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Mini Review

The role of microRNAs in copper and cadmium homeostasis

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ABSTRACT

Essential heavy metals (e.g., copper) and non-essential metals (e.g., cadmium) are both toxic to plants at high concentrations. Recently, microRNAs (miRNAs) have emerged as important modulators of plants adaptive response to heavy metal stress. Plant miRNAs negatively regulate target mRNAs by post-transcriptional cleavage. miR398 regulates copper homeostasis via down-regulating the expression of Cu,Zn-superoxide dismutase (*CSD*), a scavenger of superoxide radicals. miR393 and miR171 play an important role in cadmium stress mediation. This review focuses on the recent advance in the involvement of miRNAs in copper and cadmium stress regulatory networks in plants.

Plant metal homeostasis and tolerance

Heavy metal pollution is an increasing environmental problem worldwide. Essential heavy metals such as copper and zinc are required for a wide range of physiological processes, yet can be toxic at elevated levels. Other metals (e.g., cadmium, lead and mercury) are nonessential and potentially highly toxic. The characteristic feature of heavy-metal toxicity is the disruption of enzyme systems and the induction of oxidative stress by direct or indirectly generating reactive oxygen species (ROS) [1,2]. Thus far, a number of physiological, biochemical, and molecular studies have been made to investigate the mechanisms of plant metal tolerance and homeostasis. The best described mechanism involves the intracellular metal chelation by glutathione (GSH), phytochelatins (PCs), metallothioneins (MT), organic acids, and amino acids [3,4]. Additionally, several membrane transporters such as ATP-binding cassette (ABC) transporters, cation diffusion facilitators (CDF), and natural resistance-associated macrophage proteins (NRAMP) have been found to mediate heavy metal vacuolar sequestration, conferring metal tolerance to plants [3,5,6]. Overall, the network of metal transport, chelation, sequestration contributes to the detoxification of heavy metals. A key step in this network is heavy-metal ions sensing and the subsequent regulatory pathway [3]. Transcriptional and post-transcriptional gene regulation is important for the response to metal exposure or metal deficiency. However, the regulatory network in metal homeostasis is largely unknown. Recently, growing evidence has revealed that miRNAs function as key regulators in the alleviation of plant metal stresses [7–12]. Analysis of miRNAs and their targets involved in heavy metal stress mediation may provide a new insight into understanding of plant stress response mechanisms.

Overview of the role of miRNAs in plant metal homeostasis and tolerance

miRNAs are a newly discovered class of non-protein-coding small RNAs of roughly 21 nucleotides [13]. Predominantly the mature miRNAs are incorporated into RISC (RNA-induced silencing complex) and negatively regulate specific target genes expression by the perfect or near-perfect base pairing to their complementary mRNAs at the post-transcriptional level [14,15]. The role of plant miRNAs was initially reported to be involved in a broad range of biological processes, including growth and development patterning, cell identity, and signal transduction [16–18]. Recently, miRNAs have emerged as a class of gene expression regulators, involved in different abiotic stress responses [19–21]. miR395 and miR399 were induced by sulfate and phosphate deprivation, respectively [13,22,23]. The microarray-based analysis revealed 14 stress-regulated miRNAs in *Arabidopsis thaliana* under salinity, drought, and cold stress, among which miR168, miR171, and miR396 responded to all three abiotic stresses [21]. Many abiotic conditions including heavy metals can lead to oxidative stress by generating ROS in plants [24]. Cu and Cd are the two most intensively studied metals with regard to tolerance [3]. To date, increasing evidence points to the role of miRNAs in coping with Cu and Cd stress. miR398 is a key regulator in maintaining Cu homeostasis (Fig. 1) [7–9]. The down-regulation of miR398 expression is important for the induction of *CSD1* and *CSD2* mRNA levels under high Cu stress [7]. Conversely, when Cu is limited, decreased *CSD1* and *CSD2* transcripts abundance can be attributed to the induced miR398 expression [8,9]. Several miRNAs have been reported to

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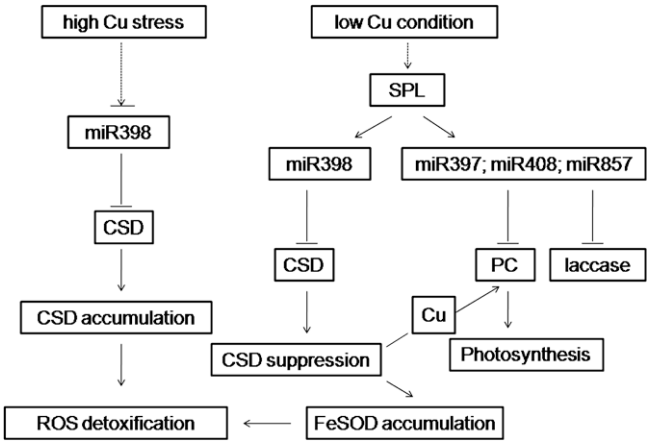

Fig. 1. Regulatory pathway of miR398 and other miRNAs in Cu homeostasis in *Arabidopsis*.

play an important role in Cd stress tolerance. miR393 and miR171 are involved in relieving Cd stress via down-regulating their target genes (Table 1); Osa-miR604 functions in the regulation of Cd tolerance through directing degradation of lipid transfer protein (*LTP*) mRNA [10–12]. The focus of this review is the recent progress in miRNAs as well as their targets involved in plant Cu and Cd homeostasis and tolerance. It extends the current view on the molecular understanding of miRNA-guided regulation of plant heavy metal adaption.

miRNAs involved in Cu homeostasis

Several heavy metals such as Cu are essential micronutrients required for a various range of physiological processes. Three most abundant copper proteins, copper/zinc superoxide dismutase (Cu,Zn-SOD), plastocyanin (PC) and cytochrome c oxidase (COX) are essential for oxidative stress alleviation, photosynthesis, and respiratory electron transport, respectively [25]. However, Cu can be toxic to plants at supraoptimal concentrations. It is a redox-active metal occurring in different oxidative states in biological systems, leading to the generation of ROS via Fenton-type reaction [26]. Superoxide dismutases (SODs) form the first line of defense against superoxide radicals. SODs can be classified into three groups: iron SOD (FeSOD), manganese SOD (MnSOD) and copper, zinc SOD (Cu,Zn-SOD; CSD) based on the metal cofactors [27]. *Arabidopsis* possesses three isozymes of CSD, which are localized to the cytoplasm (CSD1), chloroplast stroma (CSD2), and peroxisome (CSD3) [28]. CSD1 and CSD2 are the major isoforms in green tissue. miR398 was identified by computational prediction and cloned from a small RNAs library from *Arabidopsis* under abiotic stresses [13,19]. On the basis of computational prediction, miR398 targets two closely related *CSDs*: cytosolic *CSD1* (At1g08830), chloroplastic *CSD2* (At2g28190), and *COX* (At3g15640). These targets were further confirmed experimentally by cleavage site analysis [13].

Cu is detrimental to plants at supraoptimal concentrations. Excess Cu inhibits a wide range of physiological and biochemical processes, including interference with photosynthesis, pigment synthesis, fatty acid and protein metabolism and membrane integrity. The most important consequence may be the inhibition of photosynthetic electron transport, inducing radicals which start peroxidative chain reactions [29,30]. Cu,Zn-SOD (CSD), arguably as the most important SOD, functions in catalyzing the dismutation of superoxide radicals into H_2O_2; on the other hand, CSD as a major copper protein may serve as Cu sinks under conditions where Cu content is higher than the normal level [8]. *CSD1* and *CSD2* transcripts were reported to increase in response to oxidative stress induced by excess Cu [7]. To investigate the potential function of miR398-directed *CSD1* and *CSD2* regulation, *CSD1*, *CSD2* and miR398 transcripts were detected by RNA gel blot analysis in *A. thaliana* seedlings treated with 100 μM Cu [7]. Results showed that miR398 expression was down-regulated transcriptionally under high Cu stress and this down-regulation was important for the post-transcriptional induction of *CSD1* and *CSD2* mRNAs. The induction of *CSD* under high Cu stress can effectively detoxify superoxide radicals and accumulate more Cu as the main Cu sink. *CSD1* and *CSD2* mRNA expressions were fine-tuned by miR398-guided mRNA cleavage. The functional significance of miR398-mediated regulation of *CSD* was further investigated by overexpressing a miR398-resistant form of *CSD2* in *A. thaliana*. Results showed this transgenic plants accumulated more *CSD2* mRNA than plants overexpressing a regular *CSD2* and were consequently much more tolerant against heavy metal stress [7].

Cu is an essential mineral nutrient element in plants and Cu deficiency leads to reduced photosynthetic electron transport, plastoquinone and pigment synthesis, and a disintegration of the thylakoid membrane [31]. Thus its concentration must be controlled within a narrow range. Under Cu limited conditions, *CSD* transcript abundance decreased and the iron Superoxide dismutases (*FeSOD*) were up-regulated in *A. thaliana* [8,9]. At the same time, the expression of miR398 was induced. Of note, miR398 mediated the down-regulation of *CSD*, by directing the *CSD* mRNA degradation when Cu was limited [8]. When Cu is limiting, the

Table 1
miRNAs involved in Cd tolerance.

miRNAs	Targets	Targets functions	Reference
miR171	SCL transcription factors	Floral development	[10–12]
miR393	F-box proteins (TIR1); bHLH transcription factors	Auxin signaling	[10–12]
miR319	TCF transcription factors	Morphogenesis of shoot lateral organs	[10]
miR529			[10]
miR398	CSD	Cu stress response	[10]
miR166	HD-Zip transcription factors	Dorsoventral leaf polarity	[10]
miR156	SBP transcription factors	Vegetative phase change; Root development	[11]
miR396	GRL transcription factors; Rhodenase-like proteins; Kinesin-like protein B		[11]
miR159	MYB and TCP transcription factors	Floral development	[12]
miR160	ARF10, ARF16 and ARF17	Auxin signaling	[12]
miR164	NAC domain containing proteins	Lateral root development; Axillary meristem development	[12]
miR167	ARF6 and ARF8	Auxin signaling	[12]
miR168	ARGONAUTE	Plant development	[12]
miR169	CAAT binding factor		[12]
miR806			[12]

down-regulation of *CSD* mRNA along with the induction of *FeSOD* maintains SOD activity in plastid; on the other hand, the down-regulation of *CSD* expression allows efficient delivery of limited Cu to PC, which is essential for photosynthesis [8]. These observations indicate that miR398 is a key modulator in maintaining Cu homeostasis and miR398-guided *CSD1* and *CSD2* transcripts regulation seems to be a flexible strategy to control Cu availability.

Interestingly, in addition to miR398, another three miRNAs, miR397, miR408, and miR857 have been reported to be involved in response to low Cu availability [9]. They were computationally predicted to target genes encoding PC and laccase in *Arabidopsis* and these predicted targets were further validated by cleavage site analysis [9]. Abdel-Ghany and Pilon demonstrated *Arabidopsis* accumulated miR397, miR408, and miR857 mRNAs when Cu was limiting. The transcripts for *PC* and *laccase* decreased under a reciprocal regulation by the three miRNAs. Furthermore, the expression of other laccases that were not predicted targets for known miRNAs was also Cu-regulated [9]. These results suggest a strong link between miR398, miR397, miR408, and miR857 and Cu homeostasis.

The *cis*-acting elements and *trans*-acting factors involved in stress-induced gene expression have been analyzed extensively [32]. Further investigation of the regulatory pathways upstream of these Cu-responsive miRNAs provided new insights into understanding of Cu homeostasis regulation. Yamasaki et al. found that GTAC motifs exist in the promoter region of *MIR398* gene, and are essential for the response to Cu deficiency [33]. SPL7 (SQUAMOSA promoter binding protein-like 7) is homologous to Copper response regulator1, the transcription factor required for Cu deficiency response in *Chlamydomonas reinhardtii* [34]. The SPL7 transcription factor directly binds to GTAC motifs in the *MIR398* promoter, activating transcription of *MIR398* gene. Additionally, SPL7 is required for the expression regulation of other Cu-related miRNAs such as miR397, miR408, and miR857 [33]. These imply the crucial role of SPL7 in the transcriptional regulation of Cu-responsive miRNAs including miR398.

miRNAs involved in Cd tolerance

Cd is a widespread heavy metal pollutant with high toxicity to plants. It is well known that Cd can induce oxidative stress by generating ROS. Cd-induced H_2O_2 accumulation has been observed in several plants. Cd accumulation causes visible injury to plants including chlorosis, browning of root tips, growth inhibition, and even death [35,36].

The legume *Medicago truncatula* is among the few plants that cope with the adverse conditions such as heavy metal in soils [37]. Zhou et al. identified a total of 38 potential miRNAs in *M. truncatula* on the basis of bioinformatics prediction [10]. To verify the prediction of the miRNAs and test whether the miRNAs function in Cd stress mediation in *M. truncatula*, a qRT-PCR-based assay was performed. Results showed six miRNAs displayed altered expression profiles in response to Cd (80 μM). Among those, miR393, miR171, miR319, and miR529 were up-regulated, whereas miR166 and miR398 were down-regulated [10]. The down-regulation of miR398 expression implied the induction of *CSD1* and *CSD2* transcripts which can alleviate oxidative stress induced by Cd. In addition to Cd stress, expression of these miRNAs were up- or down-regulated exposed to Hg (20 μM) or Al (50 μM). These results indicate the importance of some miRNAs in the regulation of plant heavy metal stress response. In *Brassica napus*, miR156, miR393, miR171, and miR396a transcripts have been reported to be down-regulated exposed to Cd by RT-PCR analysis [11]. These miRNAs target genes encoding transcription factors involved in development regulation and signal transduction pathways (Table 1) [13,19].

Huang et al. constructed a library of small RNAs from rice seedlings exposed to 80 μM Cd for 0–24 h, identifying 19 novel Cd stress-regulated miRNAs and 9 known miRNAs from miRBase including miR171 and miR393 (Table 1) [12]. A total of 34 miRNA targets were predicted for the new miRNAs based on sequence complementarity, and most targets were associated with plant heavy metal stress response. Osa-miR604 was predicted to target wall-associated kinase (WAK) like protein. Some WAK members were reported to be involved in plant defense and heavy metal (such as Al, Cu, and Zn) responses [38,39]. In addition to WAK, Osa-miR604 targets a gene encoding lipid transfer protein (LTP), which was responsive to environmental stresses [40]. Furthermore, *LTP* transcript was induced in rice seedling roots under Cd stress. At the same time the expression of Osa-miR604 was decreased based on semi-quantitative RT-PCR analysis [12]. These results suggest that Osa-miR604 functions in the regulation of Cd tolerance via directing degradation of *LTP* mRNA in plants.

Among the Cd stress-responsive miRNAs described above, miR393 and miR171 are involved in Cd stress mediation in *M. truncatula*, *B. napus* and rice. miR393 targets four closely related F-box genes, including E3 ubiquitin ligase/TIR1 (transport inhibitor response1), which acts as a component of the ubiquitination pathway [13]. TIR1 positively regulates auxin signaling by promoting the degradation of AUX/IAA proteins [41]. Thus, the induction of miR393 expression under Cd stress in *M. truncatula* decreases *TIR1* mRNA level, which, on one hand, leads to the down-regulation of auxin signaling; on the other hand, possibly leads to less proteolysis of E3 ubiquitin ligase targeting proteins (probably positive regulators or determinants of Cd tolerance) [42]. It suggests a potential cross-talk between Cd stress signaling and auxin signaling pathway. miR171 is most highly expressed in flowers, and its target genes encode scarecrow-like transcription factors which regulate floral development [14]. miR171 functions as a developmental regulator and Cd stress modulator as well. Microarray data from *Arabidopsis* implied that many of miRNA target genes involved in the regulation of growth and development might also be responsive to cold stress [42]. These findings suggest that a number of miRNAs play important roles in plant response to abiotic stresses as well as in development regulation.

Conclusions and perspectives

Heavy metal stress affects many physiological and biochemical processes; thus, plants evolve complex mechanisms to control the correct concentrations of essential metal and to minimize the damage from exposure to non-essential metal. The network of metal uptake, transport, chelation, sequestration, and detoxification contributes to the alleviation of heavy metal toxicity and has been studied extensively. However, the regulatory network in heavy metal mediation is just beginning to be explored. More recently, miRNA-guided post-transcriptional gene regulation has been found to play a crucial role in heavy metal response. miR398-directed *CSD* mRNA repression is essential for maintaining Cu homeostasis (Fig. 1) [7–9]. miR393 and miR171 have an important role in Cd stress response in diverse plant species via regulating their targets negatively; manipulation of Osa-miR604 abundance in rice elucidates the cascade of miRNA, LTP, and Cd tolerance [10–12]. Several heavy metal responsive miRNAs such as miR171 target transcription factors which then regulate the expression of their downstream specific protein coding genes. Notably, most of the known miRNAs target genes encoding transcription factors, implying that miRNAs are key participants in gene regulatory networks. Transcriptional and post-transcriptional gene regulations are important for plant development and abiotic stresses mediation. Hence, it is conceivable that miRNA-mediated gene regulation

might serve as a major mode of regulatory networks during metal response.

The function of miR398 and its target *CSD* mRNA in Cu homeostasis has been relatively well elucidated from previous studies, whereas more evidence needs to be provided for the role of Cd-stress-responsive miRNAs as well as their targets in Cd stress regulation. The Cd stress-related miRNAs were identified by direct cloning together with PCR based assays in previous researches [10–12]. Liu et al. performed microarray-based analysis of stress-regulated miRNAs in *A. thaliana* and detected 10 high-salinity-, 4 drought-, and 10 cold-regulated miRNAs [21]. Microarray analysis in combination with computational prediction will largely widen the scope of Cd-related miRNAs. Moreover, further functional analysis of Cd-stress-responsive miRNAs such as miR171, miR393 will help to address their involvement in heavy metal mediation. Genetic or biochemical analysis of miRNA and its target mRNA or protein levels, together with transgenic strategy for over-expressing miRNA or expressing miRNA-resistant form of target mRNA will further explain their role in heavy metal tolerance in plants. Additionally, *cis*-acting elements and *trans*-acting factors have been reported to be important for stress response. The fact that miR398 is regulated by Cu stress combing the existence of GTAC motifs in *MIR398* promoter provides support for the role of miR398 in Cu stress response. The SPL7 transcription factor binds to GTAC motifs and then regulates *MIR398* gene expression [33]. However, the knowledge of Cd stress-relevant *cis*-elements in the promoter of *MIRNA* genes is limited. Thus, exploration of the upstream regulatory pathways of these miRNAs including metal signal sensing and *cis*-regulatory element analysis, as well as the regulatory pathways downstream of the miRNA target proteins will provide a better understanding of miRNA-mediated regulatory mechanisms of plant heavy metal adaptation.

Still, we are beginning to understand the functions of miRNAs in regulating plant responses to metal stress. To date, research has revealed several miRNAs are involved in two heavy metal stresses responses, Cu homeostasis and Cd tolerance. With regard to other essential or non-essential metals responses, more miRNAs might participate in this regulation. miR398 was also reported to be down-regulated in *Arabidopsis* treated with 100 μM Fe [7]; miR319 and miR393 was up-regulated by Al and Hg, respectively, in *M. truncatula* [10]. Thus, more and more miRNAs involved in regulating more metal stresses responses need to be identified and their role needs to be explored. This review would be a good starting point for further elucidating the miRNAs associated with metal homeostasis.

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