that 18 selected genes were copper responsive, half of the isolated genes encode putative organellar proteins, the identified proteins are principally involved in antioxidant metabolism and in cellular and organellar repair, and these proteins may act in a coordinated and additive or synergistic manner to ensure copper acclimation and tolerance. Metallothioneins are small cysteine-rich proteins (50–70 amino acids) and are known to play a role in detoxifying heavy metals by sequestration using cysteine residues (Bertini et al. 2000). Zhang et al. (2009) identified that 16 Cu-responsive proteins of low molecular weight will increase our understanding of plant mechanisms of Cu toxicity and tolerance. Two rice-seed embryo proteins, RicMT (OsMT2c) and CYP90D2, were among those most markedly affected by Cu treatment. The level of RicMT increased fivefold, whereas CYP90D2 decreased sixfold. Three proteins, a putative small cytochrome P450 (CYP90D2), a putative thioredoxin, and a putative GTPase, were downregulated by Cu stress. In plants, metallothioneins are thought to play an important role in metal tolerance and homeostasis (Cobbet and Goldsbrough 2002). They reported that C2 domain protein and LysM receptor-like kinases are more responsive to heavy metal stress and leaf senescence. Two possible pathways, i.e., senescence signals by the membrane-bound lysine receptor-like kinase 1 (LysMR 1) by interaction with specific factors and consequently activating downstream signaling cascades. The phosphorylation of kinase domain of the LysMR1 may activate a signaling protein such as a mitogen-activated protein kinase known to be induced during heavy metal stress or phosphatase to trigger a cascade to the nucleus and finally activate a target gene. In the second pathway, once heavy metals are in the cytoplasm, a redox signal may also be generated leading to the production of reactive oxygen species (ROS). It is known that reactive oxygen species such as H_2O_2 activate the membrane Ca^{2+} channels and mediate the influx or release of Ca^{2+} from internal stores. This generates an increase in Ca^{2+} concentration. In addition, ROS activate directly the mitogen-activated protein kinase cascade. Furthermore, the Ca^{2+} signal may activate a C2 domain protein and its Ca^{2+} binding triggers interaction with the transcription factor to form a protein complex and their translocation to the nucleus. Finally, the transcription factor interacts with its corresponding *cis*-acting element on target of the promoter to regulate gene expression (Fig. 11.2).

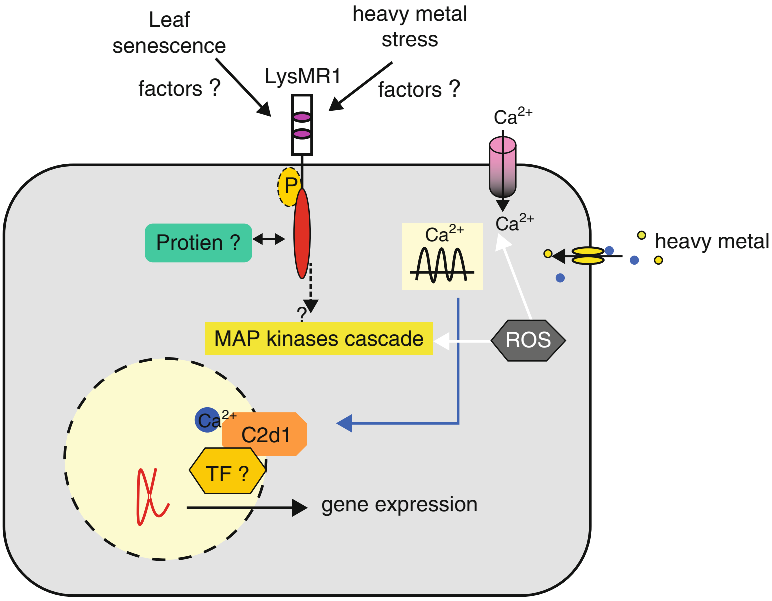

Fig. 11.2 Model for the functional role of LysMR1 and C2 domain protein in plant perception of heavy metal and senescence signals

11.4 Functional Genomics of Heavy Metal Tolerance in Plants: Approaches and Achievements

Crop plants are being constantly exposed to heavy metal toxicity; they respond differentially at hostile environments in concern with several abiotic and biotic stresses, the composition of ions and its concentration, plant habit, and developmental phase of plant growth. These plant responses mostly include a coordinated network of molecular processes such as reduced uptake or increased plant internal sequestration along with multiple metal-detoxifying mechanisms, overexpression of numerous stress-related proteins, glutathione-mediated tolerance pathways, repair systems, and cascade of signaling molecules; as a consequence, the plant became either heavy metal stress tolerant or stress susceptible. The intricacy of heavy metal tolerance response involves the set of molecular regulation, which need to be understood to yield heavy metal-tolerant plant. This would require analysis of the function of numerous genes involved in stress responses imparting tolerance by way of genomic approaches that would help to assign cellular function to each gene. Information about such stress-responsive genes has been obtained largely using conventional approaches. However, the challenge still remains to integrate the function of these genes logically to generate a global understanding of the stress response process (Bohnert et al. 2006; Valliyodan and Nguyen 2006; Vij and Tyagi 2007;

Sheldon and Roessner 2013). Integrating genome sequencing, transcriptomics, proteomics, metabolomics, ionomics, and phenomics allows large-scale gene function analysis with high-throughput technology and incorporates interaction of gene products at cellular and organism level. The complete genome sequence of rice and *Arabidopsis* and emerging sequence information for several other plant genomes, such as *Populus*, *Medicago*, lotus, tomato, pigeon pea, and maize, have enriched the genetic information to employ the tools for the determination of the function of many genes simultaneously (The Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005; Bell et al. 2001; Nakamura et al. 2002; Rensink and Buell 2005; Vij et al. 2006; Ranjan et al. 2012; Singh et al. 2012; Varshney et al. 2012). The use of in silico gene discovery, high-throughput gene expression, altered gene expression by transgenesis, functional characterization of genes of interest via gene-inactivation techniques, and genetic and genomic approaches in understanding the basis of abiotic stress tolerance has been initiated in several plant species (Vij and Tyagi 2007; Sreenivasulu et al. 2007; Sheldon and Roessner 2013). Although these functional genomic tools have been utilized for molecular depiction of abiotic stress response in various plant species, the exploitation of these tools is in its infancy for heavy metal response in plant species. Among the heavy metals reported, cadmium is considered the most phytotoxic and hazardous due to its high solubility, absorption by plants, and radical introgression into the food chain (Lux et al. 2010; Gill et al. 2011; Thapa et al. 2012). Hence, several studies were undertaken in expression genomics, proteomics, metabolomics, positional cloning, and ectopic gene expression and in depicting miRNA target to elucidate the molecular basis of cadmium tolerance in plants.

Gene expression is highly affected under cadmium stress and is up- or downregulated. Hence, the expression of a group of gene(s) during different stages reflects on functional relevance. Expression genomic approaches such as array-based transcript profiling technologies, serial analysis of gene expression (SAGE), differential display and subtractive hybridization, and quantitative real-time PCR (qRT-PCR) are employed to depict high-throughput expression of various genes in control and Cd stress-treated tissues at various developmental stages. It also hypothesized that various genes involve as key players of Cd tolerance, viz., Cd sensors, Cd-induced signaling protein kinases, aquaporins/transporters, *cis*-regulatory elements, transcription factors, hormone biosynthesis, etc., in different plants (Table 11.1).

Proteomics, the systematic analysis of stage-specific protein complements expressed by the genome, is a powerful tool for describing complete proteomes of the cell, cell organelle, and plant tissue (Porubleva et al. 2001; Agrawal and Rakwal 2006) and also for comparing proteomes under different stresses imposed by the environment on plant systems (Vij and Tyagi 2006). The combination of 2-DE and mass spectrophotometry (ESI and MALDI-TOF) proved to be very efficient for proteome characterization (Mann et al. 2001) and were recently upgraded with fluorophores (DIGE) and LC-MS for more precise characterization of proteome. All these techniques were used for proteomic studies in *Arabidopsis*, rice, barley, soybean, barley, and several medicinal plants (Table 11.2).

Table 11.1 Cadmium-responsive genes and transcripts identified using tools of expression genomics

Plants	Name of the gene/proteins	Regulator group	Functions assigned	Tools of expression genomics used	References
Arabidopsis thaliana and *A. halleri*	MT2A, MT2B, MT3	Metallothionein	Hyperaccumulation, sequestration, and ROS scavenging	cDNA microarray (comparative)	Chiang et al. (2006)
	WBC11	ABC transporter			
	P_{1B}-ATPase (HMA3, HMA4)	Efflux transporter			
	CDF (ZAT, MTPa12), IRT3, ZIP3, ZIP6, ZIP9, ZIP12, Nramp3, Nramp5	Uptake transporter			
	ZIP-9, EXP-17, CytP-450, CAM-9, curucilin like lectin, MYB-43,48,124, HSF7, ERF, endochitin, etc.	Protein kinase, metal transporter, calmodulin, energy metabolism, etc.	Hyperaccumulation through signal transduction, sequestration, and transportation to vacuole	qRT PCR and microarray (comparative)	Weber et al. (2006)
A. thaliana and *A. lyrata*	CLE-41, CXIP-4, and other 134 genes	Signaling (kinase), transcription regulators, and proteasome	ROS detoxification, cellular repair, metal sequestration, signal transduction, water transport, etc.	cDNA AFLP	Craciun et al. (2006)
A. thaliana	Cluster of genes for sulfur assimilation, glutathione metabolism, cysteine synthase, phytochelatin	Protein kinase, metal transporter, calmodulin, glutathione, oxidoreduction, etc.	ROS detoxification, cell wall metabolism, signal transduction, water transport, etc.	Whole-genome CATMA microarray, qRT PCR	Herbette et al. 2006
	Ca-dependent protein kinase, ERF2/ERF5				
	ACC oxidase synthase, calmodulin, etc.				
	IRT1 and 42 genes	Transporter	Transportation cross plasma membrane, NO synthesis, and Cd hyperaccumulation	qRT PCR and microarray	Besson-Bard et al. (2009)
	At PDR8	Efflux transporter and ABC transporter	Efflux pump at plasma membrane	qRT-PCR	Kim et al. (2007)

(continued)

Table 11.1 (continued)

Plants	Name of the gene/proteins	Regulator group	Functions assigned	Tools of expression genomics used	References
O. sativa	Ospdr9	ABC transporter	Cd stress tolerance by redox change	DD PCR and RT PCR	Moons (2003)
H. vulgare	HvlysMR1	Receptor-like protein kinase	Signal transduction and leaf senescence	DD RT-PCR, LC-ESI-MS	Ouelhadj et al. (2007)
	Hv C2-binding domain	Phytochelatin	Ca-dependent signaling, ROS detoxification, metal sequestration	qRT-PCR	Tamas et al. (2008)
	Phytochelatin synthase	Metallothionein metal transporter (uptake, efflux, and ABC)			
	Metallothionein, PIP (aquaporin), DHN1,4 (dehydrin), ascorbate peroxidase, glutathione peroxidase, DHAP				
Lotus japonicus	LjPCS2-7R (root)	Phytochelatin synthase	Phytochelatin synthase expression	qRT-PCR	Ramos et al. (2007)
	LjPCS-2-7 N (nodule)				
Lycopersicon esculentum	LePCS1	Phytochelatin synthase	Hyper-tolerance through sequestration and transportation to vacuole	Subtractive hybridization, RT PCR	Ouzoid et al. (2005)
	LeMT2	Metallothionein			
	LeNramp1,2,3	Nramp transporter			
Brassica juncea	19 genes (auxin-responsive GH3, ARF-like GTPase, ARD/ARD, APS reductase, NOP, catalase, zinc finger diacyl glycerol kinase and RAMP4)	Protein kinase, hormone biosynthesis	Dehydration stress signal transduction	Differential display	Dormer et al. (2000)
		Metallothionein, glutathione	Sulfur assimilation		
		Oxidoreduction, etc.	ROS detoxification		
			Metal binding		
	24 genes	Transcription factors	Hyper-tolerance	cDNA AFLP	Fusco et al. (2005)
		Transporters and photosynthetic process			

Solanum nigrum	Calmodulin-5,6,7,8	Calmodulin	Hyperaccumulation, vacuolar transport, ionic sequestration	Differential display	Xu et al. (2009)
	Ascorbate peroxidase like mRNA, catalase mRNA, glutamine synthase	Transporters			
		Cellular metabolism			
Solanum torvum	2,049 tag upregulated (HM chaperones, antioxidative and sulfur assimilation enzymes) and 2,022 tag downregulated (transcription factors and aquaporin); AtFRD-3	Xylem loading	Dehydration stress and ion sequestration through membrane transport	RT-PCR (EST library)	Yamaguchi et al. (2010)
		Transporters		Serial analysis of gene expression (SAGE)	
Datura innoxia	Four CD-specific cDNA	Sulfur transferase gene family	Replenishment of sulfate demand under Cd stress	RT-PCR	Louie et al. (2003)
Camellia sinensis	γ-Glutamyl cysteine synthetase, glutathione reductase, GSH	Glutathione	Oxidative stress	qRT-PCR	Mohanpuria et al. (2007)
			Vacuolar sequestration of metal ion		
Salix carpera	ScMT2B, ScMT3	Metallothionein	Metal homeostasis	cDNA and subtractive hybridization	Konlechner et al. (2013)
	ScSAT1	Serine-o-acetyl transferase	Metal perception signaling		
	ScHMAD1, ScMCT1, ScMT2A, ScZIP6, ScHMA1	Metal transporter/chelator			
	ScPMP1	Metal perception and signaling (kinase)			
	ScWalk1, ScRALFL	Alkalinization			

Table 11.2 Cadmium-responsive proteins identified using tools of proteomics and their putative functions

Plant (tissue)	Tools used	No. of proteins identified	Functions assigned	References
A. thaliana (leaf, thylakoid)	2-DE, MALDI-TOF	20 (up) and 15 (dn)	Proteins related to photosynthesis and energy metabolism; oxidative stress response; protein metabolism; sulfur assimilation	Bashir et al. (2011)
			(Retaining photosynthesis efficiency by stabilizing MCPs)	
A. thaliana (leaf, thylakoid)	2-DE, LC-ESI-MS	20 (up) and 15 (dn)	Photosynthesis and energy metabolism	Brahim et al. (2010)
			Oxidative stress response; protein metabolism	
A. thaliana (leaves)	2-DE, MALDI-TOF	25	Metabolic enzyme (ATP sulfurylase, glycine hydroxyl methyl transferase, trehalose 6-P-phosphatase, glutathione-s-transferase) Latex allergen proteins synthesis	Roth et al. (2006)
			Generation of internal link for sensing sulfur reduction	
O. sativa (roots)	2-DE	27	Carbon metabolism, chaperons, metal uptakers, and transporters	Kim and Lee (2009)
O. sativa (roots and leaves)	IPG 2-DE, MALDI-TOF	18 (root)	ROS scavengers (GST, APX, NADH-ubiquinone oxidoreductase) primarily upregulated to prevent oxidative stress damages	Lee et al. (2010)
		19 (leaf)		
O. sativa (root)	2-DE, MALDI-TOF	21	Transporter-like proteins promoting stress tolerance	Aina et al. 2007
B. juncea (leaves)	2DE/CID	17(up) and 23 (dn)	Photosynthetic gene products, energy metabolism, and Calvin cycle	D'Alessandro et al. (2013)
			Carbonic anhydrase regulated CO_2 homeostasis; RNA binding protein, plastid associated protein, chelating proteins, oxidative stress proteins, and chaperonins	
B. juncea (root)	2-D DIGE, iTRAQ	DIGE-102	O-Acetylserine sulfhydrylase, glutathione-s-transferase, and glutathione conjugate membrane transporter; Cd hyperaccumulation and tolerance	Alvarez et al. (2009)
	Nano LC-MS/MS	iTRAQ-585		
H. vulgare (leaf vacuoles)	iTRAQ, MALDI-TOF-TOF	56	Tonoplast intrinsic protein (CAX1 and ABC transporter) and it transports Cd into the vacuole and led to Cd detoxification of barley mesophyll cells	Schneider et al. (2009)

Glycine max (leaf and root)	IPG 2-DE, nano LC-MS-MS, MALDI-TOF	102 (leaf)	Activation of SOD, APX, and CAT ensures cellular protection from ROS-mediated damage; enhanced expression of molecular chaperones which stabilize protein structure and function, and cellular homeostasis	Hossain et al. (2012a)
		16 (root)		
G. max (leaf)	IPG 2-DE, nano LC-MS-MS, MALDI-TOF	78	High abundance of HSP-70 helps BABA primed protein folding to maintain normal protein functions; higher abundance of peroxidase indicated BABA potentiated antioxidant defense system to combat Cd stress	Hossain et al. (2012b)
G. max (root microsome)	2-DE, MALDI-TOF-TOF	22	Upregulation of proteins associated with Cd-chelating pathways and increased lignification of xylem vessels lead to low root-shoot translocation	Ahsan et al. (2012)
L. esculentum (leaves)	PG 2-DE, MALDI-TOF, LIFT-TOF-TOF	Low Cd-27	Low Cd activates proteins related to glycolysis, TCA cycle, and respiration	Rodriguez-Ceima et al. (2010)
		High Cd-33	High Cd inhibits the carbohydrate metabolism and respiration and enhanced production of detoxification proteins	
Phytolacca americana (leaves)	2DE, MALDI-TOF	25	Photosynthesis, sulfur and glutathione metabolism, gene expression, and mol. chaperones, 2-cys-peroxidase, oxido-reduction reaction	Zhao et al. (2011)
Kandelia candel (root)	2-DE, MALDI-TOF	53	Upregulated oxidative response, glutathione biosynthesis, enzymes of TCA and PPP cycle indicating prompt anti-oxidant response	Weng et al. (2013)
Catharanthus roseus (root/leaves)	2-DE, MALDI-TOF	19	Impair the photosynthesis process and photosynthetic function; protein biosynthesis, protein folding assembly, protein degradation, cell defense enzymes, redox homeostasis	Kumar et al. (2011)
Populus tremula (leaves)	2D-DIGE, MALDI-TOF-TOF	52 (up) and 73 (dn)	Deleterious effect on protein expression for primary carbon metabolism and oxidative stress response	Keiffer et al. (2008)
			Proteolysis, protein folding, pathogen-related protein, carbon metabolism, photosynthesis, glutathione metabolism, ascorbate and glutamine biosynthesis, oxidoreductase action, lignin biosynthesis, ATP synthase coupled protein transport, hormone (auxin) synthesis, riboflavin metabolism	

Table 11.3 Depiction of the function of cadmium-responsive genes through ectopic gene expression and insertional mutagenesis

Plant	Gene and gene product	Salient finding	References
Transgenic approaches (ectopic gene expression)			
A. thaliana	At APR1 (APS reductase)	Higher tolerance to cadmium	Sakulkoo et al. (2005)
	Hv APX1 (peroxisomal ascorbate peroxidase)	Higher tolerance to Cd stress and more accumulation of Cd	Xu et al. (2008)
	YCF1 (vacuolar transporter)	Pump heavy metals into vacuoles	Tong et al. (2004)
	AtPCS1 (phytochelatin synthase)	Cd detoxification	Lee et al. (2003)
	Os MSR3 (HSP)	Higher tolerance to Cd, bHLH transcription factor expression	Cui et al. (2013)
		More accumulation of phytochelatin, non-proteinaceous thiol, and Glutathione	
Nicotiana tabacum	CUP1 (yeast metallothionein)	ROS scavenging	Krystofova et al. (2012)
		Cd accumulation in root	
	Pv SR2 (stress-related protein)	Higher tolerance of Cd through accumulation and transportation	Xu et al. (2008)
Populus davidiana x P. bollena hybrid	Ta LEA1 (unknown)	Higher DOD, POD, ROS scavenging; lower malondialdehyde leading to higher Cd stress tolerance	Gao et al. (2012)
Insertional mutagenesis approaches (gene knockout)			
A. thaliana	Sn RK2s (SNF1-related protein kinase-2)	ABA-dependent and ABA-independent pathways for osmotic stress response	Fujii et al. (2011)
	At HSP90-3	Cd tolerance by decreased content of phytochelatin and glutathione; inhibited activities of SOD, CAT, and POD; and increased content of malondialdehyde	Song et al. (2012)
O. sativa	LCD (low cadmium)	Cd transport	Shimo et al. (2011)
N. tabacum	Sn RK2s (SNF1-related protein kinase-2)	ROS accumulation	Kulik et al. (2012)

The cadmium-responsive genes are mostly categorized into seven groups such as sensing and signaling, osmolyte biosynthesis, antioxidant protectants, protection of cell metabolism, metal transporter, ion homeostasis, and hormone biosynthesis (Maksymiec 2007; Thapa et al. 2012). Research in genetic transformation for cadmium stress tolerance was limited in the pre-genomics era by the inadequate availability of genes and specific promoters (Zhu et al. 1997). Several studies were made pertaining to gene function assignment for cadmium stress in plants either by overexpression (Sakulkoo et al. 2005; Krystofova et al. 2012; Cui et al. 2013) or suppression of gene expression by gene knockout and gene trap systems (Shimo et al. 2011; Kulik et al. 2012; Song et al. 2012) in three model plant systems, viz., *A. thaliana*, *O. sativa*, and *N. tabacum* (Table 11.3).

It is now possible to study the expression of many genes simultaneously on a genome-wide scale using plant artificial chromosome vectors with respect to their structure and function (Tyagi et al. 2006). Thus, the present trend in cadmium stress biology is to use large-scale genomic data to scrutinize and revalidate the osmolyte biosynthesis, antioxidants, LEA proteins, molecular chaperones, cell membrane proteins, aquaporins and transporters, ion homeostasis, and transcription factors involved in Cd stress tolerance based on transgenic (overexpression and insertional inactivation) approaches (Sreenivasulu et al. 2007). In addition to overexpression and insertional inactivation, another group of tools known as "RNA interference (RNAi)" is quite promising on the depiction of gene to function assignment of complex biological processes even in the regulation/signaling of heavy metal stress response (Jones-Rhoades et al. 2006; Mendoza-Soto et al. 2012; Ding et al. 2013). Among the RNAi tools, microRNAs (miRNAs) are a group of endogenous non-protein-coding small RNAs of 21 nucleotides. These miRNA genes are originated from hairpin precursors by DICER-LIKE1 (DCL1) in plants (Reinhart et al. 2002; Jones-Rhoades et al. 2006). These miRNAs negatively regulate the post-transcriptional processes and translation of specific mRNA targets through RNA-induced silencing complex (RISC) in cells (Bartel 2004). Majority of miRNAs are conserved across the species making it possible to identify putative miRNAs in other species using comparative genomics (Ding et al. 2013). Recently, it has been reported that miRNAs act as crucial regulators of multiple physiological processes, including plant development, signal transduction, and adaption to heavy metal stresses (Sunkar et al. 2006; Zhou et al. 2008, 2012; Huang et al. 2009; Mendoza-Soto et al. 2012; Hartwig 1995, Jagadeesan et al. 2010). Some of the studies on cadmium stress regulatory microRNAs in *O. sativa* (Huang et al. 2009, Ding et al. 2011, 2013), *B. napus* (Zhou et al. 2012), and *M. truncatula* (Zhou et al. 2008) and the putative stress-related functions were annotated (Table 11.4).

With the advent of DNA markers, two major approaches have been used in exploiting the gene pool for imparting cadmium stress tolerance: first, identification of stress-tolerant genes via functional genomic approaches and introduction of stress-tolerant genes into crops of interest and second, identification of DNA markers flanking the QTLs or co-segregating with the genes conferring tolerance to cadmium stress and use in marker-assisted breeding programs (Panigrahi et al. 2013). Although by using functional genomic approaches, regulatory pathways involved in abiotic stress response have been dissected and shown to enhance abiotic stress tolerance in laboratory conditions by activating stress-responsive signal transduction and downstream transcription factor genes in transgenic plants, its success in field conditions are rather poor. Hence, it is equally important to integrate developed knowledge as an outcome of functional genomics into conventional breeding programs via genomic-assisted breeding to develop stable populations conferring both stress tolerance and yield (Sreenivasulu et al. 2007). In this respect, several genes and QTLs responsible for component traits of cadmium stress tolerance have already been identified (Table 11.5), which may be used for molecular breeding programs in the near future. More recently, a concept known to be "genetical genomics" has been developed in combining the advantage of gene expression

Table 11.4 Cadmium-responsive miRNAs and prediction of their target gene function by annotation and/or overexpression in plants

Plants	mi RNA ID	Target gene/function	References
A. thaliana	miR398b	Cytosolic CSD1 and chloroplastic CSD2 (oxidative stress tolerance)	Sunkar et al. (2006)
O. sativa	miR390	TAS3	Ding et al. (2011)
	miR156	SBP transcription factors	
	miR167	Auxin-responsive factors (ARFs)	Huang et al. (2009)
	miR118	Heat shock factor protein-2	Ding et al. (2013)
	mir59	Cadmium tolerance factor, OsWAK45 receptor-like protein kinase, OsWRKY10 superfamily of rice TFs, Ras-related protein RHN1	
	miR1004	Glutathione-s-transferase GSTU6, vacuolar protein sorting protein-72, serine/threonine protein kinase 19	
	miR361	Ubiquitin protein ligase	
	miR1060	Cytochrome P450 74A4	
	miR192	AT binding cassette subfamily2, CPRF-2, F-box domain containing protein, cysteine protease, ubiquitin protein ligase, zinc finger C3HC4-type protein, IAA amino acid hydrolase, etc.	
B. napus	miR156	SBP transcription factors, glutathione γ-glutamyl cysteinyl transferase-2, serine/threonine protein kinase Nek-3	Zhou et al. (2012)
	miR159	ABC transporter, auxin response factors	
	miR164	Monothiol glutaredoxin-S12, chalcone synthase, transcriptional factors	
	miR166, 167	WRKY transcription factor 21, Nramp-1	
	miR168	Cation homeostasis, Ap2-like TFs	
	miR172	AP2-like TFs, ERFs	
	miR396	Ulp1 protease family protein, BHLH TFs, growth regulatory TFs	
Medicago truncatula	miR319	TCP transcription factor	Zhou et al. (2008)
	miR171	SCL transcription factor	

profiling and marker-based fingerprinting of related progenies in a segregating population to analyze *cis*- and *trans*-acting factors and to delineate a trait-related genetic network (Jansen and Nap 2001; Jansen 2003; Sreenivasulu et al. 2007; Kovalchuk et al. 2005; Krämer et al. 1996). In the future, the integration of informations obtained from functional genomic approaches with conventional breeding will hasten the success for various quantitative traits including heavy metal tolerance in general and cadmium tolerance in particular.

Table 11.5 Cadmium-responsive gene(s) and QTLs identified for genomic-assisted breeding

Plant	Gene/QTL (Population)	Trait associated	Markers used	Percentage variation	References
A. thaliana	*RML1*/*CDS2*	γ-Glutamyl cysteine synthetase	Positional cloning	–	
	CAD1	Phytochelatin synthetase	Positional cloning	–	Ha et al. (1999)
A. halleri	*q Cd tol-1,2,3* (BC1)	Cadmium tolerance (cosegregate with HMA4)	*A. thaliana* anchored markers, AFLP	43, 24, 16	Courbot et al. (2007)
O. sativa	*qCDS7*, *qCDR6.1*, *6.2* (DH)	Cd tolerance and accumulation shoot (s) and roots (r)	RFLP, SSR	12.55 12.41 11.72	Xue et al. (2009)
	qcd1, *qcd2*, *qcd3* (RIL)	Cd content	RFLP	9.7, 12.4, 22.7	Norton et al. (2010)
	qcd7 (CSSLs)	Cd concentration	RFLP	–	Ishikawa et al. (2005)
	qGCd7 (RIL)	Grain Cd concentration	RFLP, SSR	35.5	Ishikawa et al. (2010)
Triticum turgidum L. var. *durum*	*qCdU1* (DH)	Grain Cd concentration	STS, ESM	–	Wiebe et al. (2010)
G. max	*Cda1* ($F_{2\text{-}3}$ and RIL)	Seed Cd concentration (colocalize with genes controlling protein kinase, putative adagio-like protein, and plasma membrane H^+-ATPase)	SSR	57.3	Souframanien et al. (2010)
Avena sativa	*Qtl1* (F_2)	Grain cadmium accumulation	RAPD, REMAP, SRAP, SCAR	–	Tanhuanpaa et al. (2007)
Populus deltoides	*qCd1*, *qCd2* (pseudo-backcross pedigree)	Cd tolerance[a]	AFLP, SSR	5.9–11.6	Induri et al. (2012)

[a] Whole genome microarray analysis led to the identification of nine Cd stress-responsive genes (NHL repeat membrane protein, metal transporter, and transcription factors)

11.5 Future Strategies

During the last couple of decades, large amount of data have been accumulated on plant's responses to heavy metal stress, both at the genome and proteome levels. Data mostly include a plethora of regulatory proteins like transcription factors and signaling molecules associated with heavy metal tolerance and its related genes, which have helped to understand the molecular mechanisms associated with plant survival and crop yields under stressed conditions. But there is an intriguing amount of cross talk and interconnections that are involved in heavy metal stress signaling (Thapa et al. 2012; DalCorso et al. 2010), such as *trans*- and *cis*-acting regulatory elements, and interdependent miRNA-based post-transcriptional and translational gene regulation including alternative splicing and cryptic splicing.

In this review chapter, a large number of functionally characterized genes, either by overexpression or by knockout studies, were introduced into crop plants to provide higher tolerance against heavy metal stress under laboratory conditions in model plant species. It is evident from research outcomes that extensive work is imperative in several fronts including transgenic approaches. In most cases, constitutive expression of stress-tolerant genes is likely to cause unwanted effects. Therefore, it is highly desirable to achieve development and tissue-specific stress-responsive expression of the transgenes by identifying specific promoters. In this regard, genomic tools identified a number of stress-inducible promoters, which can be tested for specificity. Such promoters could also be used to prevent gene silencing when gene pyramiding is sought as a feasible strategy to obtain higher tolerance levels. It has also been reported that plants exhibited higher accumulation of osmolytes, upregulation of an array of stress-related proteins (HSPs, proteinases and PR proteins, and glutathione metabolism-related proteins), and signaling proteins (receptor kinases) upon heavy metal stress that suggest the involvement of diverse networks of stress pathways ultimately leading to metal tolerance.

Due to lack of integrated and coordinated approach on extensive field, tests in the pace of scientific development in genomics, transcriptomics, and proteomics were not done so far. Therefore, we are yet to develop varieties that can overcome the heavy metal constraints and perform better either by tolerance or by phytoremediation. A large number of genomic resources are now available in the form of well-catalogued annotated genome sequence and easily accessible databases. The outcome effort will not only reveal gene function but will also identify the effective combination of genes (Tyagi et al. 2006). Genome-wide strategies have been accelerated by deciphering complex stress-responsive networks and will also help in the identification of key networks and their associated genes, which may be manipulated through either genomic-assisted breeding strategies or genetic engineering. However, there is very less effort paid on the exploitation of marker-aided breeding due to quantitative control of heavy metal stress in plants. Hence, no markers/genes have been identified to quantify the plant under heavy metal stress. There is a sanguinity that the use of recent approaches of functional genomics and genomic-assisted breeding will definitely ameliorate the heavy metal stress tolerance and could generate valuable information for engineering stress-tolerant plants for their ultimate use in sustainable agriculture. On the basis of the foregoing discussion, a strategy has been proposed by integrating tools of system biology, functional genomics, and genomic-assisted breeding to improvise our future effort on the discovery of genes and pathways for the elucidation of heavy metal stress response (Fig. 11.3).

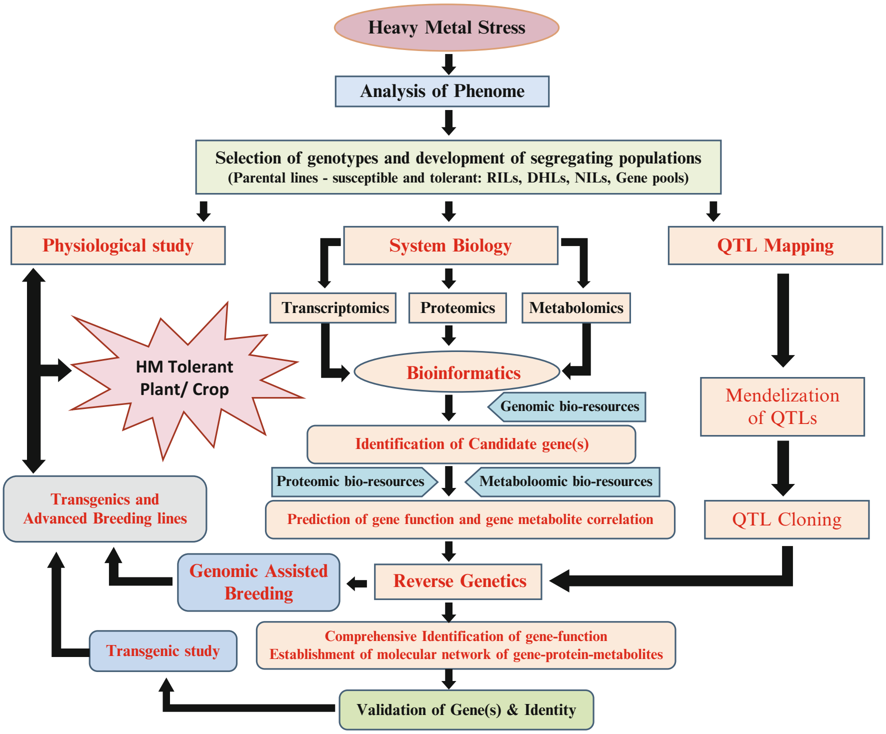

Fig. 11.3 Proposed strategy for development of heavy metal-tolerant plants by integrating functional genomics and genomic-assisted breeding

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