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Identification of manganese-responsive microRNAs in *Arabidopsis* by small RNA sequencing

Jian Gong[1¶], Dong Li[3,5¶], Hao Li[4,5], Huakun Zhou[2], Jin Xu[5]**

[1]*Pharmaceutical and Biological Engineering Department, Zibo Vocational Institute, Zibo, P.R. China*
[2]*Northwest Institute of Plateau Biology, Key Laboratory of Restoration Ecology of Cold Area in Qinghai Province, Chinese Academy of Sciences, Xining, P.R. China*
[3]*College of Horticulture, Shanxi Agricultural University, Taigu, P.R. China*
[4]*College of Forestry, Shanxi Agricultural University, Taigu, P.R. China*
[5]*CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Mengla, Yunnan, P.R. China*

**Corresponding authors: gongjiansd@126.com; xujin@xtbg.ac.cn*

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Abstract: Manganese (Mn) is an important micronutrient for growth and development in plants, however, excess Mn is harmful by disrupting photosynthesis system and inducing oxidative damage in leaves. MicroRNAs (miRNAs) play key roles in regulating Mn toxicity tolerance in plants. Here, we identified Mn toxicity-responsive miRNAs in *Arabidopsis* by using small RNA sequencing. Eighteen differentially expressed miRNAs were identified in *Arabidopsis thaliana* seedlings in response to Mn toxicity. These differentially expressed miRNAs are involved in regulating nutrition homeostasis, transport, stress response, and developmental processes. Our results indicated that these miRNAs play a key role in Mn toxicity response in plants.

Keywords: *Arabidopsis thaliana*; high-throughput sequencing; manganese toxicity; miRNA

Manganese (Mn) is an important micronutrient that regulates plant growth and development. Mn is the metal ion component of numbers of proteins and enzymes, such as superoxide dismutase (SOD) and oxygen-evolving complex in photosystem II. Mn is also involved in secondary metabolites biosynthesis (Le Bot *et al.* 1990; Lidon *et al.* 2004; Zhao *et al.* 2017). However, excess Mn disrupts photosynthesis system and induces oxidative damage in leaves, and thereby inhibiting plant growth and development (Srivastava & Dubey 2011; Millaleo *et al.* 2012). In acidic soils, aluminum (Al) and Mn toxicity are the two important limiting factors for crop growth.

MicroRNA (miRNA) is the small noncoding RNA that modulates growth, development, nutrient uptake, and heavy metal tolerance in plants (Kang *et al.* 2017; Xie *et al.* 2017). Ding *et al.* (2011) and Zhou *et al.* (2012) identified cadmium (Cd)-responsive miRNA in plants. Xie *et al.* (2017) and Kang *et al.* (2017) identified Zn- and Cd-responsive miRNAs in heavy metal hyperaccumulator *Solanum nigrum* and low Cd-accumulator *Solanum torvum*, respectively.

¶These authors contributed equally to this work.

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Valdes-Lopez *et al.* (2010) identified Mn toxicity- and nutrient deficiency-responsive miRNAs in bean (*Phaseolus vulgaris*) by microarray analysis. These heavy metal-responsive miRNAs are involved in regulating growth, development, metabolism, and heavy metal transport. However, the Mn-responsive miRNAs in *Arabidopsis thaliana* (L.) Heynh. have not been identified. Our previous study revealed that Mn toxicity inhibited root growth by disrupting auxin biosynthesis and transport in *Arabidopsis* (Zhao *et al.* 2017). In this study, we identified the Mn-responsive miRNAs in *Arabidopsis* by high-throughput sequencing. The possible mechanisms for the role of these Mn-responsive miRNAs are discussed.

MATERIAL AND METHODS

Our previous study indicated that the treatment with 4 mM $MnCl_2$ induces an approximately 50% decrease of primary root growth in *Arabidopsis* seedlings (Zhao *et al.* 2017), we thus selected this concentration in the present experiment. Five-day-old *A. thaliana* Col-0 seedlings were transferred into fresh 1/2 Murashige and Skoog (MS) medium (Murashige & Skoog 1962) containing 4 mM $MnCl_2$ for 1 day. Total RNAs were extracted from untreated- and Mn-treated *Arabidopsis* Col-0 using TRIzol (TaKaRa). Then, the RNAs of three independent biological replicates were mixed and subjected to urea polyacrylamide gel electrophoresis (15%). The 18–28 nucleotide (nt) small RNAs were isolated from the gel and purified, and then were ligated sequentially to 5' and 3' adaptors by using T4 RNA ligase 1 and T4 RNA ligase 2 (truncated) and converted to DNA. The PCR product was sequenced using the Illumina HiSeq2500 (Biomarker, P.R. China). The miRDeep2 computational software (Ver. 2.0.0.7, 2012) was used to identify and map of unique sRNA sequences to *Arabidopsis* TAIR 10 database. The DESeq R package (Ver. 1.10.1, 2010) was used to analyse the differential expressed miRNAs. The miRNA expression levels in two samples (control and Mn treatment) were normalized to obtain the expression of transcript per million. The target prediction was according to Allen *et al.* (2005).

Total RNA was extracted as described above. Stem-loop qRT-PCR was performed as previously described by Feng *et al.* (2015). SYBR® Green Realtime PCR Master Mix (Toyobo, Japan) was used to perform mature miRNA stem-loop qRT-PCR. The *U6* snRNA was chosen as a reference control and the specific primers for each miRNA gene were the mature miRNA sequences (CTGAAGTGTTTGGGGGAACTC for miR395a/d/e; TGCCAAAGGAGATTTGCCCTG for miR399a; TAGTCCGGTTTTGGATACGTG for miR826; and TTAGAGTTTTCTGGATACTTA for miR781a). The experiments were replicated three times using templates using three independent samples.

For the nutrient content analysis, the seedlings were immersed in 1 mM EDTA for 1 h and then rinsed 6–8 times with ddH_2O. Samples were dried at 75°C for 3 days and then were ground and digested in concentrated HNO_3 for 2–3 days. The samples were then boiled for approximately 1 h until completely digested. The contents of P, K, S, Fe, Mn, Cu, and Zn were determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) as described by Liu *et al.* (2016). Each experiment was repeated six times.

RESULTS AND DISCUSSION

We constructed two small RNA libraries from untreated- and Mn-treated-*Arabidopsis* seedlings. Small RNA sequencing was performed to identify *Arabidopsis* Mn-responsive miRNAs. The numbers and proportion of different types of small RNAs are shown in Table 1. Among these small RNAs, 13 254 951 total small RNAs (69.43%), representing 667 025 (11.21%) unique small RNAs, were common between the control and Mn-treated seedlings. Moreover, 2 553 379 total small RNAs (13.37%), representing 2 341 715 unique small RNAs (39.35%), were specific to the Mn-treated seedlings (Figure 1A). The majority of miRNA reads ranged from 20–24 nt in length, and the 21 nt miRNAs were the most abundant, which is the typical size range for Dicer-derived products and is consistent with the typical miRNA distribution patterns (Figure 1B, Table S1 in Electronic Supplementary Material (ESM)).

The level of miRNA enrichment could be reflected by the number of reads. In this study, we found that eighteen miRNAs were differentially expressed in the control and Mn-treated plants (Figure 2, Table S2 in ESM). Among the eighteen differentially expressed miRNAs, only two miRNA families, *miR395* and *miR399*, including seven miRNAs, *miR395a*, *miR395b*, *miR395c*, *miR395d*, *miR395e*, *miR395f*, and *miR399a* were downregulated, and the other eleven miRNAs, including *miR5026*, *miR5650*, *miR781a*, *miR781b*,

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Table 1. Number of reads obtained by Illumina sequencing and annotation of small RNAs matching transcriptome of manganese (Mn)-treated and untreated (control) *Arabidopsis thaliana* plants

Small RNA type	Control		Mn- treated	
	reads No.	(%)	reads No.	(%)
Genome	3 578 598	21.64	2 499 329	17.52
rRNA	8 257 163	49.93	7 305 413	51.20
scRNA	0	0.00	0	0.00
snRNA	2 296	0.01	2 726	0.02
snoRNA	476	0.00	235	0.00
tRNA	878 915	5.31	1 182 784	8.29
Repbase	231 482	1.40	156 698	1.10
Other	3 589 435	21.70	3 120 731	21.87
Clean reads	16 538 365	100.00	14 267 916	100.00

rRNA – ribosomal RNA; scRNA – small cytoplasmic RNA; snRNA – small unclear RNA; snoRNA – small nucleolar RNA; tRNA – transfer RNA

miR866-3p, *miR826*, *miR5595a*, *miR5995b*, *miR863-5p*, *miR2936*, and *miR861-3p*, were all upregulated in the Mn-treated seedlings (Figure 2A). The existence of several miRNA family members in differentially expressed *miR395* suggested that this family plays a role in Mn toxicity response in *Arabidopsis*.

We then analysed four miRNAs (*miR395a/d/e*, *miR399a*, *miR826*, and *miR781a*) by stem-loop qRT-PCR and found that all of the tested miRNAs show similar expression patterns to small RNA sequencing data, indicating the reliability of the small RNA sequencing data (Figure 2B).

We analysed the target genes of Mn-responsive miRNAs (Table 2). Bioinformatic analysis indicated that the predicted target genes are involved in broad spectrum of development and physiological processes. Mn toxicity repressed the expression of *miR395* and *miR399*. *MiR395* targets the *ATP sulfurylase* genes (*APSs*) and the sulfate transporter *SULTR2;1* and are involved in sulfur (S) assimilation and translocation. S starvation significantly induces the expression of *miR395*. Overexpression of *miR395* results in S over-accumulation, however, the transgenic plants show S deficiency phenotype, and the S distribution from

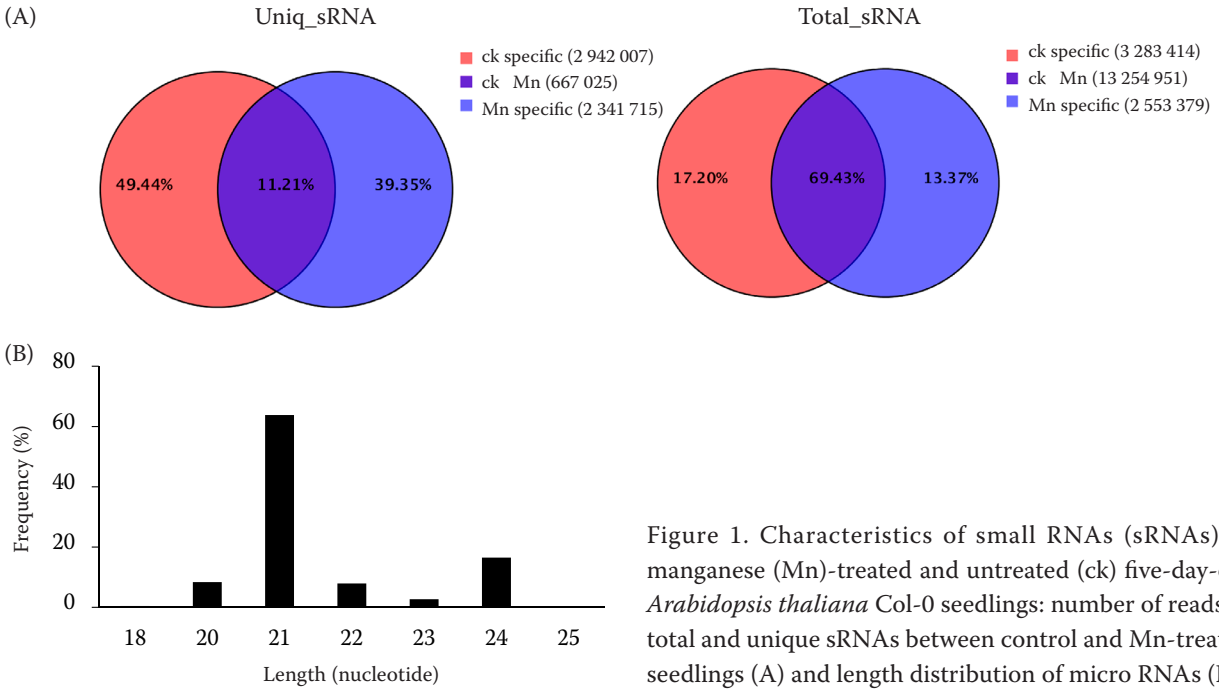

Figure 1. Characteristics of small RNAs (sRNAs) in manganese (Mn)-treated and untreated (ck) five-day-old *Arabidopsis thaliana* Col-0 seedlings: number of reads of total and unique sRNAs between control and Mn-treated seedlings (A) and length distribution of micro RNAs (B)

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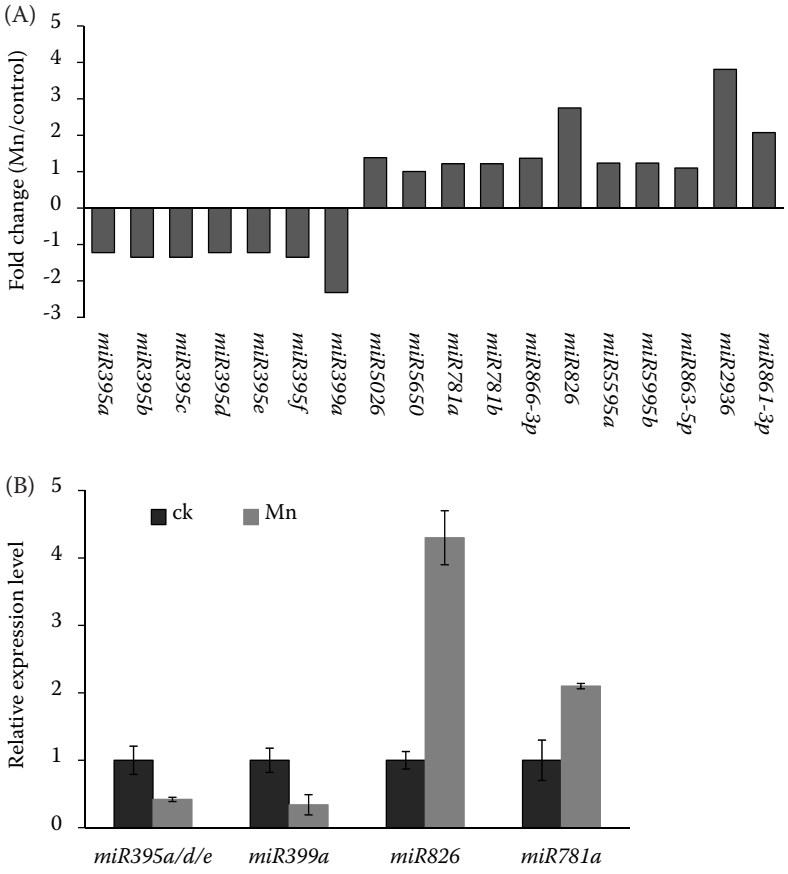

Figure 2. Characteristics of micro RNAs (miRNAs) in manganese (Mn)-treated and untreated (ck) five-day-old *Arabidopsis thaliana* Col-0 seedlings: differentially expressed miRNAs by high throughput sequencing (A) and qRT-PCR analysis of four miRNA (B)

mature or old to young leaves is impaired (Liang *et al.* 2010). All of the six members of *miR395* are significantly downregulated in Mn-treated seedlings, indicating that Mn toxicity regulates S homeostasis by modulating *miR395* expression. Mn toxicity also represses *miR399a* expression, which targets *Ubiquitin Conjugase E2* (*UBC24/PHO2*) that negatively regulates phosphate (Pi) transport and homeostasis (Aung *et al.* 2006). The downregulated expression of *miR399a* in Mn-treated seedlings suggested that

Table 2. Predicted target genes of manganese toxicity-responsive micro RNAs (miRNAs) in *Arabidopsis thaliana*

miRNA	Target genes[1]
miR395	*ATP sulfurylase* (*APS1*, *APS3*, *APS4*; *At3g22890*, *At4g14680*, *At5g43780*) *Sulfate transporter* (*SULTR2;1*; *At5g10180*)
miR399a	*Ubiquitin conjugase E2* (*UBC24/PHO2*; *At2g33770*)
miR5026	unknown
miR5650	*serine-threonine protein kinase* (*At3G21985*, *At1G66880)*; *NF-YA8* (*At1G17590*)
miR781a	*MCM2* (*At1G44900*)
miR781b	*MCM2* (*At1G44900*)
miR866-3p	*HCC2* (*At4G39740)*; *CRK28* (*At4G21400*)
miR826	*Alkenyl hydroxalkyl producing 2* (*AOP2*, *At4g03060*)
miR5595a	*MES* (*At2G23560*; *At4G37150*)
miR5995b	*MES* (*At2G23560*; *At4G37150*)
miR863-5p	*PID2* (*At2G26700*)
ath-miR2936	*RIN3* (*At5G51450*); *ASHH1* (*At1G76710*); *SUVH5* (*At2G35160*)
ath-miR861-3p	unknown

[1]Predicted according to Allen *et al.* (2005)

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Table 3. Nutrient contents in *Arabidopsis thaliana* five-day-old manganese (Mn)-treated and untreated (control) seedlings

	P	K	S	Fe	Mn	Cu	Zn
	(g/kg)					(mg/kg)	
Control	6.66 ± 0.01[a]	31.41 ± 6.75[a]	6.14 ± 0.02[a]	0.57 ± 0.01[a]	0.13 ± 0.001[a]	3.2 ± 0.13[a]	122.33 ± 0.44[a]
Mn	11.56 ± 0.07[b]	41.94 ± 3.47[a]	6.71 ± 0.04[b]	0.59 ± 0.03[a]	3.24 ± 0.03[b]	2.93 ± 0.11[a]	118.67 ± 1.11[b]

Mn toxicity affects Pi accumulation by modulating *miR399* pathways.

Mn toxicity also induces several miRNA expression. We found that excess Mn upregulates *miR826* expression. *MiR826* targets *AOP2* and its homologous genes *AOP1* and *AOP3*. *AOPs* encode 2-oxoglutarate-dependent dioxygenase that is involved in glucosinolate biosynthesis. *MiR826*-overexpressed transgenic plants display less glucosinolate and anthocyanin contents and nitrogen (N) deficiency tolerance (He *et al.* 2014). These results indicated that excess Mn affects nutrition homeostasis by modulating miRNA expression. The predicted target gene of *miR781* is *Minichromosome Maintenance 2* (*MCM2*) (Chauhan *et al.* 2017). *MCM2* modulates root meristem function and is essential to embryo development (Sabelli *et al.* 2009). Two *miR781* genes, *miR781a* and *miR781b*, were upregulated in the Mn-treated seedlings, consistent with the observed altered root growth phenotype. Predicted target gene of *miR5595* and *miR5995b* are *Methyl Esterase 7* (*MES7*) and *MES9*. *MES7* and *MES9* are involved in methyl-SA (MeSA) hydrolysis and systemic acquired resistance in plants (Yang *et al.* 2008). Both of *miR5595a* and *miR5995b* were upregulated in the Mn-treated seedlings. These data imply that Mn toxicity also affects pathogen resistance by modulating miRNA expression.

One of the important physiological processes of heavy metal toxicity is the disruption of nutrient equilibrium in plants. The above mentioned results indicated that Mn toxicity affects several miRNAs expression that modulate phosphorus (P) and S transport and metabolism. Therefore, we examined the nutrient contents in Mn-treated seedlings. We found that Mn toxicity significantly increases the accumulation of P and S, whereas it decreases zinc (Zn) content in seedlings (Table 3).

We then investigated whether the Mn-induced accumulation of P and S were also involved in Mn-induced inhibitory of primary root (PR) growth. For this purpose, five-day-old seedling were transferred to fresh 1/2 MS media containing only 1/10 of normal P or 1/10 of normal S. We found that PR growth showed a less inhibition by excess Mn in the P-deficient or S-deficient medium (Figure 3A, B). These data indicated that excess Mn could induced secondary toxicity by disturbing nutrient accumulation in plants.

Pedas *et al.* (2011) found that increased P supply in soil results in reduced leaf Mn content in barley,

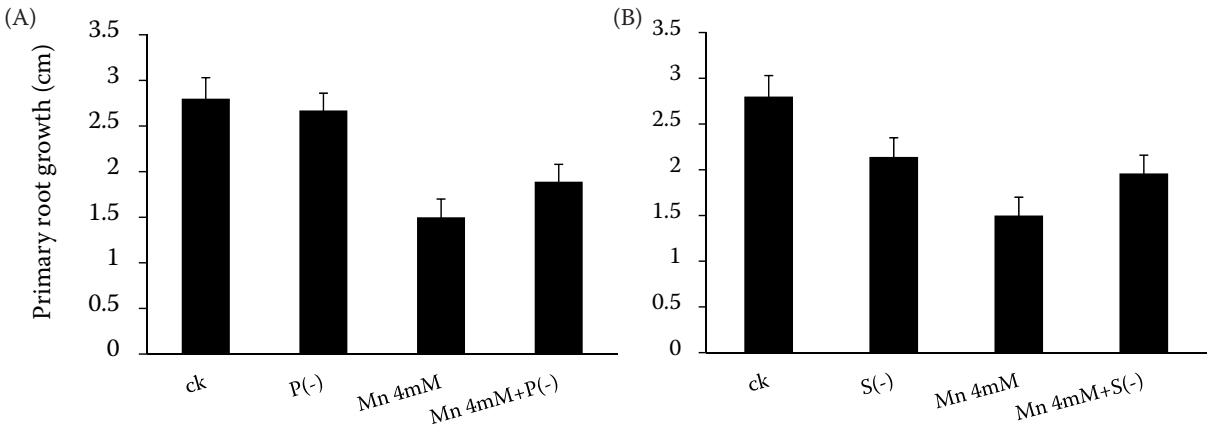

Figure 3. Primary root length of manganese (Mn 4mM)-treated and untreated (ck) five-day-old *Arabidopsis thaliana* Col-0 seedlings in phosphorus normal and deficient P (–) Murashige and Skoog medium (A) and in sulphur normal and deficient S (–) Murashige and Skoog medium (B)

The error bars represent the standard deviations; different letters indicate significantly different values ($P < 0.05$ by Tukey's test)

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suggested that the elevated P levels impeded Mn absorption in roots; and thereby resulting in Mn deficiency in leaves and shoots. In this study, we found that Mn toxicity disturbed nutrient equilibrium in *Arabidopsis* by reducing Zn accumulation whereas increasing the accumulation of P and S. Reduction of P and S content in the medium alleviated Mn-induced inhibition on root growth. Taken together, these results suggested that Mn toxicity affect the accumulation of P and S might result in P and S toxicity and might be a secondary stress to root growth, and miRNAs play a role in modulating nutrition homeostasis in this process.

In this study, we reported the identification of Mn-responsive miRNAs in *Arabidopsis*. We identified 18 differential expressed miRNAs in Mn-treated seedlings and four of them were validated by qRT-PCR. The Mn-responsive *miR395*, *miR399*, and *miR826* regulate the metabolism and transport of S and P, and N starvation response, respectively, and thereby affecting nutrition homeostasis in plants. Several miRNAs were also involved in modulating root system development (*miR781a* and *miR781b*) and phytohormone signaling (*miR5595* and *miR5995b*), suggesting that Mn toxicity also regulates developmental processes by regulating miRNA expression. These data lay a foundation for better understanding of the function of miRNAs in response to Mn toxicity in plants.

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