



# Traversing the Links between Heavy Metal Stress and Plant Signaling

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Plants confront multifarious environmental stresses widely divided into abiotic and biotic stresses, of which heavy metal stress represents one of the most damaging abiotic stresses. Heavy metals cause toxicity by targeting crucial molecules and vital processes in the plant cell. One of the approaches by which heavy metals act in plants is by over production of reactive oxygen species (ROS) either directly or indirectly. Plants act against such overdose of metal in the environment by boosting the defense responses like metal chelation, sequestration into vacuole, regulation of metal intake by transporters, and intensification of antioxidative mechanisms. This response shown by plants is the result of intricate signaling networks functioning in the cell in order to transmit the extracellular stimuli into an intracellular response. The crucial signaling components involved are calcium signaling, hormone signaling, and mitogen activated protein kinase (MAPK) signaling that are discussed in this review. Apart from signaling components other regulators like microRNAs and transcription factors also have a major contribution in regulating heavy metal stress. This review demonstrates the key role of MAPKs in synchronously controlling the other signaling components and regulators in metal stress. Further, attempts have been made to focus on metal transporters and chelators that are regulated by MAPK signaling.

**Keywords:** calcium signaling, chelators, heavy metals, hormones, MAPKs, metal transporters, metallothioneins, microRNAs

## INTRODUCTION

Heavy metals are essential to life only in trace amount while their excess amount causes cellular damage. The heavy metals present in environment affecting the growth of many organisms are iron (Fe), arsenite ( $As^{III}$ ), arsenate ( $As^V$ ), cadmium (Cd), chromium (Cr), lead (Pb), copper (Cu), mercury (Hg), aluminum (Al), etc. These metals have not only known to perturb animal kingdom but also plant kingdom. Their damaging impact on our agriculture has also been very well-documented (Tchounwou et al., 2012). At cellular level elevated quantity of heavy metals imposes damage by wide number of mechanisms. The most common mechanism is the production of reactive oxygen species (ROS) inducing oxidative stress, while others are inactivation of biomolecules by displacement of essential metal ions or by blocking essential functional groups (Stohs and Bagchi, 1995). Metals like As, Cd, Cr, Pb, Hg are able to work by displacing essential metal ions or blocking functional groups. Metals like Fe and Cu, which are redox active, generate ROS directly through redox reactions; in contrast, other metals like Pb, Cd, Ni, Al, Mn, and Zn generate ROS by indirect mechanisms. The indirect mechanism of ROS production includes stimulation of ROS producing enzymes like NADPH oxidases or displacing

essential cations from the binding sites of enzymes and inhibiting their activities. ROS at normal physiological level play essential role however its enhanced generation deteriorates functioning of cell (Cuypers et al., 2009). Plants show defense against these heavy metal ions by adsorbing them on to the chelating molecules [for e.g., phytochelatins (PCs), metallothionines, etc.] and by sequestration into the vacuoles (**Figure 1**). Many of the defense responses (not all) shown by the plants are due to the major contribution by signaling cascades, which perceive the signal from upstream receptors and transmit into the nucleus, thus regulating several defense related genes. The receptors that are known to perceive the signals and are well studied in plant stress and development include receptor like protein kinases (RLKs), flagellin sensitive 2 (FLS2), EF-Tu receptor (EFR), ethylene resistance1/2 (ETR1/2), salt intolerance 1 (SIT1), ERECTA (ER), etc. (Rodriguez et al., 2010; Sinha et al., 2011; Jalmi and Sinha, 2015). The major signaling networks working in metals stresses in addition to the other environmental stresses are calcium signaling, hormone signaling and MAPK signaling. Calcium signaling employs multitude of calcium sensing proteins like Calmodulins (CaMs), CaM like proteins (CMLs), Calcineurin B-like proteins (CBLs), and  $Ca^{2+}$ -dependent protein kinases (CDPKs) that bind to  $Ca^{2+}$  and trigger different downstream signaling pathways (Luan et al., 2002; Sanders et al., 2002; Dodd et al., 2010; Steinhorst and Kudla, 2014). In case of hormone signaling there are different plant hormones that play role in metal stress response (Cao et al., 2009; Vitti et al., 2013; Chen et al., 2014; Zhao et al., 2014). Of the several signaling modules, the most predominant and complex is the mitogen activated protein kinase (MAPK) signaling composed of three-tier phosphorylation module MAPKKKs (Mitogen Activated Protein Kinase Kinase Kinase), MAPKKs (Mitogen Activated Protein Kinase Kinase), and MAPKs (Mitogen Activated Protein Kinase) (Hamel et al., 2006). MAPKs are substantially known in providing tolerance against biotic and abiotic stress (Rodriguez et al., 2010; Rao et al., 2011; Sinha et al., 2011) (**Figure 2**).

During environmental stress plants exhibit molecular response that helps them to adapt during various environmental calamities. Plant's molecular response to metal stress is signified by the synthesis of signaling molecules and stress-related proteins like metal transporters and chelators. They tackle the heavy metal toxicity by chelating and sequestering them in the plant vacuoles, serving as temporary storage of essential as well as toxic metabolites (Sharma and Dietz, 2006; Verbruggen et al., 2009; Mendoza-Cózatl et al., 2011) (**Figure 1**). Based on this ability plants are now being widely used for removing heavy metals contamination from the environment by process of phytoremediation (Salt et al., 1998).

Transport of heavy metals required for their relocation is performed by transporters localized in the parenchyma cells of xylem and companion cells of phloem. Majority of loading and unloading of the metal ions in xylem and phloem is done by the transporters. Prominent groups of transporters maintaining physiological concentration of heavy metals are: zinc-iron permease (ZIP), heavy metal transport ATPase (CPx- and P1B-ATPase), natural resistant associated macrophage protein (NRAMP), cation diffusion facilitator (CDF), and

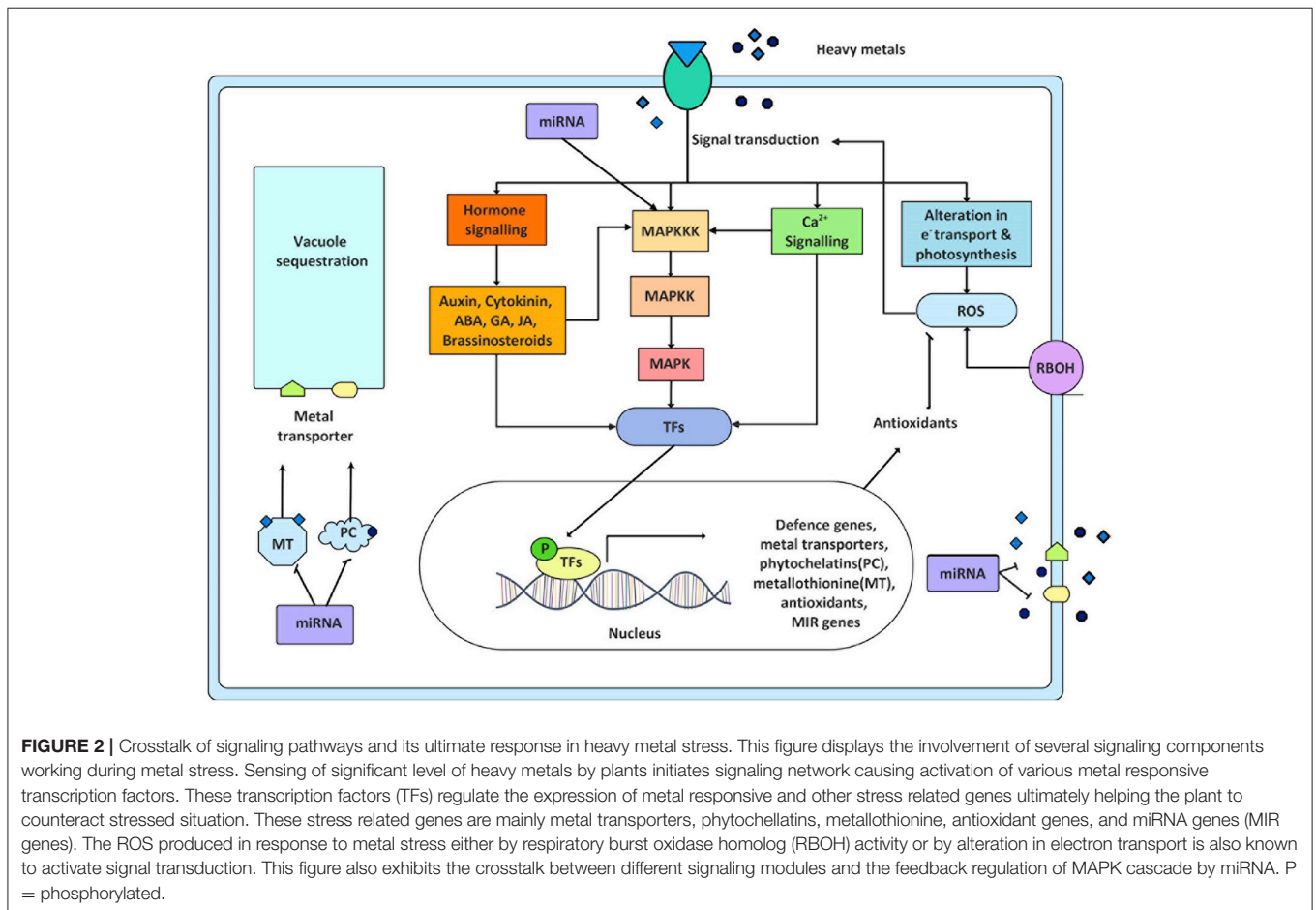
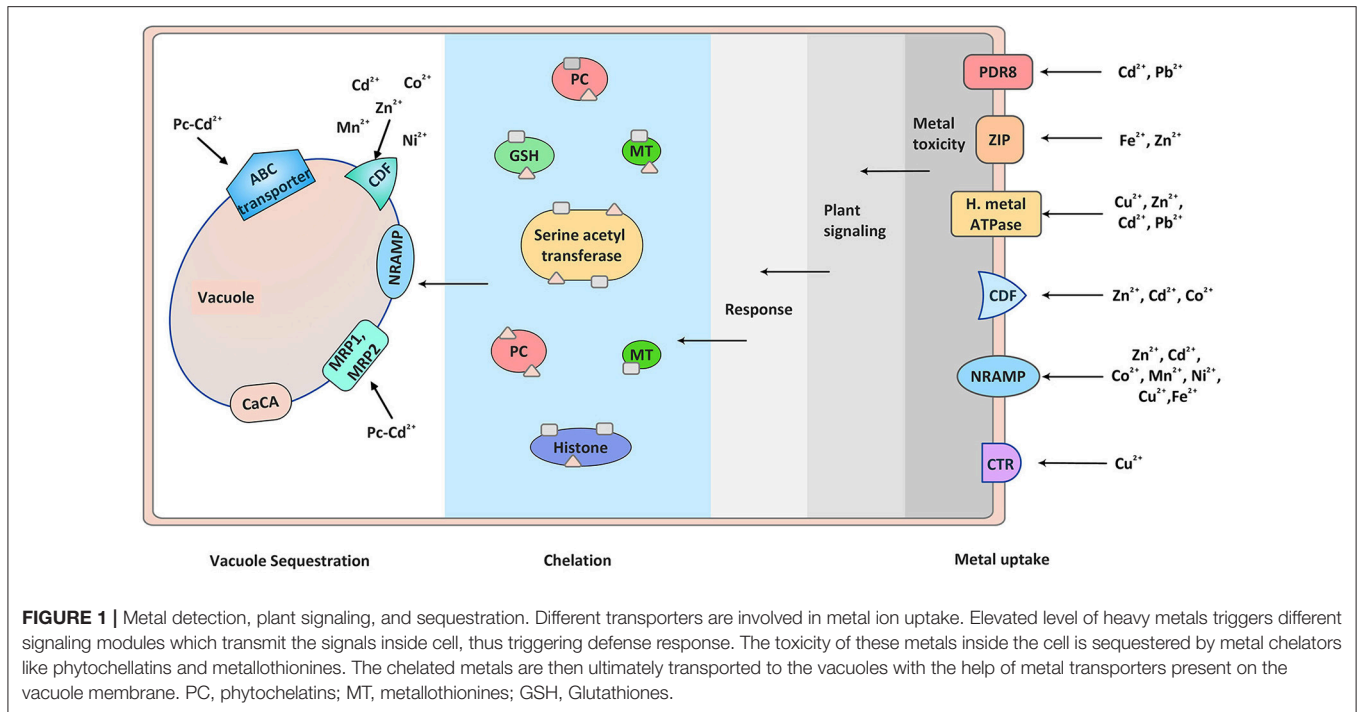
ATP-binding cassette (ABC) transporters, present at plasma membrane and on tonoplast membrane of cell (Park et al., 2012; Singh S. et al., 2015) (**Figure 1**). In addition, Cys-rich metal binding peptides like PCs or metallothionines, nicotinamide, and glutathione are also important players of metal transport (**Figure 1**). Studies suggesting the role of metal transporters and Cys-rich metal binding peptides in arsenic metal uptake, transport, and detoxification have been very well-described by Kumar et al. (2015). Apart from the transporters and chelators, vacuole sequestration capacity (VSC) is very much important in metal allocation. Interaction between membrane localized transporters and ion chelators adjust the VSC in response to changing environment (Peng and Gong, 2014). The regulation of the VSC will decide the toxicity of heavy metals to the plants. It is important to study the regulatory mechanisms of VSC and its ultimate impact on metal transport and sequestration. Additionally, study of metals signal perception and transmission by the plants in regulating the metal transport is also important.

This review will majorly emphasize on impact and mechanism of action of heavy metals, different signaling modules and other regulators triggered by heavy metal stress, the impact of plant signaling on downstream defense responses and the fragmentary work performed on regulation of metal transporters by MAPKs that still remains unexplored in plants.

## Plant Signaling in Response to Heavy Metals

The inability of plants to escape from environmental stresses such as metal pollution has driven the evolution of multiple mechanisms to efficiently sense, respond, and therefore adapt to such stresses. Sensing of heavy metals by plants generates a response such as modulation of molecular and biochemical mechanisms of cell. Certainly, this response is evoked by important signal transduction network operated in plant cell formed by several signal transduction units. The ultimate response of plant is shown by synthesizing metal transporter proteins and metal binding proteins helping the plant to counteract excessive metal stress (Maksymiec, 2007; Peng and Gong, 2014; Singh S. et al., 2015).

In many crops, the early sign of metal toxicity is known to be similar to other environmental stresses like osmotic or dehydration stress, oxidative stress in addition to defects in nutrient balance, photosynthesis, and development (Chen et al., 2001; Yadav, 2010; Rucinska-Sobkowiak, 2016). This similarity of the response reflects interconnection between intricate signaling networks. The interplay and convergence of these signaling pathways finally results in regulation of various transcription factors activating several stress responsive genes. The genes that normally get regulated in context of metal stress include the genes for metal chelators and transporters (Singh S. et al., 2015) (**Figure 2**). Several signal transduction units operate in response to heavy metal toxicity, with different signaling pathways acting in response to different species and concentrations of metals. Some of these signaling pathways are discussed in detail below.



## MAPK Signaling in Heavy Metal Stress

MAPKs are some of the most important and highly conserved signaling molecules that function in response to many diverse stresses and during many developmental pathways (Sinha et al., 2011). MAPK cascade consists of three tier components MAPKKKs, MAPKKs, and MAPKs mediating phosphorylation reactions from upstream receptor to downstream target (Hamel et al., 2006). MAPK signaling mediates the transmission of stress related signals thus regulating large number of cellular processes (Hamel et al., 2006; Rodriguez et al., 2010). Among abiotic stresses, heavy metal stress has conferred profound effect on MAPK signaling pathways. MAPKs are known to be activated by perception of specific metal ligand and also by ROS molecules produced in the metal stress (Jonak et al., 2004; Smeets et al., 2013; Jalmi and Sinha, 2015).

There are plenty of reports showing the activation of MAPKs in response to heavy metals like Cd, Cu, and As (Jonak et al., 2004; Yeh et al., 2007; Ding et al., 2011; Rao et al., 2011; Smeets et al., 2013), however studies in response to other metals such as Pb, Zn, Fe are very scant. Likewise, in depth investigation to decipher a complete MAPK signaling cascade in response to specific metal stress still remains elusive. In Arabidopsis, the best-characterized MAPKs are MPK3 and MPK6, which are activated by diverse stimuli are also known to be induced by CdCl<sub>2</sub> and CuSO<sub>4</sub> (Asai et al., 2002; Pitzschke et al., 2009; Liu et al., 2010; Takahashi et al., 2011; Sethi et al., 2014). Similarly in rice, OsMSRMK2 (OsMPK3 homolog), OsMSRMK3 (OsMPK7 homolog), and OsWJUMK1 (OsMPK20-4 homolog) transcripts increased in response to Cu<sup>2+</sup> and Cd<sup>2+</sup> treatment in leaves and roots (Yeh et al., 2007; Rao et al., 2011). In Alfalfa, activation of four distinct MAPKs: SIMK, MMK2, MMK3, and SAMK was demonstrated in response to CuCl<sub>2</sub> or CdCl<sub>2</sub>. SAMK and SIMK are the orthologs of rice OsMPK3 and OsMPK6, respectively. Higher concentrations of CuCl<sub>2</sub> induced the activity of SIMK, MMK2, and MMK3, and to a lesser extent of SAMK while CdCl<sub>2</sub> showed a similar but delayed MAPK activation (Jonak et al., 2004). Copper-mediated induction of SIMK specifically activated SIMK and SAMK and not MMK2 and MMK3 manifesting specificity in the signaling cascades in response to different metals (Jonak et al., 2004; Opdenakker et al., 2012) (Table 1).

Beside the activation of MAPKs by Cu<sup>2+</sup> and Cd metals, there are several other heavy metals that cause the activation of MAPKs, but are not studied in detailed aspect. In yeast, Al<sup>3+</sup> tolerance was provided by over-expression of a MAP kinase gene in Al<sup>3+</sup>-sensitive mutant, indicating the association of MAPK with Al<sup>3+</sup>-resistance (Schott and Gardner, 1997). Similarly, in wheat root apex Al<sup>3+</sup> treatment led to the activation of 48-kDa MAPK, playing significant role in transmitting Al related signals and Al-resistant in wheat (Mossor-Pietraszewska, 2001). In rice, a 42-kDa MAPK found to activate myelin basic protein (MBP) in response to iron. Pre-treatment of rice roots with an antioxidant, glutathione (GSH), decreased iron-induced root cell death, and MAPK activation, demonstrating the involvement of ROS induced MAPK activation in iron-triggered signaling (Tsai and Huang, 2006). Although, Zn is a non-redox metal, however MAPK activation by Zn results from the activation of oxidative stress in rice. Zn stimulates a rapid activation of MBP

by three MAPKs with approximate molecular weights of 34, 40, and 42 kDa in rice roots (Lin et al., 2005). Pb stress leads to the upregulation of four MAPKs such as MAPKKK7, MAPK6, MAPK18, and MAPK20 in radish (Wang et al., 2013). Arsenite severely affect the growth of rice seedlings. OsMKK4 and OsMPK3 transcripts were found to be induced in arsenite treated rice leaves and roots (Table 1). *In-silico* homology modeling and docking analysis supported OsMKK4–OsMPK3 interaction (Rao et al., 2011), suggesting the role of this MAPK module in arsenic stress. Accumulating evidences suggest that metal ions such as arsenic and chromium are able to induce reactive oxygen and nitrogen species, thereby altering nitric oxide (NO) induced cell signaling. NO has been shown to modulate the activity of MAPK, NO donors, and recombinant NOS were shown to cause the activation of SIPK (Rao et al., 2011).

Heavy metal induced ROS production is already known in plants and the role of these ROS molecules in activating MAPK signaling is very well-accepted. In Arabidopsis, two important completely characterized MAPK cascade MEKK1-MKK4/5-MPK3/6 (Asai et al., 2002) and MEKK1-MKK2-MPK4/6 are known to work downstream of ROS, participating in both abiotic and biotic stress signaling (Pitzschke et al., 2009; Jalmi and Sinha, 2015). Apart from this, MAPK cascades also exert positive feedback regulation on ROS production. A cascade OX11-MPK6 activated by ROS also positively regulates ROS production (Asai et al., 2008). MEKK1-MKK4-MPK3/6 is known to act upstream of NADPH oxidase stimulating ROS production in pathogen attack and H<sub>2</sub>O<sub>2</sub> produced is in turn known to activate MPK3 and MPK6 (Kovtun et al., 2000). These studies provide a speculation and link of MAPK cascades that might work in different metal stress depending upon the activation by the ROS molecules produced. Furthermore, MEKK1-MKK4/MKK5-MPK3/MPK6 module working downstream of receptor FLS2 and receptor like kinase (RLKs), eventually giving resistance against pathogen is very well known (Asai et al., 2002). These RLKs are also reported to be regulated by Cd<sup>2+</sup> speculating the involvement of similar MAPK cascade working downstream of RLK in metal stress. Recent study reported that MEKK1-MKK5-MPK6 mediates salt induced expression of iron superoxide dismutase gene further inducing ROS production. Iron superoxide dismutases (Fe-SOD) are the metal binding SOD and require Fe<sup>3+</sup> metal ion as cofactor (Myouga et al., 2008; Xing et al., 2015). These reports suggest involvement of MAPKs in mediating metal stress however a detail study of a complete MAPK cascade working in heavy metal stress is required.

## Calcium Signaling in Heavy Metal Stress

The calcium ion (Ca<sup>2+</sup>) as corroborated by different studies acts as a universal secondary messenger in the normal functioning of plants as well as in response to various environmental stresses (Sanders et al., 2002). The cytosolic free Ca<sup>2+</sup> concentration changes in response to various stress stimuli triggering complex interactions and signal transduction pathways (Rudd and Franklin-Tong, 2001). This transient increase in the cytosolic concentration is perceived by highly sensitive calcium sensing proteins that mediate this chemical signal into a biological

**TABLE 1** | Signaling components involved in metal stress.

Heavy metal	Plant	Signaling components		
		MAPK	Calcium	Hormone
Cd	Arabidopsis	MEKK1, MPK3, MPK6 (Jonak et al., 2004; Liu et al., 2010)	Unknown	<b>Auxin:</b> <sup>2</sup> PAT1, <sup>2</sup> CYP79B2, <sup>2</sup> CYP79B3, <sup>2</sup> YUCCA, <sup>2</sup> GH3, <sup>2</sup> TIR1, <sup>1,2,3</sup> PINs, <sup>2</sup> ABCB, <sup>2</sup> ARFs, <sup>3</sup> AXR3/IAA17 ( <sup>1</sup> Hu et al., 2013; <sup>2</sup> Wang et al., 2015; <sup>3</sup> Yuan and Huang, 2016) <b>Cytokinin:</b> IPT, CKX (Vitti et al., 2013) <b>Ethylene:</b> <sup>4,5</sup> ACS, <sup>5</sup> ACC, <sup>4</sup> ETR2, <sup>4</sup> ERF1,5, <sup>5</sup> GSH1, <sup>5</sup> GSH2 ( <sup>4</sup> Weber et al., 2006; <sup>5</sup> Schellingen et al., 2015)
	Rice	MAPK2, MPK3, MPK6, MSR3, WJUMK (Agrawal et al., 2003; Yeh et al., 2007; Rao et al., 2011)	Unknown	<b>Auxin:</b> MAPK3/6/7, YUCCA, PINs, ARF, and IAA (Zhao et al., 2014)
	<i>M. sativa</i>	SAMK, SIMK, MMK2, MMK3 (Jonak et al., 2004)	Unknown	Unknown
	<i>Zea mays</i>	MPK3 (Wang et al., 2010)	Unknown	Unknown
	Radish	Unknown	Ca <sup>2+</sup> /CaM (Rivetta et al., 1997)	Unknown
	<i>B. natalensis</i> , <i>R. crispus</i> Glycin max	Unknown	Unknown	<b>Cytokinin:</b> PI-55, INCYDE (Gemrotová et al., 2013) <b>Ethylene:</b> ACS, MAPK2, MAPK2 Chmielowska-Bak et al., 2014
Cu	Arabidopsis	MPK3, MPK6 (Liu et al., 2010; Schellingen et al., 2015)	Unknown	<b>Auxin:</b> <sup>2</sup> PAT1, <sup>2</sup> CYP79B2, <sup>2</sup> CYP79B3, <sup>2</sup> YUCCA, <sup>2</sup> GH3, <sup>2</sup> TIR1, <sup>2</sup> PINs, <sup>2</sup> ABCB, <sup>2</sup> ARFs, <sup>1,2</sup> DR5 ( <sup>1</sup> Peto et al., 2011; <sup>2</sup> Wang et al., 2015) <b>Ethylene:</b> <sup>4</sup> COPT5, <sup>3</sup> ACS, <sup>3</sup> ERF ( <sup>3</sup> Weber et al., 2006; <sup>4</sup> Carrió-Seguí et al., 2015)
	Rice	MAPK2, MPK3, MPK6, MSR3, WJUMK (Agrawal et al., 2003; Yeh et al., 2007; Rao et al., 2011)	Unknown	Unknown
	<i>M. sativa</i>	SIMK, SAMK, SIMK, MMK2, MMK3 (Jonak et al., 2004)	Unknown	Unknown
As	Arabidopsis	Unknown	Unknown	<b>Auxin:</b> AUX1, PIN1, PIN2 (Krishnamurthy and Rathinasabapathi, 2013) <b>Ethylene:</b> ERFs (Fu et al., 2014)
	Rice	MKK4, MPK3, MPK4 (Rao et al., 2011)	CaM, CaM kinase, CaM like protein (Chakrabarty et al., 2009; Huang et al., 2012)	Unknown
	<i>B. juncea</i>	46Kda MAPK (Gupta et al., 2009)	Unknown	Unknown
Al	Arabidopsis	Unknown	Unknown	<b>Auxin:</b> <sup>1,2</sup> PIN2, <sup>2</sup> AUX1 ( <sup>1</sup> Shen et al., 2008; <sup>2</sup> Sun et al., 2010)
	Wheat	Unknown	Myosin, Calpain, Phospholipase C, Phospholipase A <sub>2</sub> (Jones and Kochian, 1997)	Unknown
	<i>T. aestivum</i>	48Kda MAPK, 42Kda Protein kinase (Osawa and Matsumoto, 2001)	Unknown	<b>Ethylene:</b> <sup>3</sup> ALMT1, <sup>4</sup> ACS, <sup>4</sup> ACO ( <sup>3</sup> Tian et al., 2014; <sup>4</sup> Yu et al., 2016)
	<i>M. sativa</i>	Unknown	Unknown	<b>Auxin:</b> AUX1, PIN2 (Wang S. et al., 2016)

(Continued)



TABLE 1 | Continued

Heavy metal	Plant	Signaling components		
		MAPK	Calcium	Hormone
Hg	Arabidopsis	Unknown	Unknown	<b>Auxin:</b> <i>PAT1, CYP79B2, CYP79B3, YUCCA, GH3, TIR1, PINs, ABCB, ARFs</i> (Wang et al., 2015)
	Rice	MSRMK2, MSRMK3, WJUMK (Agrawal et al., 2003)	Unknown	<b>Ethylene:</b> <i>OsACS2, OsACO1, OsACO2, OsACO5 and OsACO6, 5 MAPKKK, 1 MAPKK and 2 MAPK</i> (Chen et al., 2014)
	<i>M. sativa</i>	Unknown	Unknown	<b>Ethylene:</b> <i>ACCS, ACCO, AP2, ERF1</i> (Montero-Palmero et al., 2014)
Pb	Arabidopsis	Unknown	CNGC1 (Sunkar et al., 2000)	<b>Auxin:</b> <i>PAT1, CYP79B2, CYP79B3, YUCCA, GH3, TIR1, PINs, ABCB, ARFs</i> (Wang et al., 2015) <b>Ethylene:</b> <i>EIN2</i> (Cao et al., 2009)
	Rice	34Kda, 40Kda & 42Kda MAPK (Huang and Huang, 2008)	CDPK-like Kinase (Huang and Huang, 2008)	Unknown
	tobacco	Unknown	CBP4 (Arazi et al., 1999; Sunkar et al., 2000)	Unknown
	<i>R. sativus</i>	<i>MAPKKK7, MAPK6, MAPK18, MAPK20</i> (Wang et al., 2013)	Unknown	Unknown
Zn	Arabidopsis	Unknown	Unknown	<b>Auxin:</b> <i>PAT1, CYP79B2, CYP79B3, YUCCA, GH3, TIR1, PINs, ABCB, ARFs</i> (Wang et al., 2015)
	Rice	34Kda, 40Kda & 42Kda MAPK (Lin et al., 2005)	Unknown	<b>Auxin:</b> <i>MAPK3/6/7, YUCCA, PINs, ARF, and IAA</i> (Zhao et al., 2014)
	Wheat	Unknown	Myosin, Calpain, Phospholipase A <sub>2</sub> (Jones and Kochian, 1997)	Unknown
Cr	<i>Zea mays</i>	MPK5 (Ding et al., 2009)	Unknown	Unknown
	Foxtail millet	Unknown	TPC1, MRC5, CaM (Fang H. et al., 2014)	Unknown
Mn	Arabidopsis	Unknown	ECA1 (Wu et al., 2002)	Unknown
Ni	Arabidopsis	Unknown	Unknown	<b>Auxin:</b> <i>PAT1, CYP79B2, CYP79B3, YUCCA, GH3, TIR1, PINs, ABCB, ARFs</i> (Wang et al., 2015)
	Tobacco	Unknown	CBP4 (Arazi et al., 1999)	Unknown
Ba	Faba bean	Unknown	Ca <sup>2+</sup> channels (Hamilton et al., 2001)	Unknown
B	Barley	Unknown	Calmodulin, Ca <sup>2+</sup> - binding proteins (Tombuloglu et al., 2015)	Unknown

response. Plants harbor myriads of calcium sensing proteins such as Calmodulins (CaMs), CaM like proteins (CMLs), Calcineurin B-like proteins (CBLs), and Ca<sup>2+</sup>-dependent protein kinases (CDPKs) that bind to Ca<sup>2+</sup> and trigger different downstream

signaling pathways (Luan et al., 2002; Sanders et al., 2002; Steinhorst and Jörg, 2003; Dodd et al., 2010).

Multiple studies in different plant species, such as chickpea, *Glycine max*, *Vitis vinifera*, and tomato have been carried out

to discern the contribution of  $\text{Ca}^{2+}$ -binding like proteins and  $\text{Ca}^{2+}$  sensing proteins in augmented tolerance to various abiotic stresses (Tripathi et al., 2009; Li Z. Y. et al., 2012; de la Torre et al., 2013). There have been reports demonstrating that the application of  $\text{Ca}^{2+}$  exogenously can modulate the physiological and biochemical responses in order to alleviate the heavy metal stress. The activity of antioxidant enzymes such as ascorbate peroxidase, glutathione reductase, and superoxide dismutase has been shown to be enhanced upon the application of exogenous  $\text{Ca}^{2+}$  (Ahmad et al., 2015). Though there have been several reports that substantiate the role of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -dependent signaling pathways in imparting tolerance to heavy metal stresses in plants, our understanding of the mechanisms by which these responses are regulated is still meager and invites further elaboration.

Cd is one of the heavy metals physiochemical properties very similar to that of calcium (Choong et al., 2014). This naturally results in an exchangeability of the two ions in  $\text{Ca}^{2+}$  binding proteins and studies have provided evidence that Cd displaced  $\text{Ca}^{2+}$  from its binding sites in calmodulin, sarcolemma and troponin C *in vitro* (Langer and Nudd, 1983; Chao et al., 1984; Ellis et al., 1984). The high similarity in the ionic radii of  $\text{Ca}^{2+}$  and Cd indicates a possibility of Cd uptake through receptor- or voltage-gated  $\text{Ca}^{2+}$  channels and this uptake could possibly be inhibited (at least to some extent) by blocking the  $\text{Ca}^{2+}$  channels (Choong et al., 2014). Plants exposed to cadmium exhibit a higher level of intracellular  $\text{Ca}^{2+}$ , inducing adaptive mechanisms in order to mitigate the toxic effects of the heavy metal (Yang and Poovaiah, 2003). One of the mechanisms used for the increase in  $\text{Ca}^{2+}$  level is the production of  $\text{IP}_3$  which triggers the release of sequestered calcium from the intracellular calcium reserves as indicated in few reports (Smith et al., 1989). A study of *Brassica juncea* in consistence with the above report proves that application of  $\text{Ca}^{2+}$  attenuates the toxicity and makes the plant withstand the deleterious effects of Cd consequently improving the growth and seed quality of the plants (Ahmad et al., 2015). Moreover, studies on different plant species have demonstrated that exogenous application of calcium and silica, calcium and spermidine and  $\text{Ca}^{2+}$  and/or  $\text{K}^{2+}$  can promote the alleviation of cadmium toxicity and reduction in metal accumulation (Siddiqui et al., 2012; Srivastava et al., 2015; Gong et al., 2016).

An interesting study on *Arabidopsis* seedlings has shown that  $\text{Ca}^{2+}$  mitigates the toxic effects of Cd through maintaining auxin homeostasis indicating a crosstalk between signaling pathways in order to combat heavy metal stress (Zhao et al., 2015). Moreover, studies on yeast cells have proposed the role of  $\text{Ca}^{2+}$ -ATPases (Pmr1p and Pmc1p) of vacuolar and Golgi membrane in coping with Cd toxicity. This is achieved through cooperation with a Glutathione-conjugated transporter Ycf1p whose activity is controlled by phosphorylation once again insinuating an interface between different signaling pathways in response to environmental stresses (Mielniczki-Pereira et al., 2011).

The  $\text{Ca}^{2+}$ /Calmodulin system is also involved in response to toxicity mediated by heavy metals other than Cd such as  $\text{Pb}^{2+}$  and nickel ( $\text{Ni}^{2+}$ ) (Ahmad et al., 2015). It was demonstrated by

Arazi et al. that transgenic tobacco plants expressing the plasma membrane associated NtCBP4 (*Nicotiana tabacum* calmodulin-binding protein) exhibit higher levels of tolerance to Ni toxicity. Contrastingly, the same plants were found to be hypersensitive to  $\text{Pb}^{2+}$ , depicting an exclusion of  $\text{Ni}^{2+}$  and augmented accumulation of  $\text{Pb}^{2+}$  as compared to wild type plants (Arazi et al., 1999) (Table 1).

In foxtail millet (*Setaria italica*), hydrogen sulfide was found to interact with  $\text{Ca}^{2+}$  signaling in imparting improved tolerance to Chromium ( $\text{Cr VI}^{\text{IV}}$ )-mediated heavy metal stress. It has also been discerned that  $\text{Ca}^{2+}$  provides tolerance against Cr stress by enhancing the activity of antioxidant enzymes (Fang H. et al., 2014). Additionally, an involvement of CDPKs has also been suggested through transcriptional profiling of the rice roots exposed to long or short durations of Cr stress. Increasing Cr(VI) concentration was found to be correlated with an increase in CDPK-like protein activity, reflecting the role of  $\text{Ca}^{2+}$  signaling in the stress response (Huang et al., 2014) (Table 1).

Differential expression of Calmodulins in response to arsenic stress indicates the possible role of  $\text{Ca}^{2+}$  signaling components in the stress response (Chakrabarty et al., 2009). Besides, a study has demonstrated that cytosolic free  $\text{Ca}^{2+}$  played a key role in the regulation of root activity, metal contents and biomass in close relation to lanthanum (La) dose and acid rain strength. The adverse effects on the roots caused by acid rain could be alleviated by low concentrations of  $\text{La}^{\text{III}}$  and synergistic effects on the roots were observed upon combined exposures at higher concentrations of  $\text{La}^{\text{III}}$  and acid rain (Zhang et al., 2016). The release of intracellular  $\text{Ca}^{2+}$ , the subsequent activation of  $\text{Ca}^{2+}$  channels and the generation of  $\text{H}_2\text{O}_2$  was observed in response to elevated levels of  $\text{Cu}^{2+}$  in the marine alga, *Ulva compressa*. It was evidenced that the gene expression of antioxidant system is regulated via cross-talk among the various cellular signals and levels of  $\text{Ca}^{2+}$ , NO, and  $\text{H}_2\text{O}_2$  (González et al., 2012).

It is established that in response to environmental changes CDPK work together with MAPK for transmission of signals to adapt against changing environment (Takahashi et al., 2011; Wurzinger et al., 2011; Opdenakker et al., 2012) (Figure 2). A CDPK, CPK18 was found to be an upstream kinase of MPK5 in rice, wherein MPK5 was phosphorylated on Threonine 14 and 32 by CPK18 (Xie et al., 2014). Also, MKK3 together with Ca/CaM is known to activate MPK8, which negatively regulates the expression of RBOHD (NADPH oxidase) in response to mechanical stress (Takahashi et al., 2011). A study suggest that  $\text{Ca}^{2+}$  is involved in  $\text{Pb}^{2+}$ -mediated cell death and triggering of MAPK activity via CDPK pathway by enhancing the activity of CDPK like kinase (Huang and Huang, 2008). Besides this, the role of calmodulins has been reported to modulate MAPK signaling pathway (Tebar et al., 2002), which defines a possibility of their interplay in response to metal stress. All these findings outline a vital function for the  $\text{Ca}^{2+}$  regulatory loop, which is critical for maintaining the redox homeostasis of the cell and ion balance in response to heavy metal stress. In animals this crosstalk has been elaborately studied in metal stress than in plants. Hence, it will be highly advantageous to study the importance of this signaling crosstalk and further the regulation of  $\text{Ca}^{2+}$  signaling in heavy metal stress in plants.

## Hormone Signaling in Heavy Metal Stress

The root architecture is of great importance in plant grown in metal-polluted areas, as the remodeling of root architecture in response to metals can be used as a strategy to escape from heavy metal stress. Interestingly, auxin, ethylene, and cytokinin modulate patterning (Vanstraelen and Benková, 2012) and lateral root formation (De Smet et al., 2015). Thus, there are several studies reporting the involvement of these phytohormones in remodeling of the root system architecture in response to heavy metal stress.

Auxin is an essential plant growth hormone playing role in developmental as well as environmental stress responses. It directly affects plant responses to metal stresses by modulating auxin homeostasis including auxin stability, transport, and redistribution (Potters et al., 2007). Basipetal auxin transport through the outer root cell layers is mediated by AUX1 and PIN2 (Marchant et al., 1999; Rashotte et al., 2000). The regulation of auxin signaling in heavy metal stress is evident by various studies conducted over the years. Recently, it was reported that in response to metal stress plants regulate the location and accumulation of auxin by differential and dynamic expression of auxin-related genes like Phosphoribosyl Anthranilate Transferase 1 (PAT1), CYP79B2 and CYP79B3, YUCCA (YUC), Gretchen Hagen (GH3) genes, (TIR1), PIN family, and ABCB family (Wang et al., 2015) (**Table 1**).  $\text{Cu}^{2+}$  toxicity in *Arabidopsis* leads to changes in auxin and cytokinin accumulations and mitotic activity within the primary and secondary root tips (Lequeux et al., 2010). It is also reported that in excess of  $\text{Cu}^{2+}$  lack of auxin leads to an increase in NO levels thereby diminishing root elongation (Peto et al., 2011). This inhibition of primary root elongation by Cu is also due to the modulation of auxin redistribution by PIN1 (Yuan and Huang, 2016). Further, Al was also studied to inhibit root growth by inhibiting the transport of PIN2 vesicles from plasma membranes to endosomes, further disturbing IAA synthesis in apical buds and imbalance of IAA transportation and distribution in roots (Shen et al., 2008; Wang S. et al., 2016). It was further revealed that *aux1-7* and *pin2* mutants exhibited better tolerance to  $\text{Al}^{3+}$  than wild-type plants implying the plausible involvement of AUX1 and PIN2 proteins in  $\text{Al}^{3+}$  induced inhibition of root elongation. Apart from this, Cd disrupts the maintenance of auxin homeostasis in *Arabidopsis* seedlings by increasing IAA oxidase activity and altering the expression of several auxin biosynthetic and catabolic genes (Hu et al., 2013). Cd-mediated up-regulation of biosynthetic gene *NITRILASE* (*NIT*) resulted in increased IAA concentration in *Arabidopsis* roots promoting lateral root growth, thus protecting roots from Cd (Vitti et al., 2013). Moreover, recent report revealed the inhibition of root meristem growth through Cd-induced NO accumulation, which in turn represses auxin transport and stabilizes AUX/IAA proteins to repress auxin signaling (Yuan and Huang, 2016). A positive role for auxin transport through AUX1 on plant tolerance to As stress via ROS-mediated signaling was also disclosed in a study (Krishnamurthy and Rathinasabapathi, 2013) (**Table 1**).

MAPK signaling is established in influencing auxin signaling and its transport (Mockaitis and Howell, 2000). A captivating

report unveiled the interplay of auxin/cytokinin and MAPKs, in which OsMKK4/5-OsMPK3/6 was elucidated as key player in auxin/cytokinin interaction regulating the expression pattern of OsPIN1b/9 (Singh P. et al., 2015). However, the involvement of MAPK signaling regulating auxin response in metal stress is still uncertain. Appealing study performed in rice displayed relationship between auxin signaling and MAPK signaling in Cd stress. It was analyzed that expression of most of the key genes of auxin signaling including YUCCA, PIN, ARF, IAA, and cell cycle related genes was negatively regulated by MAPK in Cd stress (Zhao et al., 2014). This certainly implicates the major role of MAPK signaling in regulating auxin signaling in heavy metal stress.

Cytokinins (CKs) are N<sup>6</sup>-prenylated adenine derivatives involved in the regulation of plant growth and development and in biotic and abiotic stresses (Perilli et al., 2010). There are reports of CKs in plants activated upon heavy metal stress that are able to alleviate heavy metal induced toxicity. The inhibition of photosynthetic pigment and chloroplast membranes by Cd was restored by CKs, increasing photosynthetic capacity and primary metabolite levels (Piotrowska-Niczyporuk et al., 2012). Exogenous kinetin application can also modulate antioxidant enzyme activity, proline, free amino acids, and soluble sugars that counteracted Cd caused inhibitory effects on growth and photosynthesis (Al-Hakimi, 2007).

Ethylene (ET) is a gaseous plant hormone regulating various important growth aspects. It is biosynthesized by ACC synthase (ACCS) that convert AdoMet to ACC, while ACC oxidase (ACCO) catalyzes the conversion of ACC to ethylene. ACCS and ACCO are encoded by multigene families and regulated by many biotic and abiotic factors (Kende, 1993). Several reports support the role for ethylene in the regulation of plant metal stress responses. The effects of metal stress on ethylene production in plants are both metal and concentration dependent (Thao et al., 2015; Keunen et al., 2016) (**Table 1**). Major five ET synthesis genes from rice OsACS2, OsACO1, OsACO2, OsACO5, and OsACO6 (Chen et al., 2014) along with transcription factors AP2 and ERF1 from *Medicago sativa* (Montero-Palmero et al., 2014) were found to be upregulated in Hg treatment. However in rice, genes involved in cytokinin signaling (OsRR1, 3, 4, 6, and 11) were down regulated, suggesting both ET and CK may regulate the Hg-induced inhibition of rice root growth (Chen et al., 2014). Additionally, Cu and Al were also found to increase ACS transcript level in *Arabidopsis*, *Medicago truncatula* and *Lotus japonicus* (Weber et al., 2006; Sun et al., 2010). There was inhibition in root growth under Al stress which was correlated to enhanced ethylene production upon Al treatment (Sun et al., 2010). Recently, it was revealed that in wheat ET negatively regulates Al-induced efflux of malate ions using ET8, which is an important mechanism for Al tolerance (Tian et al., 2014; Yu et al., 2016) (**Table 1**).

Besides the effect of heavy metals on ethylene synthesis, they even exert effect on ethylene signaling. Cu treatment increases expression of number of ethylene responsive factors like *ERF1*, *ERF2*, and *ERF5* (Weber et al., 2006). Apart from these, Cd was exhibited to establish its role in regulating ethylene synthetic genes (ACS2 and ACS6) along with MAPK cascades,



NO generation, and polyamine metabolism (Chmielowska-Bak et al., 2014; Schellingen et al., 2015) (**Table 1**).

Like the interplay of MAPK with auxin signaling, there are also evidences of involvement of MAPKs in ethylene biosynthesis and signaling, however its importance in metal stress is still unknown (Opdenakker et al., 2012). Two important MAPKs, MPK3 and MPK6 are known to be responsible for phosphorylation of ACS2 and ACS6, thus increasing ethylene production (Liu and Zhang, 2004; Li G. et al., 2012). This ACS2/6 is very well reported to be induced by metal stress. Further, putrescine, an essential signaling molecule involved in modulating plant resistance to Al stress by inhibiting ACS and ET production (Yu et al., 2016), was found to be regulated by AtMPK3/6 (Kim et al., 2013). Moreover, a MAPK cascade MKK9-MPK3/6 acting downstream of ethylene receptor CTR1 was found to control a key transcription factor EIN3 involved in ethylene biosynthesis (Xu et al., 2008; Yoo et al., 2008). Roots of rice plants exposed to Cr showed an increased expression of the *EIN3* and *EIN4* genes (Trinh et al., 2014) suggesting the putative role of this MAPK cascade heavy metal induced ET biosynthesis. Recently, Chmielowska-Bak et al. (2014) revealed that cadmium causes induction of ethylene responsive genes and MAPKs in soybean seedlings also suggesting an elevation in MAPKK2 gene expression. Further, Schellingen et al. (2015) suggested a link between MPK3/6 mediated ROS production and ethylene signaling during Cd stress in Arabidopsis.

## Regulation of microRNAs during Heavy Metal Stress

Besides the contribution of signaling pathways in transmitting heavy metal related stimuli and regulating the plant response, other regulators like small RNAs are majorly found to have profound effect on metal stress response. Small RNAs such as microRNAs are a 20–24 nucleotide non-coding RNAs that regulate the gene expression at post-transcriptional level by targeting mRNA degradation or by translation repression (Raghuram et al., 2014). It has been shown that different miRNA families are differentially regulated temporally as well as spatially, differing in concentration from species to species (He et al., 2016). All these data indicate that differential regulation of any miRNA depends upon the function of miRNA target, physiology, and metabolism of the plant.

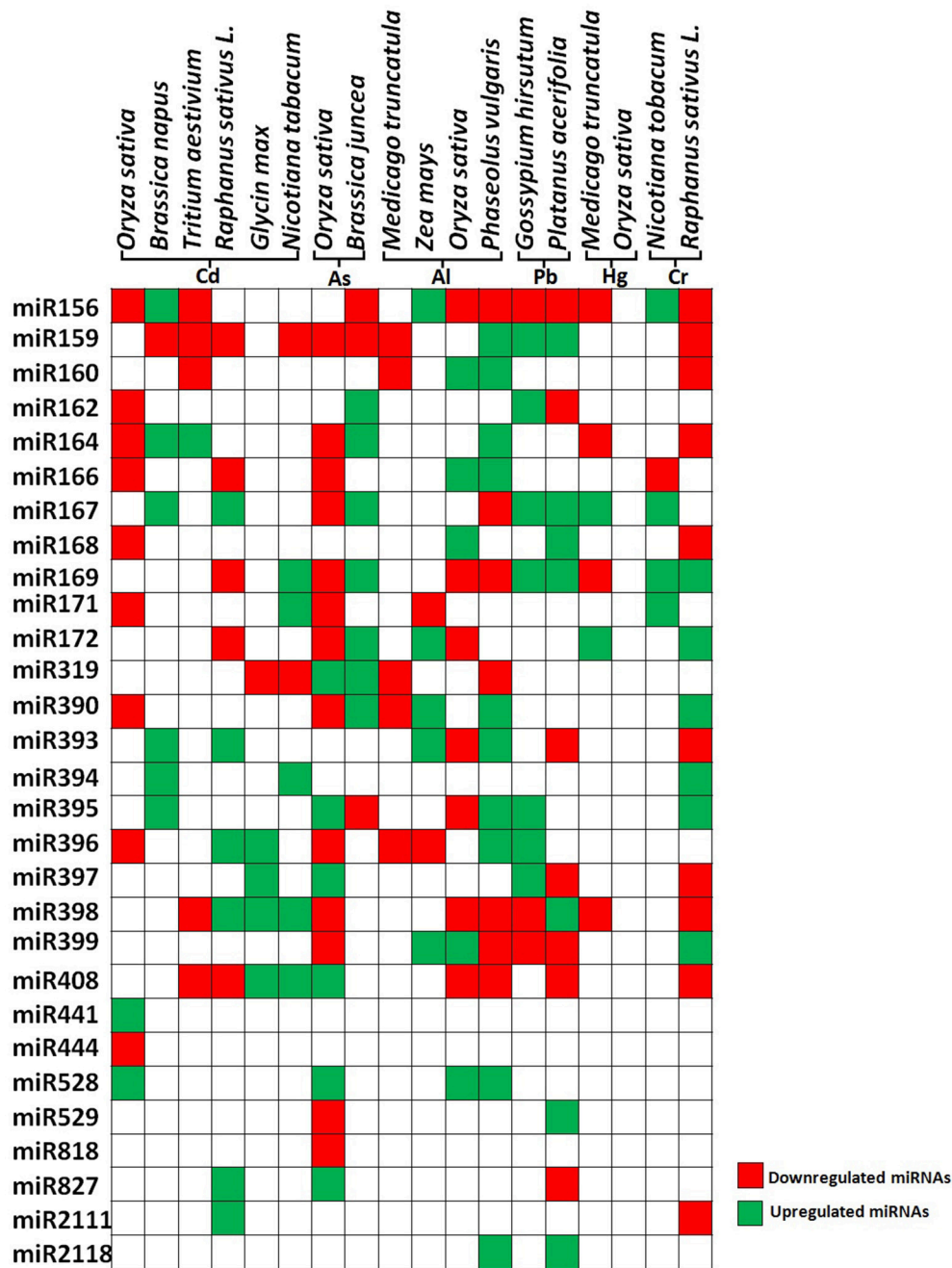
Recently genome wide, transcriptome analysis, and high throughput sequencing have been used to identify the microRNAs, which are responsive to heavy metal stress in many plant species. It has been shown that various conserved miRNAs are differentially regulated during the normal and stress conditions (**Figure 3**). Differential expression of miRNAs in heavy metal stress indicates the possible involvement of miRNAs in heavy metal stress detoxification and tolerance (Ding et al., 2011; Liu and Zhang, 2012; Zhou et al., 2012; Bukhari et al., 2015; Noman and Aqeel, 2017). Here, we have focused on studies showing differential expression of conserved miRNA in metal stress and regulation of signaling pathway by miRNAs or vice versa in response to heavy metals.

Plant exposed to excess concentration of Cd, employed differential regulation of miRNAs. For example in rice, Cd exposure modulates expression of various novel and evolutionarily conserved miRNAs. Upon exposure to Cd, miR441 expression was significantly upregulated while 12 miRNAs were found to be down-regulated. Among the down-regulated miRNAs, miR192 predicted to target ABC transporter, which is shown to be involved in heavy metal transport. Overexpression of miR192 significantly reduced rice seed germination and seedling growth under Cd stress compared to wild-type plants. This suggests that decrease in the amount of miR192 leads to the accumulation of ABC transcripts which eventually leads to Cd sequestration by ABC transporter during Cd stress (Ding et al., 2011; Tang et al., 2014; He et al., 2016) (**Figure 3**).

Contemplating these studies carefully we found that cadmium stress downregulates major miRNAs like miR159 and miR166 in most of the plant species (**Figure 3**). The targets of several Cd responsive miRNAs, including miR398 and miR408 have been shown to target the heavy metal detoxification genes. The Cu–Zn superoxide dismutase (CSD), is an essential enzyme for the detoxification of superoxide radicals and reduced accumulation of respective miRNAs lead to the accumulation of these scavengers during stress, hence protecting the plants against heavy metal induced oxidative damage. Recent studies have identified many As responsive miRNAs by deep sequencing in rice and mustard (Liu and Zhang, 2012; Srivastava et al., 2013; Pandey et al., 2015; Sharma et al., 2015) (**Figure 3**). They have reported that the expression of miR172 was significantly down-regulated whereas miR393, miR397, and miR408 were upregulated. Studies revealed that miR408 has a direct role in targeting Cu containing proteins or superoxide dismutase (Ma et al., 2015). Also, it has been reported that during heavy metal stress, ROS leads to the induction of lipid peroxidation and downregulation of miR397, which has been shown to target laccase. This may lead to positive regulation of lignin biosynthesis through the accumulation of laccase enzymes (Jones-Rhoades and Bartel, 2004) (**Figure 3**).

Aluminum (Al) is being considered as a major limiting factor for plant development interfering with cellular redox equilibrium. Similar to other metals, Al stress also downregulates most of the miRNAs in rice such as miR156, miR395, miR398, miR159 except miR399, miR166, miR168 which showed upregulation (Lima et al., 2011). Contrastingly, in maize the similar miRNAs showed upregulation except miR171 and miR396 which showed downregulation (Kong et al., 2014) (**Figure 3**). MicroRNAs also play a key role in metal complexation wherein two important genes ATP sulfurylase (APS) and SULTR2:1 were identified as targets of miR395 which is reported to induce by Al stress, Cd stress, and sulfur deficiency. Both of these genes lead to the production of GSH and PCs which are chief molecules in metal chelation (Matthewman et al., 2012) (**Table 2**).

Other metals such as mercury, lead, and chromium have also been shown to affect miRNA expression. Mercury treatment differentially regulated miRNAs in *M. truncatula* where miR156, miR172, miR164, miR169, miR398 were downregulated whereas miR167 and miR172 were upregulated (Zhou et al., 2012). Cotton



**FIGURE 3 |** Differential expression of heavy metal responsive microRNAs in plants. The figure represents the data taken from genome wide study of differentially expressing miRNAs in different plant species. Green color and red color indicates up regulated and down regulated miRNAs respectively.

seedlings treated with Pb showed downregulation of miR156, miR398, miR399 and upregulation of miR162, miR167, miR169, miR395, miR396, and miR397 (He et al., 2014). Chromium showed upregulation of miR156, miR167, miR169, miR171, and downregulation of miR166 (Bukhari et al., 2015) (Figure 3). Among the heavy metal responsive miRNAs, miR156, miR159, miR166, and miR398 were shown to be differentially regulated. The currently available data about miRNA targets suggests

that most of the miRNAs such as miR169, miR390, miR394, miR395, miR397, miR399, and miR528 are directly involved in the heavy metal stress tolerance by regulating the transcripts of ROS scavenging enzymes, laccases, or metal transporters. Apart from their direct involvement in heavy metal stress, some of the miRNAs play important role in plant growth and development. For example, miR156 regulate the important transitions in shoot development while miR159 is known to

**TABLE 2** | Heavy metal responsive conserved miRNAs, their targets, and functions.

miRNAs	Targets	Target functions	References
miR156	SQUAMOSA-PROMOTER BINDING PROTEIN (SBP)-like proteins (SPL)	Floral development	Schwab et al., 2005
miR159	MYB transcription factors	Floral development	Achard et al., 2004
miR160	ARF transcription factors	Auxin signaling, Floral organ development	Wang et al., 2005
miR162	DCL1	Micro RNA biogenesis	Xie et al., 2003
miR164	NAC, CUC genes	Drought resistance, Leaf margin serration	Nikovics et al., 2006; Fang Y. et al., 2014
miR165/166	HD-ZIP transcription factors, KANADI	Root development	Singh et al., 2017
miR167	ARF transcription factors	Auxin signaling	Wang et al., 2015
miR168	AGO1	MicroRNA pathway	Vaucheret et al., 2004
miR169	Nuclear factor Y	Drought resistance	Li et al., 2008
miR171	GRAS domain transcription factors/SCARECROW-like (SCL)	Floral development	Ma et al., 2014
miR172	APETELA2-like transcription factors	Transcriptional regulation, Developmental phase transition	Aukerman and Sakai, 2003
miR319	TCP family transcription factor	JA biosynthesis, Senescence	Schommer et al., 2008
miR390	Stress-responsive leucine-rich repeat receptor-like kinase(SRK), ARF	Cd stress tolerance	Fahlgren et al., 2006; Ding et al., 2016
miR393	TIR1, AFB family	Auxin signaling	Chen et al., 2011
miR394	LEAF CURLING RESPONSIVENESS (LCR)	Abiotic stress tolerance	Song et al., 2016
miR395	ATP sulfurylases (APS), ARABIDOPSIS SULFATE TRANSPORTER 68 (AST68)	Sulfate assimilation	Matthewman et al., 2012
miR396	GROWTH-REGULATING FACTOR(GRF) TFs, bHLH74	Cell proliferation regulation	Debernardi et al., 2012
miR397	Laccase	Lignin biosynthesis	Jones-Rhoades and Bartel, 2004
miR398	Cu-Zn superoxide dismutase (CSD)	ROS response	Jones-Rhoades and Bartel, 2004
miR399	ubiquitin-conjugating enzyme E2 24 (UBC24)/PHOS2	Phosphate starvation	Chiou et al., 2006
miR408	Cu containing proteins, Cu/Zn superoxide dismutase, Cu chaperon	Abiotic stress tolerance	Ma et al., 2015
miR441	Unknown	-	-
miR444	MADS-box TFs	Root development	Wang H. et al., 2016
miR528	MATE transporter family, Cu binding protein(CBF)	Enhances Tolerance to Salinity Stress and Nitrogen Starvation, Arsenite Tolerance	Liu et al., 2015; Yuan et al., 2015
miR529	SPL family	Phase transition	Morea et al., 2016
miR818	Serine/threonine kinase	Flowering time regulation	Liu and Zhang, 2012; Ding et al., 2013
miR827	ubiquitin E3 ligase	Suppress immune responses	Hewezi et al., 2016
miR2111	PHO2 and GmPT5	Responses to phosphate starvation	Xu et al., 2013
miR2118	MEL1 gene, TIR-NBS-LRR	Determinate fate acquisition of panicle meristems, drought stress responses	Wu et al., 2015; Ta et al., 2016

inhibit growth and promote programmed cell death by regulating R2R3 MYB transcription factors (Alonso-Peral et al., 2010; Xu et al., 2016) (Table 2). miR166 regulate diverse aspects like formation of apical and lateral meristem, leaf polarity vascular development, and floral development while miR398 is a major plant stress regulator (Jung and Park, 2007). Differential regulation of these important miRNAs by heavy metals might severely affect plant development by altering various mechanisms (Table 2).

A connection between miRNA and MAPK signaling was deciphered by a study which showed regulation of miR398b/c

by OXI upon Cd and Cu treatment (Smeets et al., 2013). OXI is a component of MAPK cascade working upstream to MPK6, regulating ROS production (Asai et al., 2008) (Figure 2). Apart from this, several transcription factors which are known to be downstream target of MAPKs have also been found to be target of miRNA. Transcription factors of Squamosa promoter binding like protein (SPL) family, known to be involved in flower development, were studied to be the targets of miR156/157 and are regulated by Cd, Hg, and Al (Zhao et al., 2014) (Table 2). Additionally, SPLs binds to Cu responsive elements in the promoter of miR398 gene (Cu-responsive gene),

regulating the expression of miR398 in Cu stress (Yamasaki et al., 2009). These studies uncover the concept of how plants exhibit interaction among different components in responding against the environmental stress.

## Modulation of Transcription Factors during Heavy Metal Stress

Heavy metal toxicity is the serious problem of the modern world. For combating heavy metal stress, plants have evolved numerous detoxification and mobilization mechanisms as described earlier. Beside these, activation of complex signaling network is another important factor in heavy metal stress tolerance. Genome wide expression analysis have also reported modulation in the expression of transcription factor families upon exposure to heavy metals (Yanhui et al., 2006; Ogawa et al., 2009; Shim et al., 2009; Farinati et al., 2010; Wang et al., 2010; Smeets et al., 2013). Several studies have reported that upon heavy metal exposure, MAPK signaling cascade activates the downstream transcription factor targets (Figure 2). The transcription factors such as MYB (MYeloBlastosis), WRKY (containing a conserved WRKYGQK domain and a zinc finger-like motif), ZAT (C<sub>2</sub>H<sub>2</sub>-type zinc finger transcription factor), bZIP (basic region leucine ZIPper), AP2 (Activator Protein 2), ERF (ethylene-responsive factor), and DREB (dehydration responsive element-binding protein), have been identified as potential downstream targets of MAPKs (Roelofs et al., 2008; Li et al., 2016).

Transcription factors are important regulators of gene expression affecting many developmental processes and defense responses in plants (Yanhui et al., 2006). Studies have showed that upon exposure to Cd, expression of most of the transcription factors belonging to MYB, AP2, DREB, WRKY, and NAC up-regulates at different time intervals in rice (Ogawa et al., 2009). The MYB family is one of the largest families of transcription factors having diverse functions in eukaryotes (Dubos et al., 2010). Previous report have shown that MYBs such as MYB4, MYB28, MYB43, MYB48, MYB72, and MYB124 were highly induced in Cd and Zn metal stresses in Arabidopsis (van de Mortel et al., 2008). They have also found that the MYB72 loss of function mutant exhibits increased metal sensitivity in Arabidopsis than the related Zn/Cd-hyper accumulator *Thlaspi caerulescens*. In another study, it was reported that Cd inactivates MYB2 by induction of NO production which causes nitrosylation of cysteine residues in the MYB2 transcription factor in Arabidopsis (Serpa et al., 2007). Recent reports have also established role of OsMYB45 in Cd toxicity (Hu et al., 2017). They found that mutation in OsMYB45 resulted in Cd hypersensitive phenotype with significant increase in H<sub>2</sub>O<sub>2</sub> content in the leaves of mutant and decrease in CAT activity as compared to the wild-type. In recent times, Wang F. Z. et al. (2017) have established the role of rice MYB transcription factor OsARM1 (ARSENITE-RESPONSIVE MYB1) that regulates As-associated transporters genes. He found that OsARM1 binds to the conserved MYB binding sites in the promoters of *OsLsi1*, *OsLsi2*, and *OsLsi6*, which encode key As transporters and affects their expression. Several studies

suggest MYB TFs to be the downstream targets of MAPKs. Most recently, Li et al. (2016) found that MPK4 induced by light, regulates photoprotective anthocyanin biosynthesis by regulating MYB75/PAP1 transcription factor (Li et al., 2016). An altered gene expression and activity of MYBs as well as MAPKs in Cd stress gives us a clue of MYB being potential substrates of MAPKs during heavy metal stress.

WRKY transcription factors specifically bind to W-box in the promoters of many genes that are responsive to many biotic and abiotic environmental stress factors. Opdenakker et al. (2012) have reported significantly higher expression of members of WRKY family upon Cu and Cd metal exposures. They found that the transcription factors WRKY22, WRKY25, and WRKY29 were overexpressed in response to short-term exposure of roots to Cu. In contrast, only the expression of WRKY25 and WRKY29 affected upon exposure to Cd over a period of 24 hr (Opdenakker et al., 2012). In Cd treated *T. caerulescens*, expression of WRKY53 was found to be highly induced (Wei et al., 2008). Previous reports also showed that the flagellin induced MAPK cascade MEKK1-MKK4/MKK5-MPK3/MPK6 activates WRKY22 and its close homolog WRKY29 (Asai et al., 2002). Also there are reports showing SA dependent activation of WRKY25 and WRKY33 by MKS1 that directly interacts with MPK4 and negatively regulates defense responses in plants (Andreasson et al., 2005). In accordance with this, a tobacco WRKY1 was found to be phosphorylated by the defense-activating MAP-kinase SIPK (Menke et al., 2005). Also, it was reported that MPK3 and MPK6 phosphorylates WRKY33 and induces phytoalexin biosynthesis in Arabidopsis (Mao et al., 2011). Previous studies revealed that MEKK1 directly interact with WRKY53 on the protein level and also bind to its promoter (Miao et al., 2007). Most recently, expression of WRKY25, a downstream target for MPK4 was found to be up-regulated following Cd exposure (Smeets et al., 2013). Overall, these reports suggest that WRKY transcription factors can work coordinately with MAPK cascade during heavy metal stress tolerance.

Plant bZIP transcription factors are another class that provides defense against various environmental stresses including heavy metal stress. Reports have suggested induced expression of bZIP transcription factors upon Cd exposure (Ramos et al., 2007). Previous studies showed that the bZIP transcription factor from *B. juncea*, BjCdR15, is a regulator of Cd uptake, transport and accumulation in shoots and confers cadmium tolerance in transgenic plants (Farinati et al., 2010). In Soybean, Cd treatment significantly up-regulates bZIP62 expression while the expression of bZIP44 and bZIP78 is down-regulated (Chmielowska-Bak et al., 2014). Likewise, bZIP1 from *Tamarix hispida* showed increased expression in Cd stress in tobacco (Wang et al., 2010). Recent study on a novel bZIP gene, *BnbZIP3* from ramie (*Boehmeria nivea*) plant has showed that it positively regulates heavy metal stress tolerance by improving root growth upon overexpression (Huang et al., 2016). Though the direct link for bZIP transcription factors with MAPKs was not discovered in context to heavy metal stress but a report suggests that Arabidopsis bZIP transcription factor VIP1 (VirE1-Interacting Protein 1) localization and VirE2/T-DNA



complex nuclear import may require phosphorylation by MPK3 (Djamei et al., 2007).

Apart from the above mentioned transcription factors, Cd also modulate expression of AP2/ERF family members, namely, ERF1 and ERF5 in *Arabidopsis* (Herbette et al., 2006). Similarly, Cd induces ERFs in *A. thaliana* and *A. halleri* (Weber et al., 2006). It has been reported that dehydration-responsive element-binding protein (DREB) transcription factors which are members of ERF family of transcription factors gets up-regulated upon heavy metal treatment. Cd leads to elevated expression of DREB1A and DREB1B in rice (Ogawa et al., 2009) while inhibited expression of transcription factors belonging to DRRB family was found in *Solanum torvum* plants (Yamaguchi et al., 2010). Recent studies have demonstrated that MPK3 and MPK6 regulates ethylene signaling by regulating ERF104 and Ethylene-Insensitive 3 (EIN3) which enhances the expression of ERF104 (Yoo et al., 2008; Bethke et al., 2009). In a recent study, a zinc finger transcription factor (C<sub>2</sub>H<sub>2</sub>-type), ZAT12 expression modulated upon short-term exposure to Cu while no such influence was observed upon long-term Cd exposure (Opdenakker et al., 2012). Recently, *Arabidopsis* ZAT6 was found to be positive regulator of Cd tolerance through the glutathione-dependent pathway (Chen et al., 2016). They identified that ZAT6 positively regulates expression of PCs synthesis pathway genes such as *GSH1*, *GSH2*, *PCS1*, and *PCS2*. Studies through protein-protein interaction also showed ZAT10 as direct substrate for MPK3 and MPK6 (Nguyen et al., 2012), suggesting involvement of MAPKs in regulation of heavy metal stress via ERFs and ZAT transcription factors.

Altogether, heavy metal stress activates various signaling components including MAPK cascades. Though, the reports on involvement of MAPKs upstream to transcription factors are rather scarce under metal stress but the above data demonstrated that they indeed interacts with transcription factors and mediates heavy metal stress tolerance response in plants.

## MAPK Signaling in Metal Sequestration and Transport

Encounter of heavy metal by plant roots generates many responses. This starts with binding of metal to the root cell wall and exudates, followed by metal influx across the plasma membrane. The high degree of metal influx is taken care by efflux of metal ions into the apoplast and chelation in the cytoplasm by PCs, metallothionines, organic, and amino acids. These metal ligand complexes are transported to the tonoplast and sequestered into the vacuoles (Sharma and Dietz, 2006; Verbruggen et al., 2009; Mendoza-Cózatl et al., 2011) (Figure 1). There are several molecules involved in this whole process of metal uptake, transportation, chelation, and sequestration. These are metal transporters and chelators accomplishing their task and protecting plants in metal toxicity (Singh S. et al., 2015).

MAPKs are one of the important signaling modules transmitting various stress related signals and are also known to get activated by heavy metal stress as discussed previously. The best characterized MAPKs MPK3 and MPK6 are the ones that are known to get expressed and activated by wide range of metals. Cadmium and copper have shown profound effect

on these MAPKs in number of species. Up-regulation of MPK3 and MPK6 in these heavy metals gives us a clue about their function in metal homeostasis by either regulating downstream metal transporters or chelators that function in response to Cd and Cu. However, studies on ultimate effect of their activation on regulation of metal transporters, other TFs and proteins are still elusive in plants. Even though, there are significant reports on regulation of metal transporters by MAPKs in animals which gives an idea about this crosstalk occurring even in plants.

A number of metal transporters involved in metal ion homeostasis have been identified from different plants. The major groups of metal transporters studied are ZIP, heavy metal transport ATPase (CPx- and P1B-ATPase), NRAMP, CDF, and ABC transporters (Park et al., 2012; Singh S. et al., 2015). Several studies report their role in metal translocation and uptake based on expression pattern in different heavy metals. ZIP members were the first to be reported in plants, having ability to transport divalent cations like Zn<sup>2+</sup>, Fe<sup>2+</sup>, Mn<sup>2+</sup>, and Cd (Eide et al., 1996). IRT1 gene from *Arabidopsis* belonging to ZIP family is major transporter of iron leading to high affinity Fe uptake. In iron limiting environment IRT1 is present only in roots and is studied to be induced within 24 h of iron deficient conditions. Plants overexpressing IRT1 accumulate high levels of Cd and Zn along with Fe (Connolly et al., 2002). Other ZIP members ZIP1 and ZIP2 were studied to be Zn and Mn transporters in roots contributing to remobilization of Mn/Zn from vacuole to cytoplasm in root stellar cells and further movement from root stele to xylem parenchyma. According to their role, the expression of both the genes is mainly localized to the root stele (Milner et al., 2013). An interesting study on iron deficiency induced ethylene production in *Arabidopsis* reports the role of MPK3 and MPK6 in iron transport. The expression of iron transport and chelator genes (IRT1, FRO2, and FIT) was down regulated in *mpk3* and *mpk6* mutants under Fe deficiency (Ye et al., 2015), which suggests a possibility of these iron responsive genes working downstream of MAPK cascade (Table 3). This also suggests a crosstalk of MAPK signaling and hormone pathways in metal translocation in plants. Whilst in plants, there is single report of MAPK involvement in regulating metal transporter; in animals this concept is well-explored. It is not only the MAPKs known for regulating metal transporters but metal transporters are also equally involved in activating MAPKs. A report on chicken cell line suggest a role of ZIP transporter ZIP9/SLC39A9 in elevating intracellular zinc level and thereby regulating the activation of Erk MAPK signaling (Taniguchi et al., 2013) (Table 3).

Other metal transporter family NRAMP functions in diverse organisms ranging from bacteria to humans. In plants there are two subfamilies of NRAMP genes and several of them upregulate in Fe, Mn, and Cd deficiency. NRAMP proteins are studied to be localized on intracellular membranes of plastid and vacuolar membrane (Thomine and Schroeder, 2004). Expression analysis of NRAMP in plants suggests that unlike ZIP family (expressed mainly in roots) these metal transporters are expressed both in root and shoot, thus participating in metal homeostasis in all plant tissues. However, functional characterization of plant NRAMP transporters remains limited. Couple of studies

**TABLE 3** | Metal transporters regulating MAPK signaling and vice versa.

Family	Metal transporter	Metal ions	MAPKs	Model organism	Reference	
<b>METAL TRANSPORTERS REGULATING MAPKs</b>						
1	ZIP family	ZIP9/SLC39A9	Zn	ERK MAPKs	Chicken	Taniguchi et al., 2013
2	NRAMP family	NRAMP1 (SLC11A1)	–	P38MAPK	Mammals	Moisan et al., 2006
3	CTR family	CTR1	Cu	MEK1-ERK1	Mammals	Tsai et al., 2012; Turski et al., 2012
4	CDF family	ZnT1	Zn, Co, Cd	Raf1-MEK-ERK	<i>C. elegans</i>	Jirakulaporn and Muslin, 2004
5	Metallothioneins	Zn	P38 MAPK	Mammals	Rice et al., 2016	
<b>MAPKs REGULATING METAL TRANSPORTERS</b>						
1	ZIP family	IRT1	Fe	MPK3, MPK6	Arabidopsis	Ye et al., 2015
2	NRAMP family	NRAMP1	–	P38MAPK, p42/44 MAPK	Mammals	Zhang et al., 2000
3	ABC family	MRP1	–	ERK/MAPK pathway	Mammals	El Azreq et al., 2012
4		ABCA1, ABCG1		Ras-MAPK pathway	Mammals	Mulay et al., 2013
5	Zn transporter family	Zrc1	Zn	Pbs2-Hog1-RCK1/RCK2	Yeast	Bilsland et al., 2004
6	Phytochelatin	–	Unknown MAPK	<i>S. mansoni</i>	Rigouin et al., 2013	

in animal suggest the regulation of NRAMP1 transporter by MAPKs. This ion transporter was known to work downstream of p38 and p42/44 MAPK pathway activated upon proinflammatory mediators and bacterial infection in mammalian cells (Zhang et al., 2000). Also, another study implied the role of NRAMP1 in modulation of MAPK pathway (Moisan et al., 2006) (**Table 3**).

ABC transporters comprises of largest family, classified into eight subfamilies playing roles in diverse cellular processes like nutrient uptake, osmotic homeostasis, hormone transport, pathogen resistance, fatty acid import, and metal tolerance (Park et al., 2012). Arabidopsis ABC transporter, AtPDR8 is identified as cadmium extrusion pump conferring resistance to heavy metal Cd and Pb (Kim et al., 2007). Owing to their metal transportation capacity ABC C-type transporters AtABCC1 and AtABCC2 have been identified as major phytochelatin-heavy metal(oid) complex transporters. In recent study in wheat, expression of 13 ABC transporter genes was analyzed in different metals, which suggested that these genes were differentially regulated by Cd, indicating their participation in Cd uptake, transport, and sequestration (Wang X. et al., 2017). Yet another transporter of ABC family named as MRP1 (multidrug associated protein) is known to be regulated in ERK/MAPK pathway dependent manner in leukemic T cells (El Azreq et al., 2012). ABC transporters are known for their role in vacuole sequestration in plants. Besides this Ras/MAPK pathway is also reported to regulate ABC metal transporters (ABCA1 and ABCG1) in human hepatic cells (Mulay et al., 2013) (**Table 3**). A study in yeast proves the fact of activation of metal transporter by MAPKs more firmly. A MAPK cascade consisting of Pbs2-Hog1-Rck1/Rck2 in yeast is studied to activate a transcription factor (Yap2) and Zn transporter (Zrc1) thereby providing oxidative stress resistance (Bilsland et al., 2004) (**Table 3**).

Another metal transporter involves CTR transporter having an important role in maintaining Cu homeostasis in various species. CTR transporters are either plasma membrane proteins transporting Cu from extracellular spaces to cytosol or lysosome membrane proteins transporting Cu from lysosome to cytosol.

In mammals, a study on high affinity copper transporter CTR1 was reported to activate MAPK cascade, wherein the mutation of CTR1 and Cu chelators reduces the activation of Erk1 (MAPK) by MEK1 (MAPKK) (**Table 3**). This is due to the fact that Erk1 phosphorylation by MEK1 requires Cu binding which diminishes in *ctr1* mutant (Tsai et al., 2012; Turski et al., 2012). Likewise, another report suggest that CDF proteins which are famous for the transport of Zn<sup>2+</sup>, Co<sup>2+</sup>, Cd in plants, modulates the activity of Raf1-MEK-ERK pathway in *C. elegans*. Its homolog in mammals, ZnT1 binds directly to the Raf1 in its regulatory domain thus activating it (Jirakulaporn and Muslin, 2004) (**Table 3**). Besides this, there are wide array of metal transporters that mediate Ca transport and its sequestration into the vacuole. Cation/proton exchangers (CAX) and isoforms have broad specificity and are widely implicated in Ca transport and other heavy metals like Mn<sup>2+</sup> and Cd. CAX have been identified in salt tolerance, cadmium transport and tolerance. In mammals NHE1, one of the CAX was found to regulate MAPKs wherein it inhibited ERK1/2 and stimulated JNK1/2 activity (Pedersen et al., 2007).

Apart from the transporters activating MAPKs or vice versa, there are also studies suggesting the activation of MAPKs by metal chelators. The important metal chelators are PCs and metallothioneins and these are small cystein rich peptides with metal binding capacity (Singh S. et al., 2015). Two reports suggest MAPK activation by metallothioneins mainly by the release of the metal ions chelated (Chung et al., 2008; Rice et al., 2016). Additionally, PCs and phytochelatin synthase are hypothesized to be acting downstream of MAPK pathway in plants and human parasite respectively (Rigouin et al., 2013) (**Table 3**).

## CONCLUSION

From this review it is implied that metal exerts tremendous effect on plant by modulating its functioning at various levels. Metal stress activates several signaling pathways, known to have important role in imparting resistance against environmental stresses. Of these, an important signaling pathway contributing

majorly in managing stress response is MAPK signaling pathway. Activation of signaling pathways magnifies the activation and functioning of various downstream components like transcription factors and other cytosolic protein thereby altering the expression of genes. In this review, the impact of heavy metals on activation of MAPK, calcium and hormone signaling along with other regulators like transcription factors and miRNAs is certainly reported. Several studies performed throughout different scientific groups suggest important role of MAPK signaling in heavy metal stress. However, a detailed evaluation of complete MAPK cascade working in combating heavy metal stress is required. Further, this review compiles the results revealing interplay of MAPK signaling with calcium, auxin, and ethylene signaling in response to heavy metal stress. All these findings outline the regulatory function of MAPKs acting either upstream or downstream to other signaling molecules. In animals this regulatory network has been elaborately studied in metal stress than in plants. Hence, it will be highly advantageous to study the importance of this signaling crosstalk in heavy metal stress in plants. Additionally, this review also summarizes interplay between MAPK signaling and other regulators like miRNAs and transcription factors in conveying a response against metal stress. However, scarce reports on regulatory network of MAPKs with transcription factors suggest a need for more in depth experiments in response to heavy metal stress in plants. Though, there are ample numbers of reports on activation of different signaling components, studies on deciphering of a complete regulatory network in heavy metal stress in plants are

still lacking. Furthermore, the studies on activation of MAPKs by metals and metal transporters and in turn their regulation by MAPKs in animals and yeast, suggests occurrence of this phenomenon even in plants. However, the fragmentary work performed keeps this area mysterious in plants. Exploring the regulators of these metal transporters will contribute significantly in unraveling the mechanisms of metal stress tolerance in plants.

## AUTHOR CONTRIBUTIONS

SJ, PB, DV, SN, ST, and DS: wrote the manuscript; SJ: conceptualize the overall structure, prepared the illustrations, and edited the manuscript; KS and PB: prepared figures; AS: conceptualized, edited and approved the final manuscript.

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

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